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Stroke and migraine: Translational studies into a complex relationship

Mulder, I.A.

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STROKE & MIGRAINE

TRANSLATIONAL STUDIES INTO
A COMPLEX RELATIONSHIP

INGE MULDER

Inge Mulder
STROKE & MIGRAINE - Translational studies into a complex relationship
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STROKE & MIGRAINE
TRANSLATIONAL STUDIES INTO
A COMPLEX RELATIONSHIP

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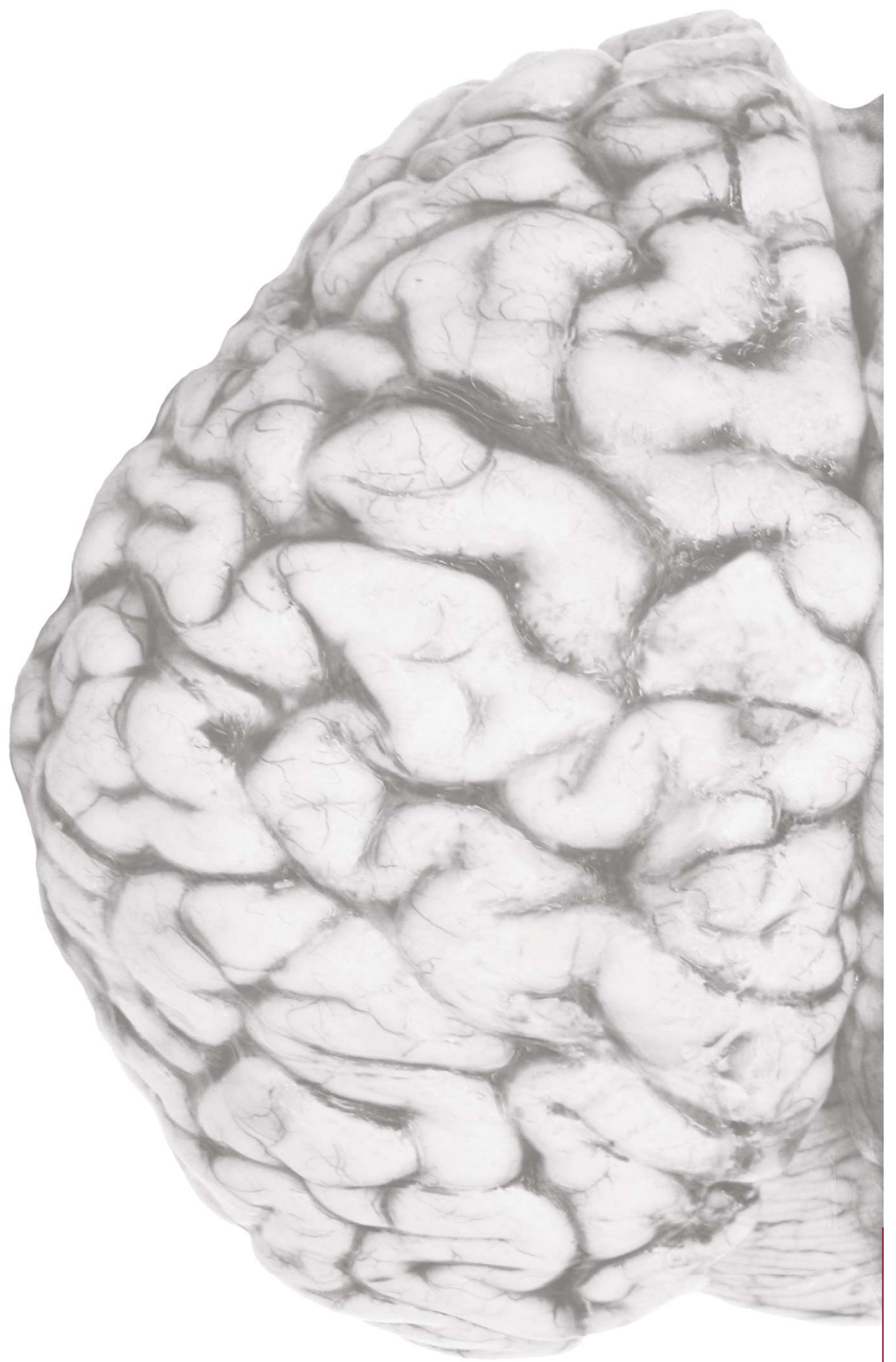
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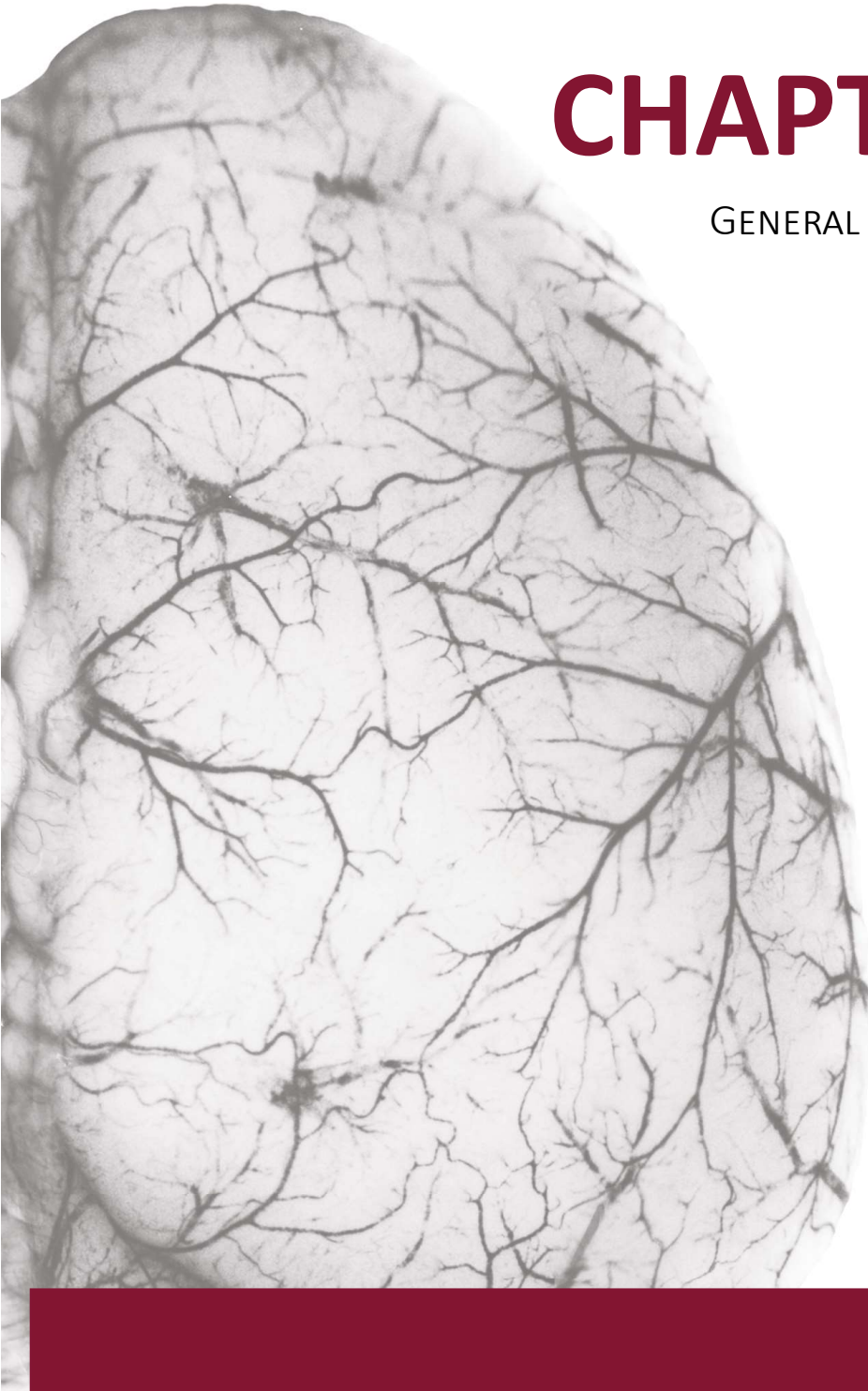
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VOOR MIJN OUDERS EN ZUS

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CHAPTER 1

GENERAL INTRODUCTION



GENERAL INTRODUCTION

Although stroke and migraine are generally considered to be two very different disease entities, they actually are closely connected and their pathophysiological overlap becomes increasingly clear. In this thesis we investigate, in a translational manner in patients and experimental animal models, the relationship between migraine and ischemic stroke and between migraine and delayed cerebral ischemia after subarachnoid hemorrhage.

1. Epidemiology of stroke and migraine

1.1 Ischemic stroke

Stroke is the second most frequent cause of death and the most frequent cause of disability worldwide,¹ with a global incidence of more than 10 million and a prevalence of almost 26 million.² In the Netherlands, every year approximately 46,000 people get a stroke. According to 'The Stroke Council of the American Heart Association / American Stroke Association' criteria, the definition of stroke is "an episode of neurological dysfunction caused by focal cerebral, spinal, or retinal infarction".³ Roughly, there are two main stroke subtypes: ischemic stroke and hemorrhagic stroke. Ischemic stroke is the most common (about 80%) type of stroke. Although hemorrhage stroke is less common its long term consequences are often severe and therefore both subtypes have great impact on a patient's daily life.

Ischemic stroke occurs when blood flow to the brain is restricted due to occlusion of a cerebral artery, typically by a local thrombus or an embolus (Figure 1). The global incidence is almost 7 million with a prevalence of 18 million.² In the Netherlands, the incidence is almost 20,000

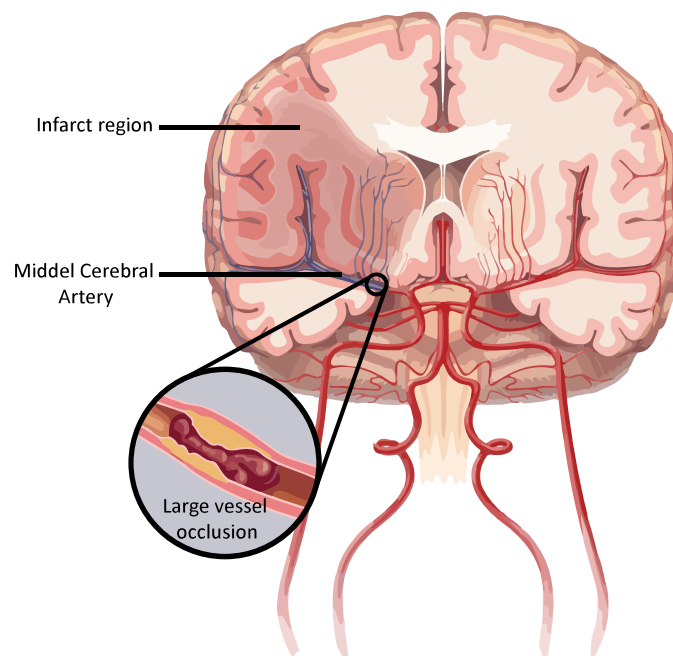


Figure 1. Schematic illustration of an ischemic stroke (modified from strokecenter.org)

per year.⁴ Different subtypes are described, according to their underlying cause, which affects stroke management. The 'Trial of Org 10172 in Acute Stroke Treatment' (TOAST) classification⁵ describes five subtypes: (I) large-artery atherosclerosis (embolus / thrombosis), (II) cardioembolism, (III) small-vessel occlusion (lacunar infarct), (IV) stroke of other determined etiology, and (V) stroke of undetermined etiology. There is a remarkable gender difference in stroke with men having an overall higher risk for first-ever stroke at medium age,⁶ where women have a higher risk in young (< 55 year) and older (> 75 year) ages.⁷ Also, the prevalence and average age of first-ever stroke is higher in women.^{6,7} Women have a higher burden after stroke, with more often physical impairment and depression than men.⁷

1.2 Migraine

Migraine is a common episodic brain disorder affecting approximately 15% of the population.⁸ Migraine is characterized by attacks of severe, usually throbbing unilateral headache that are accompanied by nausea, vomiting, photo-, and / or phonophobia.⁹ Attacks typically last 4 to 72 hours. Because of its high prevalence and major social and economic burden migraine was rated one of the most disabling common chronic neurological disorders.¹⁰ Two main types of migraine can be distinguished: migraine without aura and migraine with aura.⁹ The latter is present in about one third of patients and is characterized by an aura that can precede the headache. An aura consists of transient focal neurological symptoms affecting mainly the visual system but that can also include sensory, aphasic and motor symptoms. Migraine is a heterogenic disease with an attack frequency that can vary between and within patients from a few attacks per year up to a few per week; also the same patient can suffer from migraine with and without aura attacks. Migraine affects more women than men in a 3:1 ratio.^{9,11}

1.3 The stroke-migraine connection

Evidence is accumulating that migraine, especially migraine with aura, is an independent risk factor for ischemic stroke,¹¹⁻¹⁵ especially in women. At first this seems unexpected given the clinical disease characteristics that are quite different between both disorders, including the sex difference. Whereas migraine is a chronic disorder most common in young to middle-aged women (age 25 to 55 years), stroke is an acute event that typically occurs in middle aged men. Regardless, additional clinical evidence for the co-morbidity of stroke and migraine comes from: (I) the possible existence of migraineous infarctions,^{16,17} (II) the co-occurrence of migraine and cervical artery dissection,¹⁸ (III) shared risk factors like hypercoagulability¹⁹ and endothelial dysfunction,²⁰ (IV) the fact that certain drugs to treat migraine, such as triptans and ergotamines, have been associated with increased stroke risk,²¹ and (V) genetic evidence linking stroke and migraine in multiple monogenic diseases.^{22,23}

2. Primary and secondary ischemic damage in stroke; core and penumbra

Multiple complex mechanisms are responsible for infarct maturation during and after vessel occlusion. Although the exact mechanisms are still largely unknown, a typical temporal pattern seems to occur after a focal perfusion deficit.²⁴ Within this temporal and spatial continuum the infarct territory can be divided into two main areas: the ischemic core and the penumbra, or 'tissue at risk' (Figure 2).^{25,26} Within minutes after the ischemic event, cells contributing to the core become necrotic with membrane breakdown, dysfunctional cellular metabolism and energy supply, disturbed ion homeostasis, and loss of cell integrity. The tissue surrounding the core, however, is 'struggling to survive' due to collateral blood supply being borderline

sufficient. Cells in the penumbra are metabolically active for some time, until the disruption of the cellular homeostasis in these cells also leads to cell death. It is increasingly clear that inflammatory factors²⁷ and blood-brain-barrier (BBB) breakdown²⁸ play an important role in the transition of penumbra tissue into core tissue. The transition process can take up to several hours, which has direct impact on the ‘time-to-treat’ window of stroke patients. The first destructive cascade that is activated after a perfusion deficit is cellular excitotoxicity, which contributes to a large extent to the tissue damage. Excitotoxicity includes the production of reactive oxygen and nitrogen species (ROS and RNS) and acidosis. Within minutes, and lasting up to several hours, multiple pathophysiological events take place: (I) a rise of extracellular K^+ , (II) presynaptic terminal depolarization, (III) excessive extracellular neurotransmitter accumulation, (IV) N-methyl-D-aspartate (NMDA)-receptor activation, (V) loss of ion homeostasis (Ca^{2+} , K^+ , Na^+ , H^+ , Cl^- , HCO_3^-), and (VI) a rise of neuronal and glial intracellular Ca^{2+} resulting in cytotoxic edema.²⁹⁻³¹ Secondary mechanisms contributing to increased tissue damage are BBB breakdown, reperfusion injury, inflammation and apoptosis.³² Protective and regenerative mechanisms to prevent and repair damage of a stroke also occur actively in the peri-acute and chronic phase after ischemic onset.³² However, the molecular pathways involved in these events are still to be unraveled. Investigating at the molecular level, by elucidating the peptides, (amino-) metabolites and lipids that show changes in the various, especially the early, stages of a stroke can help us to dissect the pathophysiology of stroke and can eventually lead to new therapeutic targets to treat patients.

3. The role of spreading depolarization and neurovascular coupling in the shared pathophysiology between stroke and migraine

3.1 Spreading depolarization

Spreading depolarization (SD) is the generic term for a self-propagating wave of membrane depolarization in neuronal and glial cells which travels through cerebral grey matter of the central nervous system and which is accompanied by a period of electrical silencing. These depolarization waves have been described in humans in ischemic stroke,³³ in subarachnoid hemorrhage (SAH)³⁴ and in traumatic brain injuries³⁵ at the site of the injury where they are referred to as anoxic depolarizations (ADs) and peri-infarct depolarizations (PIDs). SDs are also considered to be the underlying pathophysiological mechanism of a migraine aura.^{36,37} In migraine aura a SD that originates in the visual cortex and spreads to frontal cortical regions is referred to as cortical spreading depression (CSD), named after the neuronal depression that follows the sharp wave front of hyperexcitation (with concomitant neuronal and glial cell depolarization). SDs can therefore be seen as a spectrum that includes PID, AD and CSD depending on the disease type and is also referred to as the stroke-migraine depolarization continuum.^{24,38}

3.1.1 Spreading depolarization in ischemic stroke

In a pathological condition, for example during ischemic stroke onset, depression of spontaneous neuronal activity is seen 10-20 seconds after a blood flow reduction below a certain threshold (15- 23 mL / 100g / min).²⁵ Neurobiological mechanisms that are active in this initial period are neuronal hyperpolarization, loss of synaptic activity, reduction of vesicular transmitter release by adenosine mediation, and reduced energy consumption (that acts as a survival mechanism of the tissue to cope with the ischemia). Within 2-5 minutes, AD occurs resulting in an even further reduced blood flow (5-10 mL / 100 g / min) and depression of

activity.^{38,39} The AD originates from the core, and spreads via the penumbra into healthy tissue. This wave is triggered by loss of membrane integrity due to hypoxia and energy depletion. Notably, after this first depolarization wave, multiple PID waves erupt from the penumbra, spreading into the penumbra, core and healthy tissue. Due to the energy mismatch created by these PIDs, each wave will turn part of the penumbra tissue into a permanently depolarized and necrotic state,⁴⁰⁻⁴² and therefore co-determines the severity of stroke outcome.

3.1.2 Spreading depolarization in migraine

Under normal conditions, neurons and their dendrites have a membrane potential that enables them to fire action potentials, which is the way neurons communicate. This membrane potential is maintained by active ion pumps. During CSD in a migraine aura, this homeostasis is disrupted, resulting in: (I) a near-complete breakdown of ion gradients,⁴³ (II) increased extracellular K^+ level, (III) loss of electrical activity,⁴⁴ (IV) swelling of neurons,⁴⁵ (V) sustained

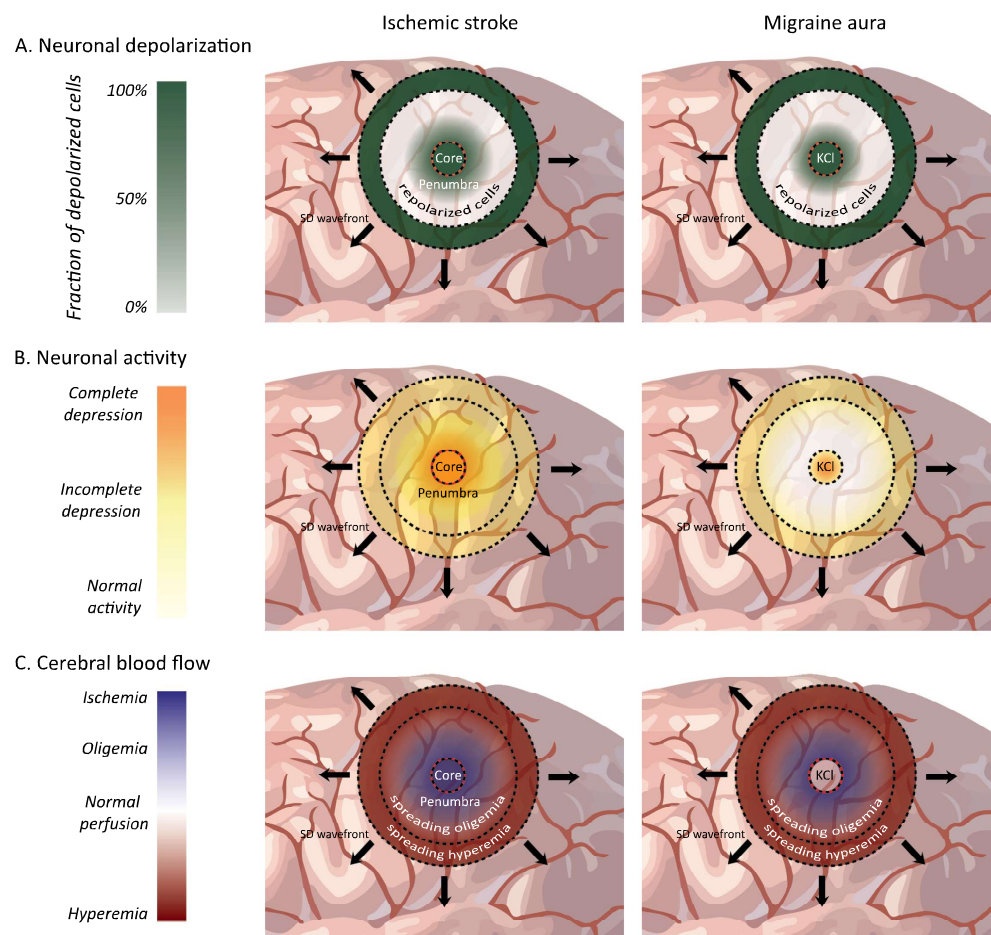


Figure 2: Schematic illustration of SD waves triggered by occlusion of a cerebral vessel (left) as seen during ischemia and triggered by high potassium (right) occurring during a migraine aura, with (A) neuronal depolarization, (B) Neuronal activity and (C) Cerebral blood flow. (Modified from Dreier *et al.* 2015²⁴ with permission). SD – Spreading Depolarization.

depolarization,⁴⁶ and (VI) a hemodynamic response.⁴⁷ Unlike SD events in stroke, CSDs in migraine are considered rather benign transient disturbances of (cortical) brain function without permanent damage. Whether SD also occurs in migraine without aura, also referred to as ‘silent aura’, is debated.^{48,49}

3.2 Neurovascular coupling

Vessels are a major player in the pathology of stroke and likely also in migraine, in the first place due to their involvement in neurovascular coupling. As a result of neurovascular coupling, a hyperemic response occurs to meet the increased energy demand when PIDs circle around the ischemic core (in the case of stroke) or when a CSD wave spreads through the cortex (in the case of a migraine aura).⁵⁰ Briefly before and prolonged after this phenomenon, hyperemia and oligemia are present.⁵¹ In pathological (ischemic) tissue, AD and PIDs are accompanied with paradoxical vasoconstriction resulting in oligemia.^{52,53} The AD / PIDs and additional decrease in resting cerebral blood flow (rCBF) is also called spreading ischemia,⁵⁴ due to inverse neurovascular coupling (Figure 2).⁵² In such pathological tissue, the hypoperfusion wave travels, in contrast with CSD, together with the spreading depolarization at the same time through the tissue, where it enters the vicious cycle of an inverse hemodynamic response and energy supply / demand mismatch. The trigger for PIDs is the massive misbalance in ion homeostasis that induces a vicious circle of ischemia, depolarization and vasoconstriction with an increased infarct territory as the devastating result.^{39,55} PIDs under ischemic conditions are seen in numerous experimental animal models.⁵⁶⁻⁵⁸ CSD is thought to occur in patients that have migraine with aura, although the most convincing evidence thus far in humans came from analyzing indirect vascular responses seen with imaging techniques³⁷ and correlations with clinical characteristics,⁵⁹ adding to the debate on how relevant CSD is in humans.⁶⁰ In contrast, CSD has been studied widely in animal models in which it has been shown that it indeed is the likely cause of the aura.^{61,62}

3.3 Vascular dysfunction

The connection of stroke and migraine has also been attributed to multiple vascular pathologies, such as endothelial dysfunction and coagulation abnormalities.⁶³ Endothelial dysfunction includes reduced vasodilatation, increased endothelial derived vasoconstriction (vasospasm) and subsequent impairment of cerebral vascular reactivity. These processes can subsequently lead to an increase in coagulation factors, increased release of inflammatory factors that eventually can lead to atherosclerosis and increased stroke risk. Coagulation abnormalities (primarily or secondarily due to endothelial dysfunction) are found in stroke^{64,65} as well as in migraine⁶⁶⁻⁶⁸ patients and include increased platelet-activating factor (PAF), increased VonWillebrand Factor (VWF), both of which are released by or triggered by endothelial cells.

4. Monogenic disorders in stroke and migraine

There are a number of monogenic diseases in which ischemic stroke and migraine are part of the clinical spectrum.⁶⁹ Understanding the genetics and molecular mechanisms of these diseases provides an unique opportunity to further unravel the pathophysiology of the stroke-migraine association. Here three monogenic diseases will be discussed: (I) Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL),⁷⁰ (II) Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S),⁷¹ and (III) Familial Hemiplegic Migraine (FHM).⁷² Both CADASIL and RVCL-S belong to

the group of small vessel diseases,⁶⁹ a condition in which the walls of small arteries in the brain are damaged.⁷³ In contrast, FHM is considered more a disease of neurologic than of vascular dysfunction.

4.1 CADASIL

CADASIL is the most common type of hereditary small vessel disease and characterized by progressive development of subcortical infarcts, starting at middle age⁷⁴⁻⁷⁷ with cognitive decline even before first stroke onset.^{78,79} Remarkably, approximately 40% of CADASIL patients suffer from migraine with aura,⁸⁰ which in many is the first presenting symptom, sometimes decades before the onset of other disease characteristics. As the disease progresses, accumulation of lacunar infarcts, microbleeds and brain atrophy result eventually in severe vascular dementia. CADASIL is caused by mutations in the *NOTCH3* gene, which encodes the NOTCH3 protein that is mainly expressed in vascular smooth muscle cells.⁸¹ CADASIL mutations typically alter the number of cysteines that are responsible for correct folding of the protein's extracellular domain (NOTCH3^{ECD}).^{82,83} Misfolding eventually leads to accumulation of mutant protein in vascular smooth muscle cells (VSMC),^{77,84} degeneration of these cells, vessel wall thickening, and the occurrence of dense deposits of granular osmophilic material (GOM) in the vessel wall. Typically the abnormalities are observed in small- and medium-sized arteries.⁸⁵ Various transgenic mouse models are available that express NOTCH3 protein with CADASIL mutations, either from a human cDNA overexpression construct,⁸⁶⁻⁸⁸ a rat⁸⁹ or human⁹⁰ genomic overexpression construct, or a mouse knock-in construct.^{91,92} To more or lesser extent, these animal models exhibit key features of the disease.^{90,93} However, brain imaging abnormalities seen in CADASIL patients, have not yet been found in these mice.

4.2 RVCL-S

RVCL-S is a systemic small vessel disease with prominent vasculopathy of, most profoundly, retina, brain and kidney that may lead to visual loss, cognitive disturbances, depression and kidney dysfunction, which starts at middle-age.^{71,94-96} About half of RVCL-S patients also suffer from migraine (with or without aura), as became clear from investigating all 11 known RVCL-S families in the world.⁷¹ RVCL-S patients also have an increased ischemic stroke risk as evidenced by the small white matter infarcts seen in many patients.⁷¹ RVCL-S is caused by heterozygous C-terminal frameshift mutations in the *TREX1* gene,⁹⁵ which encodes the major mammalian 3' - 5' exonuclease that has multiple possible functions such as acting as cytosolic DNA sensor to prevent autoimmunity.^{97,98} A study of mutant cells and a transgenic mouse model that expresses a *TREX1* mutation pointed at an aberrant release of free glycans due to abnormal oligosaccharyltransferase (OST) function as a possible mechanism for the vasculopathy.⁹⁹

4.3. FHM

FHM is a monogenic subtype of migraine with aura and evidence is accumulating that it is linked to stroke.⁹ FHM is characterized by long-lasting hemiparesis during the aura phase,⁹ with headache features¹⁰⁰ and trigger factors¹⁰¹ that are similar to those in common migraine with aura. Three genes, FHM1 to FHM3, have been identified that all encode ion transporters.⁷² FHM1, the gene that is most prominently linked to stroke, is caused by certain missense mutations in the *CACNA1A* gene,¹⁰² which encodes the $\alpha 1A$ subunit of voltage-gated $Ca_v2.1$ (P / Q-type) calcium channels. These channels are located at most, if not all, synaptic terminals of the central nervous system where they regulate neurotransmitter release.^{103,104} FHM1

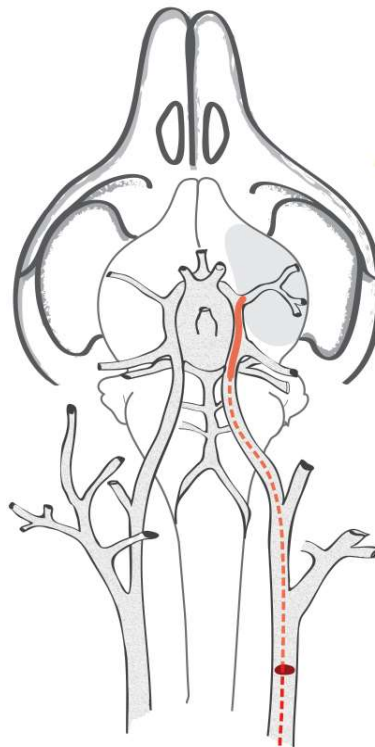


Figure 3. Experimental transient intraluminal suture model for middle cerebral artery occlusion (MCAO) in mice.

mutations cause a left-shift in the action voltage, prolongation of opening of $\text{Ca}_v2.1$ channels, and increased neurotransmitter release. In the cortex, this results in an enhanced glutamate release that explains the increased SD sensitivity seen in transgenic mice that express $\text{Ca}_v2.1$ channels with FHM1 mutations.¹⁰⁵⁻¹⁰⁹ These transgenic mice also were shown to be a relevant model to study the relation of stroke and migraine.^{56,110}

5. Techniques to investigate the relation between stroke and migraine

5.1 Experimental stroke model in mice

Various cerebral stroke models are described in literature, ranging from global (transient whole circulatory arrest) to focal (transient or permanent occlusion of a cerebral artery) occlusion of cerebral blood flow. These models give us the opportunity to study stroke-induced mechanisms with the final goal of reducing patient burden after an infarct.¹¹¹ One of the most common causes of ischemic stroke seen in patients is the occlusion of the middle cerebral artery (MCA) by a thrombus or embolus.¹¹² This stroke subtype is best mimicked by the experimental middle cerebral artery occlusion model (MCAO) with reperfusion, which therefore, is the most widely used model in experimental stroke research (Figure 3). With this model, the MCA is occluded by the temporary introduction of a filament into the intracerebral artery (ICA) that is maneuvered towards the origin of the MCA where it blocks blood flow. The MCAO model allows for ischemic core and penumbra development, of which the ratio and severity is directly dependent on the occlusion time. Occlusion of the MCA for 30-60

minutes will make the lateral striatum (caudoputamen) ischemic with or without ischemia of the frontoparietal cortical region. Advantages of this model over other models, such as distal transient / permanent MCAO, is that it is minimally invasive concerning the research target area (the brain), since skull integrity is maintained and the occlusion is more stable compared to for instance embolic stroke models.¹¹³ Therefore, MCAO reduces the amount of confounding factors of massive surgery and thus mimics the clinical situation as accurate as possible.

5.2 State-of-the-art imaging techniques in mice

5.2.1 Magnetic Resonance Imaging

Using magnetic resonance imaging (MRI) as a readout technique for infarct characteristics, avoids disadvantages such as: (I) histological validation (the current golden standard) that introduces errors as there will be changes in brain morphology from processing brain sections (swelling / shrinkage of tissue), (II) the manual-labor-intensive nature of infarct volume analysis, and (III) the necessity to sacrifice the animal making longitudinal studies and multiple readout times unfeasible. Anatomical spin-spin relaxation time contrast T2 MRI sequence can detect ischemic lesions in a way that they can be analyzed in a longitudinal manner.¹¹⁴⁻¹¹⁶ This T2 sequence is shown to be sensitive to vasogenic edema which is one of the mechanisms active during infarct development.¹¹⁷ In clinical research, multiple algorithms for automatic detection, segmentation and classification of stroke areas in the brain have been developed.¹¹⁸ However, segmentation of brain lesion in mouse MRI data still heavily relies on manual time-consuming protocols.¹¹⁹

5.2.2 Mass Spectrometry Imaging

To simultaneously analyze the distribution of hundreds of molecules from a tissue sample¹²⁰ within its histological context,¹²¹ mass spectrometry imaging (MSI) can be used.¹²² MSI can distinguish molecules from different classes such as peptides, (amino-) metabolites, proteins and lipids. The identity of molecules is determined using their unique mass-to-charge ratio (m/z). Matrix-Assisted-Laser-Desorption / Ionization (MALDI) MSI is a method to ionize molecules in the target tissue. MSI involves matrix deposition onto a tissue section, where after a laser beam allows desorption and ionization of molecules that subsequently are detected by the mass analyzer. From this data, 2D images are reconstructed that provide detailed information on the spatial distribution of the respective metabolites. To avoid confounding distortion of sections from different samples by the various procedures (e.g. cutting, processing), 2D MSI images can be co-registered with for example histological images, MRI images or brain atlases.^{123,124} Arguably, tissue preparation is the most important factor determining the success of a MSI experiment, especially for molecular classes that are highly susceptible to *post mortem* changes, foremost ATP and ADP,^{120,121,125,126} that are important to evaluate molecular mechanisms relevant to stroke. Multiple tissue preparation methods have been reported that have their advantages and limitations concerning different molecular classes,¹²⁰ but at present none of them is ideal.

5.3 State-of-the-art CT techniques in patients

Initial triage and management in ischemic stroke is crucial in patients who come to the emergency room with signs and symptoms of acute ischemic stroke, since time to reperfusion is highly important for the outcome of the patient. Along with the neurologic exam, radiological imaging is eminent for diagnostic and therapeutic purposes. In today's

clinic, a CT-scan is made to visualize the possible infarct territory. Non-contrast CT (NCCT) is used to differentiate between ischemic and hemorrhagic stroke, and to exclude other possible causes for the presenting symptoms, such as a subdural hematoma. Additionally, CT angiography (CTA) is increasingly performed, which gives important information concerning the presence and location of a thrombus and functional collateral and anastomotic function, which is crucial information for mechanical thrombectomy management.^{127,128} Upcoming is the opportunity for CT perfusion (CTP) in acute stroke management. This relatively new technique can provide additional information on the viability of the infarcted tissue. CTP includes information concerning tissue perfusion, such as cerebral blood volume and flow (CBV and CBF, respectively), mean transit time (MTT), time-to-peak (TTP) and blood-brain-barrier permeability (BBBP).¹²⁹⁻¹³²

6. Scope and outline of the thesis

In this thesis, studies of experimental ischemic infarct rodent models and results from epidemiological human studies investigating ischemic stroke patients are combined to investigate relevant mechanisms that (possibly) underlie migraine and stroke. Understanding the molecular mechanisms underlying this comorbidity will eventually help us to identify possible therapeutic targets to reduce infarct size and improve clinical outcome.

Part I of the thesis describes advances in the methodology to obtain and analyze infarct data of experimental stroke in multiple monogenetic stroke and migraine mouse models. [Chapter 2](#) describes a renewed sacrificing method, which is now used for mouse tissue collection after experimental stroke in order to reduce post-mortem molecular degradation as much as possible. This method is applied in [Chapter 3](#) to investigate, with state-of-the-art MALDI-MSI techniques, brain tissue of transgenic mice with an FHM1 missense mutation in the CACNA1A gene that underwent experimental MCAO. Lipids are analyzed with respect to the core and penumbra at different time points after experimental infarct induction in order to find potential altered molecular pathways in these infarct areas which might be responsible for infarct enlargement and maturation. In [Chapter 4](#) an automated method for MRI lesion segmentation in mice is developed to overcome current obstacles of tedious manual segmentation that, in principle, is error-prone. The segmentation tool is used for data analysis in [Chapters 5 and 6](#). In [Chapter 5](#) the tool is used to investigate infarct volume, in addition to parameters of vascular functionality, in transgenic mice with a human RVCL-S mutation to investigate whether, and to what extent, these mice show vascular dysfunction seen in patients with RVCL-S. In [Chapter 6](#) transgenic RVCL-S, CADASIL, and FHM1 mice are investigated and compared, aimed to identify possible stroke vulnerability changes in these animal models, as seen in patients with the same mutation. Here we also included neuronal hyper-excitability experiments by examining CSD characteristics as possible mechanism for stroke vulnerability.

Part II describes data of clinical studies in which state-of-the-art CT techniques are used to detect radiological infarct characteristics in patients with migraine or headache and stroke. In [Chapter 7](#) we used modern CTA and CTP techniques to investigate whether radiologic stroke features and occurrence of secondary brain damage differed in stroke patients with and without migraine and whether this resulted in different outcomes after intravenous-thrombolysis and / or thrombectomy. In [Chapter 8](#) we investigated the association between migraine and cerebrovascular atherosclerosis in patients with acute ischemic stroke. A general discussion about the interpretation of the experimental and clinical studies and suggestions

for future research is presented in [Chapter 9](#).

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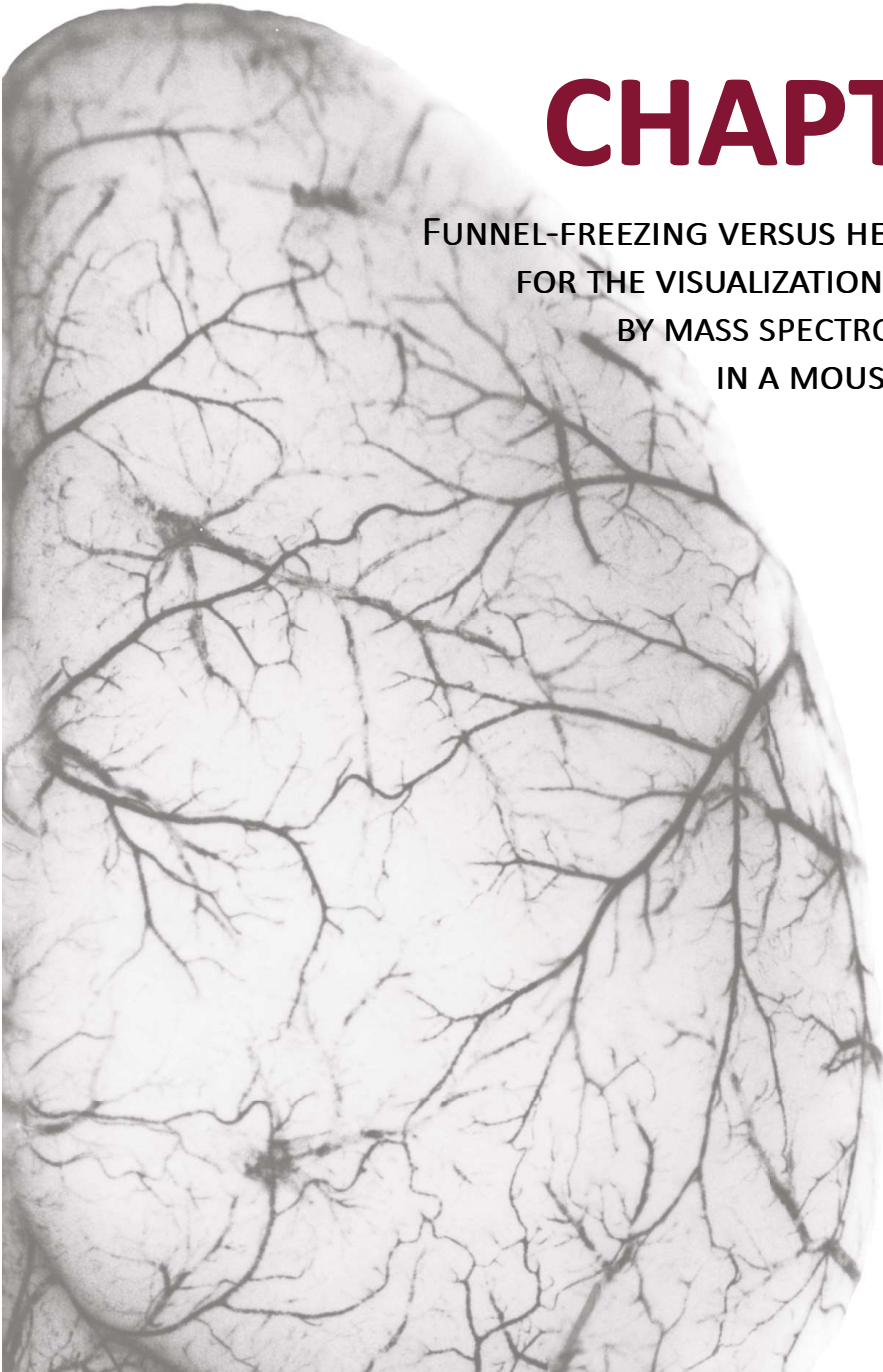
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CHAPTER 2

FUNNEL-FREEZING VERSUS HEAT-STABILIZATION
FOR THE VISUALIZATION OF METABOLITES
BY MASS SPECTROMETRY IMAGING
IN A MOUSE STROKE MODEL

Mulder IA*, Esteve C*, Wermer MJH, Hoehn M, Tolner EA,
van den Maagdenberg AMJM and McDonnell L

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**Authors contributed equally*

ABSTRACT

Tissue preparation is the key to a successful matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) experiment. Rapid *post mortem* changes contribute a significant challenge to the use of MSI approaches for the analysis of peptides and metabolites.

In this technical note we aimed to compare the tissue fixation method *ex vivo* heat-stabilization with *in situ* funnel-freezing in a middle cerebral artery occlusion (MCAO) mouse model of stroke, which causes profound alterations in metabolite concentrations. The influence of the duration of the thaw-mounting of the tissue sections on metabolite stability was also determined. We demonstrate improved stability and biomolecule visualization when funnel-freezing was used to sacrifice the mouse compared with heat-stabilization. Results were further improved when funnel-freezing was combined with fast thaw-mounting of the brain sections.

INTRODUCTION

MALDI mass spectrometry imaging (MALDI MSI) can simultaneously record the distributions of hundreds of molecules directly from tissue samples¹ and within their histological context.² MSI is used to analyse metabolites, drugs, peptides, proteins, lipids and glycans, and is applied to diverse biomedical and biological applications. Tissue preparation is arguably the single most important factor that determines the success of a MALDI MSI experiment, and this is especially true for metabolites on account of their high susceptibility to *post mortem* changes.¹⁻³ For example, for the analysis of mouse brain tissue obtained by post-euthanasia freezing, the procedure of decapitation, brain excision and snap-freezing takes one to several minutes, during which time the remaining activity of endogenous enzymes is known to lead to post-mortem degradation.² Similar results have also been reported for neuropeptides.⁴

Several strategies have been reported for the reduction of post-mortem changes of metabolites in brain tissue:

- I. Heat-stabilization of *ex vivo* tissues (HS) - enzymes are inactivated by heating the tissue to 95°C using high power heating blocks.²⁻⁶ Blatherwick *et al.*³ have demonstrated that *ex vivo* heat-stabilization is able to halt the rapid *post mortem* degradation of adenine nucleotides that otherwise occurs in *ex vivo* snap-frozen tissue.
- II. *In situ* freezing (ISF) under anaesthesia is based on freezing the tissue using liquid nitrogen while maintaining blood flow and oxygenation.^{2,7,8} Hattori *et al.*⁷ have demonstrated that ISF is superior to *ex vivo* snapfrozen tissue for maintaining metabolite integrity, including adenine nucleotides that are prone to rapid *post mortem* change.
- III. *In situ* focused microwave irradiation (FMW) - uses focused microwaves to very rapidly, <2 s, heat the tissue to deactivate enzymes.² Sugiura *et al.*² have compared FMW with ISF and *ex vivo* snap-freezing and reported that, while ISF and FMW provide similar results for most metabolites, there are several metabolites that are best analyzed using FMW on account of their very rapid *post mortem* changes.² However, the high expense and negative aesthetics of animal sacrifice via focused microwave irradiation has severely limited its use.

ISF and *ex vivo* heat-stabilization have been reported to be superior than *ex vivo* snap-freezing for preserving metabolic integrity but have not yet been compared. With *in situ* funnel-freezing under anaesthesia⁹ blood flow is still present until the tissue is frozen. It has previously been demonstrated that warm ischemia times lead to greater *post mortem* changes than cold ischemia times.¹⁰ Accordingly, it may be reasoned that *in situ* funnel-freezing may better preserve metabolites in their pre-sacrifice state than *ex vivo* heat-stabilization, which takes 1–2 min to excise the brain and another 1–2 min (of warm ischemia) to stabilize the tissue, time which is critical for metabolite stability. A caveat is that with *in situ* funnel-freezing enzymes are not inactivated. Accordingly, preparation of the tissue sections for MSI analysis must be very carefully controlled after *in situ* funnel-freezing as the enzymes can reactivate, continuing metabolite degradation, as soon as the tissue is thawed.¹¹

In this paper we have systematically compared *in situ* funnel-freezing, *ex vivo* heat-stabilization and subsequent thaw-mounting methods for the analysis of metabolites by MALDI-MSI in a mouse model for ischemic stroke. Ischemic stroke is an often disabling event caused by interruption of blood supply to part of the brain.¹² Understanding the biomolecular profiles in the infarct core and penumbra¹³ may help explain differential vulnerability and recovery of brain regions to metabolic stress and to search for potential neuroprotective or neurorestorative therapies.¹⁴ In this context, a discriminating factor between core and

penumbra is the level of ATP;¹⁵ previous investigations utilizing both bioluminescence and MALDI MSI (using *in situ* freezing) have reported a localized increase in ATP. As ATP is very quickly degraded post-mortem, it also represents an excellent model system to assess how well the tissue's metabolic status has been preserved.⁷

MATERIALS AND METHODS

Animal protocol

Male 2- to 4-month-old C57BL/6J mice were used. Experimental stroke was induced using a slightly modified middle cerebral artery occlusion model (MCAO) first described by Longa *et al.*¹⁶ Mice were anesthetized using isoflurane (3% induction, 1.5% maintenance) in 70% pressurized air and 30% O₂. Carprofen 5mg/kg, *s.c.* (Carporal, 50 mg/mL, AST farma B.V., Oudewater, the Netherlands) was given before surgery. During surgery the mouse body temperature was maintained at 37°C using a feedback system. Briefly, the surgical procedure; a silicone-coated nylon monofilament (7017PK5Re, Doccol cooperation, Sharon, MA, USA) was introduced into the internal carotid artery, via a small incision in the right common carotid artery, to block the middle cerebral artery (MCA) at its origin, for 30 min. During the occlusion period, the mouse was allowed to wake up in a temperature-controlled incubator (V1200, Peco Services Ltd, Brough, United Kingdom) maintained at 33°C. After surgery, the animal was placed in the incubator again for 2 hours with easy access to food and water.

On a subset of animals, used for the preliminary experiments, SHAM surgery was performed using the same protocol, only without blocking the MCA. At 24 hours after MCAO mice were scanned *in vivo* using a 7T small animal MRI system (Bruker Pharmascan; Bruker, Ettlingen, Germany), under Paravision 5.1 software (Bruker). A Multi SliceMulti Echo (MSME) sequence protocol was run with TR/TE of 4.000 ms/9 ms, 20 echoes, two averages, matrix 128×128 mm, FOV of 2.50 cm, bandwidth 59523.8Hz, slice thickness of 0.5 mm and 16 slices (no gap) and quantitative T2 maps were calculated from the multi-echo trains.

For *ex vivo* heat-stabilization the mouse was sacrificed by decapitation and the brain was quickly isolated (<1.5 min). The brain was immediately stabilized using a tissue heat-stabilizer device (StabilizorTM, Denator AB, Göteborg, Sweden) at 95°C in 60–90 seconds depending on brain volume. Thereafter, brains were frozen on dry-ice and stored at -80°C.

In situ funnel-freezing was based on previous reported studies.^{9,17-19} Briefly, the mouse was anesthetized using isoflurane (3% induction) and 1.5–2% isoflurane in 30% O₂ and 70% pressurized air was used to maintain deep anaesthesia. A skin incision was made from the level of the eyes to the occiput exposing the skull. A funnel was placed onto the skull, with the posterior rim of the funnel at the lambdoidal suture. The skin was pulled up around the funnel and secured with four sutures to prevent leaking. Liquid nitrogen was continuously poured into the funnel for 3 minutes. Thereafter, for easier removal of the brain, the whole animal was frozen in liquid nitrogen. Next the animal was put into dry-ice and the brain dissected using a scalpel and a dental drill. The excised and frozen brain was stored at -80°C. All animal experiments were approved by the Animal Experiment Ethics Committee of Leiden University Medical Center.

MALDI MSI

Coronal sections (12 µm, between -0.10 and +0.40 from Bgma) were cut using a cryostat microtome (Leica Microsystems, Wetzlar, Germany) at -21°C. The brain sections were thaw-

mounted onto ITO glass slides (Delta Technologies, Stillwater, MN, USA) coated with 0.05% poly-L-lysine (poly-L-lysine coating used for greater adherence of tissue sections, protocol used as reported in Aichler *et al.*²⁰). Sections were thaw-mounted onto the slides by localized warming of the reverse side of the MALDI target using a finger for max 3 seconds (fast) or for 1 minute (slow).^{1,10} The slide-mounted tissues were stored at -80°C. For analysis the slide-mounted tissue sections were first brought to room temperature in a desiccator for 5 minutes. For matrix application a uniform coating of 9-AA (2 mg/ml in 70% MeOH) was added using a SunCollect automated deposition system (SunChrom, Napa, CA, USA). Brain sections from three animals per group were analyzed in technical duplicate on a 9.4-Tesla Solarix MALDI-FTICR (Bruker Daltonics, Bremen, Germany), equipped with a SmartBeam II laser system that consists of a frequency tripled Nd:YAG laser operating at 355 nm, at repetition rates up to 1 kHz, and using a spatially modulated laser profile. MS data were acquired in negative mode by first accumulating the ions from 500 laser shots in an external hexapole ion trap before transferring them to the ICR cell for detection. Ions were detected in the range 50–1000 m/z and MSI was performed with a spatial resolution of 125 µm. Data acquisition, processing, and data visualization were performed using the Flex software suite (FlexControl 3.4, ftmsControl 2.0, FlexImaging 4.1 and DataAnalysis 4.2) from Bruker Daltonics.

After MSI data acquisition the matrix was washed off with 70% ethanol and the tissue samples stained with cresyl violet (Nissl stain).^{21,22} High-resolution histological images were obtained with a digital slide scanner (3D Histech MIDI) and were registered to the MSI datasets using FlexImaging. A scheme of the work flow is presented in Figure 1.

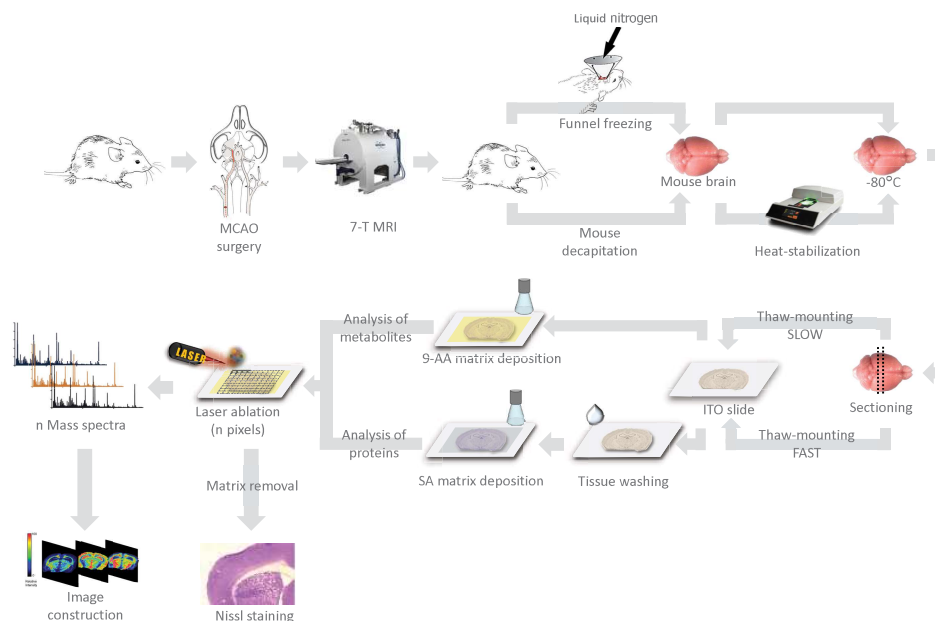


Figure 1. Schematic of the workflow used to analyze the effect of brain tissue sampling protocol. Mice first underwent MCAO surgery, were scanned using a 7T MRI at 24 hours and directly thereafter sacrificed by either *in vivo* funnel-freezing, or by decapitation followed by *ex vivo* heat-stabilization. Coronal tissue sections were then cut and mounted onto poly-lysine coated slides using slow (1 min) or fast (3 sec) thaw-mounting. Metabolites were analyzed by MALDI-FTICR-MSI using 9-AA as the matrix. Each section was stained with Nissl reagent after matrix removal.

Data analysis

The RMS normalized intensities of six mouse brain sections were measured for each group (two technical duplicates of three biological replicates). MS data were extracted from each MSI dataset for statistical analysis: (I) a non-paired Student's t-test (one tailed) was used for comparisons between *ex vivo* heat-stabilized and *in situ* funnel-freezing brains and (II) a paired Student's t-test (one-tailed) was used for comparisons (fast versus slow thaw-mounting) within each mouse brain. Statistical analysis was performed in Microsoft Excel 2010. Metabolite identities were assigned on the basis of the very high-mass accuracy of the high-field MALDI FTICR mass spectrometer used for the experiments (<1 ppm), in conjunction with the results of previous metabolite MALDI MSI experiments (it is now broadly established that MALDI MSI samples a consistent set of molecules) and the isotope profiles.^{2,3,7,8,23,24} For selected metabolites, in which the ion intensity was sufficient for MS/MS, the ID's were confirmed by MS/MS.

RESULTS AND DISCUSSION

When seeking to investigate the metabolite/peptide content of tissues it is vital that the tissue collection protocol limits the sometimes rapid *post mortem* changes that follow animal sacrifice. In a preliminary experiment, we compared the metabolite MSI signatures from brain tissue obtained using *ex vivo* heat-stabilization and *in situ* funnel-freezing with *ex vivo* snap-freezing, to check if the results we obtained were consistent with those previously reported;^{3,7} it was indeed found that both methods were more effective at retaining labile metabolites but *in situ* funnel-freezing appeared to lead to more intense labile metabolite signals (Supplemental Figure 1). The results described herein describe an experiment designed to compare *ex vivo* heat-stabilization and *in situ* funnel-freezing. The comparison included both tissue stabilization methods as well as the time used for thaw mounting because *in situ* funnel-freezing does not deactivate the tissue enzymes, and thus *post mortem* changes may still occur during any subsequent tissue processing step (Figure 1). Here we used the MCAO model for ischemic stroke because the localized increase of ATP previously reported by Hattori *et al.*,⁷ a molecule highly sensitive to *post mortem* degradation,² provides an excellent *in situ* measurement of metabolite stability and the unaffected contralateral hemisphere provides an internal control. Mouse brain tissue samples were obtained by *in situ* funnel-freezing and *ex vivo* heat-stabilization, from which 12 μ m thick coronal tissue sections were placed onto the ITO-coated glass slides using fast (<3 sec) and slow (1 min) thaw-mounting. All experiments were performed in technical duplicate and biological triplicate. Figure 2A shows example MSI images recorded for AMP, ADP, and ATP together with their corresponding T2-weighted MRI and histological images. The localized increase of ATP in the ischemic penumbra was consistently detected in the MSI datasets of the tissues obtained via *in situ* funnel-freezing (Figure 2A) but not from those obtained via *ex vivo* heat-stabilization. Furthermore, it can be seen that when using *ex vivo* heat-stabilization the tissue is deformed during the process (Figure 2A and B, bottom rows) because of the pressure applied to the tissue by the thermal blocks to ensure a high thermal contact. When comparing the MS images with the T2-weighted MR image and the histological section, comparisons are more straightforward using the *in situ* funnel-freezing technique.

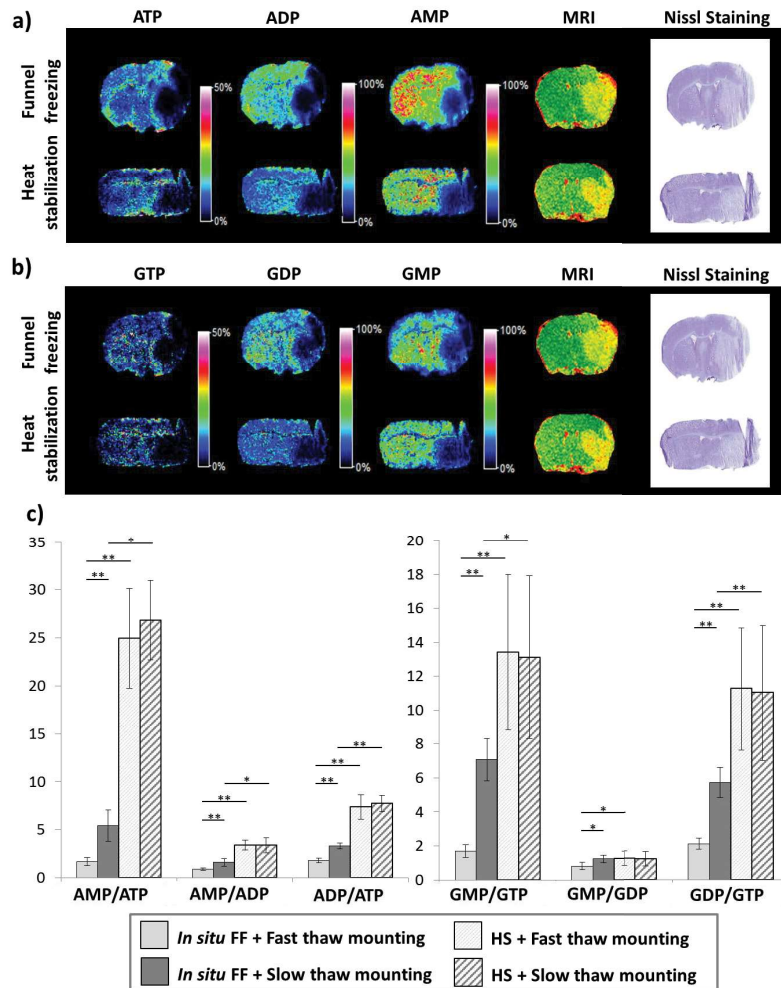


Figure 2. Metabolite MSI dataset showing the effect of the brain tissue sampling protocol on the visualization of adenosine and guanosine metabolites in mouse brain by MALDI-FTICR. (A) Images showing the visualization of the average distribution of AMP (m/z 346.055), ADP (m/z 426.022) and ATP (m/z 505.988) for mouse brain obtained via *ex vivo* heat-stabilization and *in vivo* funnel-freezing. (B) Images showing the visualization of the average distribution of GMP (m/z 362.050), GDP (m/z 442.016) and GTP (m/z 521.982) for mouse brain obtained via *ex vivo* heat-stabilization and *in vivo* funnel-freezing. (C) Average intensity ratios of AMP:ATP, ADP:AMP, ADP:ATP (left) and GMP:GTP, GDP:GMP, GDP:GTP (right) from the control hemisphere, for *ex vivo* heat-stabilization (HS) and *in vivo* funnel-freezing (FF) with the two thaw-mounting times (slow and fast). Each group consists of three biological replicates each analyzed with two technical replicates. Error bars represent standard deviation across technical replicates. (** = $p < 0.01$ and * = $p < 0.05$)

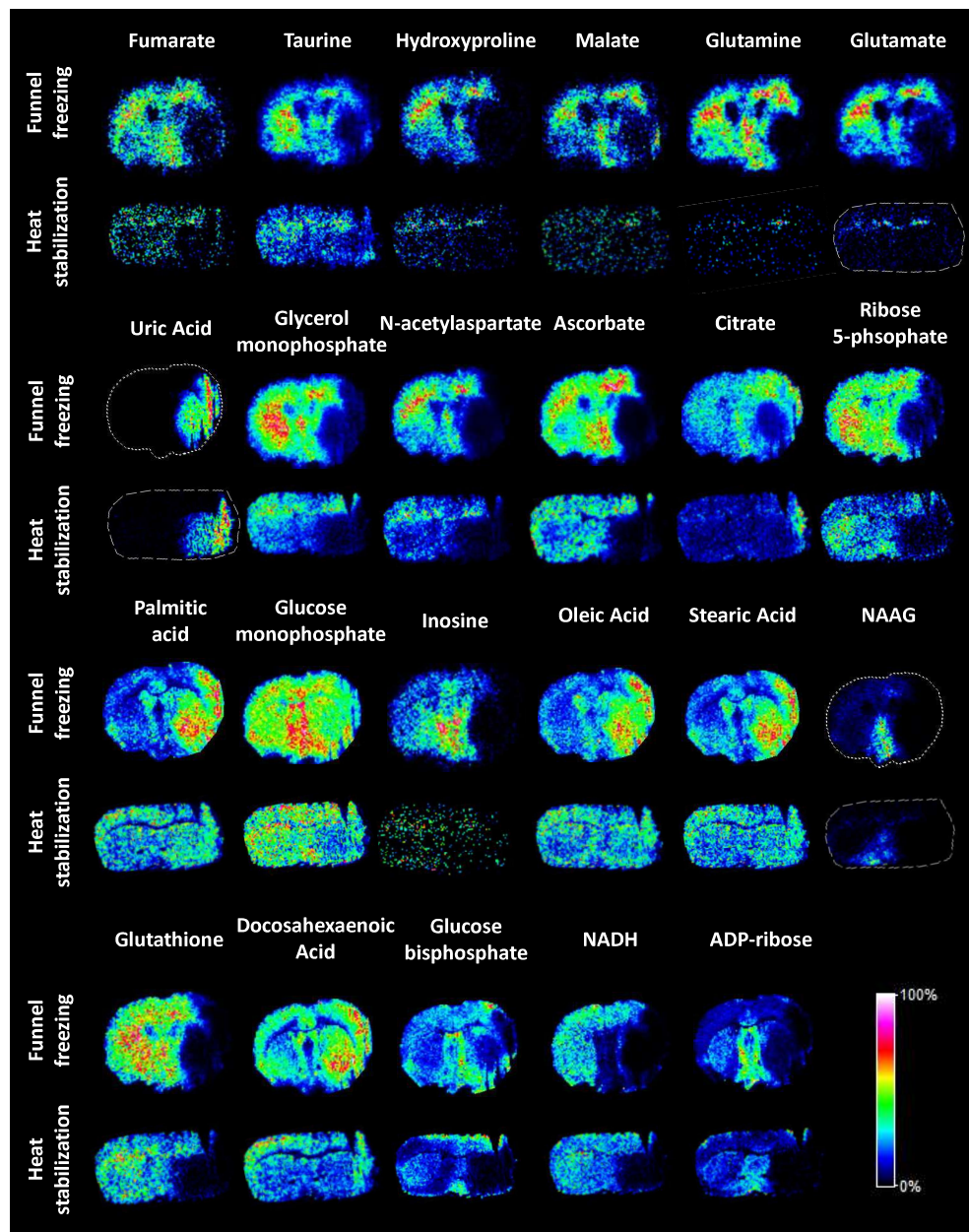


Figure 3. Metabolite MSI images of mouse brain metabolites found in the range m/z 50–1000 and analyzed by MALDI-FTICR.

A significant reduction in AMP, ADP, and ATP signals in the ischemic region was observed for all preparations, in agreement with previous reported results.²³ The ATP signals from the control hemispheres obtained from the *ex vivo* heat-stabilized tissues were lower than those obtained using *in situ* funnel-freezing; conversely the AMP signals from the control hemispheres were greater from the *ex vivo* heat-stabilized tissues. Similar results were obtained for the nucleotides GTP, GDP, and GMP as shown in Figure 2B. However, it is known that MCAO (stroke hemisphere) and heat-stabilization (control hemisphere) can lead to increased salt levels, which can cause a global increase in ionization bias and thus lower MSI signal intensities. To circumvent differences in global ionization bias the AMP:ATP, ADP:ATP, and AMP:ADP intensity ratios were calculated (Figure 2C) from the control hemisphere. The bar chart shows that the ratio of AMP to ATP was 5-fold higher for *ex vivo* heat-stabilized tissue than for those obtained using *in situ* funnel-freezing when slow thaw-mounting was used, and around 15-fold higher when fast thaw-mounting was used. For both thaw-mounting methods (compared with heat-stabilization) the difference in AMP to ATP ratios were statistically significant. Accordingly, the results demonstrate that irrespective of the mounting method used, the *in situ* funnel-freezing method leads to less *post mortem* changes of the adenine nucleotide metabolites, which is also supported by the observation that the increase in ATP seen in the penumbra was only observed with tissues obtained using *in situ* funnel-freezing.

While the *in situ* funnel-freezing method was found to lead to reduced *post mortem* changes, it is more sensitive to subsequent tissue processing methods. A comparison of the fast and slow thaw-mounting methods on the adenine nucleotide metabolite ratios within the *in situ* funnel-freezing brain group revealed a statistically significant increase in metabolite degradation that was not present in the *ex vivo* heat-stabilized group, Figure 2C. This result indicates that for *ex vivo* heat-stabilization all ATP degradation takes place during brain excision and during heat treatment; after heat-induced denaturation the enzymes are no longer active and further metabolite degradation is limited. For *in situ* funnel-freezing the enzymes are not deactivated and so once the tissue is thawed *post mortem* metabolite degradation may continue. Nevertheless, it should be noted that ATP degradation was always lower when using *in situ* funnel-freezing. This is consistent with previous reports that *post mortem* changes occur more rapidly when the tissue is still at physiological temperatures.^{10,25} Similar results were obtained for GTP, GDP and GMP, except for the GMP:GDP ratio (Figure 2C).

Sample treatment not only influences the stability of adenine nucleotide metabolites. Figure 3 shows the distribution of several metabolites that were detected by MALDI-FTICR-MSI using 9-AA as matrix. In several cases metabolites showed similar distribution for both methods. However, some of them could only be detected using *in situ* funnel-freezing or had very weak signal for *ex vivo* heat-stabilized brains, including fumarate (m/z 115.003), hydroxyproline (m/z 130.050), malate (m/z 133.014), glutamine (m/z 145.061), glutamate (m/z 146.045), citrate (m/z 191.019), and inosine (m/z 267.073). Furthermore, the intensity of metabolite peaks was a lot higher when *in situ* funnel-freezing was used, and also exhibited greater contrast with the stroke region, e.g. taurine (m/z 124.007), glycerol monophosphate (m/z 171.006), N-acetylaspartate (m/z 174.040), palmitic acid (m/z 255.232), or glutathione (m/z 306.076). In their comparison of *in situ* freezing, focused microwave irradiation and *ex vivo* snap-freezing (no heat-stabilization) Sugiura *et al.*² used capillary electrophoresis to compare *post mortem* metabolite stability. One group, primarily consisting of amino acids, was stable to *post mortem* changes. Using the on-tissue derivatization methods recently reported by Shariatgorji *et al.*²⁶ we also compared the amino-metabolites obtained from tissues processed via *in situ* funnel-

freezing with those obtained via *ex vivo* heat-stabilization, Figure 4. It can be seen that the distributions are more comparable than those of the less stable metabolites shown in Figures 2 and 3. Again indicating that the changes are due to *post mortem* degradation.

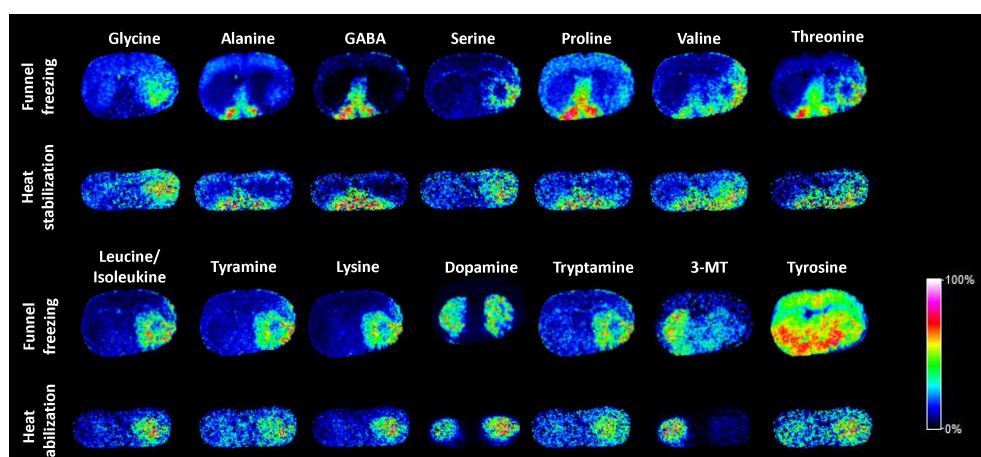


Figure 4. Amino-metabolite MSI images of mouse brain sections analyzed using MALDI-FTICR and the on-tissue derivatization agent 2,4-diphenyl-pyranilium tetrafluoro-borate.²⁶

CONCLUDING REMARKS

These results again highlight the crucial role of the tissue in MSI experiments, how it was sampled and how it was mounted. Two different tissue sampling strategies, *in situ* funnel-freezing and *ex vivo* heat-stabilization, as well as fast and slow thaw-mounting, were investigated for their impact on metabolite levels and distributions using MALDI-FTICR-MSI. The results demonstrated that *in situ* funnel-freezing has lower degradation compared to heat-stabilization irrespective of the thaw-mounting method, but care should be taken to keep the thaw-mounting short (and thus reproducible) to limit metabolite degradation and variation between experiments. *In situ* funnel-freezing has the additional advantage of causing little deformation to the mouse brain, allowing alignment to other imaging datasets and reference tissue atlases.

ACKNOWLEDGEMENTS

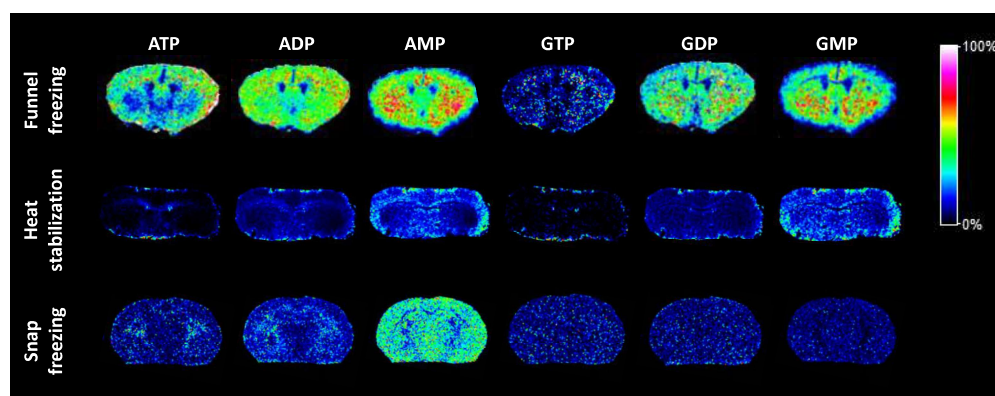
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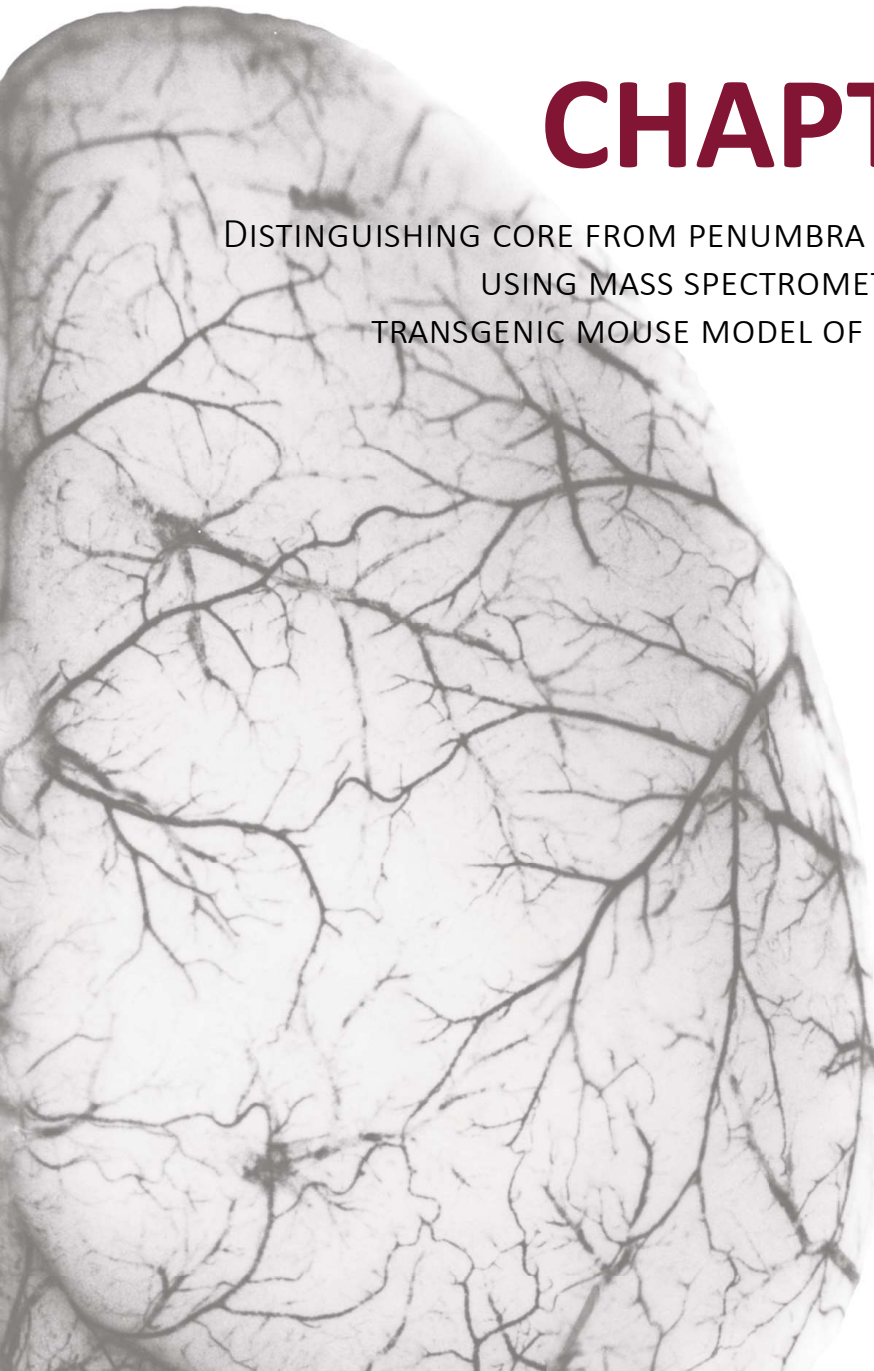
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SUPPLEMENTAL MATERIAL



Supplemental Figure 1. Metabolite MSI dataset of control brain tissue after SHAM surgery showing the effect of the tissue sampling protocol on the visualization of adenosine and guanosine metabolites in mouse brain by MALDI-FTICR. Images showing the visualization of the average distribution of AMP (m/z 346.055), ADP (m/z 426.022), ATP (m/z 505.988) GMP (m/z 362.050), GDP (m/z 442.016) and GTP (m/z 521.982) for mouse brain obtained via snap-freezing, *ex vivo* heat-stabilization and *in vivo* funnel-freezing.



CHAPTER 3

DISTINGUISHING CORE FROM PENUMBRA BY LIPID PROFILES
USING MASS SPECTROMETRY IMAGING IN A
TRANSGENIC MOUSE MODEL OF ISCHEMIC STROKE

Mulder IA*, Ogrinc Potočnik N*, Broos LAM, Prop A, Wermer MJH[#],
Heeren RMA[#] and van den Maagdenberg AMJM[#]

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[#]Authors contributed equally

ABSTRACT

Detecting different lipid profiles in early infarct development may give an insight on the fate of compromised tissue. Here we used Mass Spectrometry Imaging to identify lipids at 4, 8 and 24 hours after ischemic stroke in mice, induced by transient middle cerebral artery occlusion (MCAO).

Combining linear transparency overlay, a clustering pipeline and spatial segmentation, we identified three regions: infarct core, penumbra (i.e. comprised tissue that is not yet converted to core), and surrounding healthy tissue. Phosphatidylinositol 4-phosphate ($m/z = 965.5$) became visible in the penumbra 24 hours after MCAO. Infarct evolution was shown by 2D-renderings of multiple phosphatidylcholine (PC) and Lyso-PC isoforms.

High-resolution Secondary Ion Mass Spectrometry, to evaluate sodium/potassium ratios, revealed a significant increase in sodium and a decrease in potassium species in the ischemic area (core and penumbra) compared to healthy tissue at 24 hours after MCAO. In a transgenic mouse model with an enhanced susceptibility to ischemic stroke, we found a more pronounced discrimination in sodium/potassium ratios between penumbra and healthy regions.

Insight in changes in lipid profiles in the first hours of stroke may guide the development of new prognostic biomarkers and novel therapeutic targets to minimize infarct progression.

INTRODUCTION

Ischemic stroke is a severe neurological event and its incidence in Western society is increasing.¹ Ischemic stroke generally occurs due to an interruption of blood supply, drastically decreasing glucose and oxygen concentrations within seconds. ATP levels drop rapidly, resulting in random ion leakage across the neuronal cell membrane. As a consequence, the ion balance in the tissue is severely disrupted, ultimately leading to cerebral ischemia. The ischemic territory consists of a core (necrotic tissue) that is surrounded by a border zone, the “penumbra”. Penumbra tissue has only partially reduced blood flow, just above the ischemic threshold level.² When the ischemia is not resolved in time, the penumbra will be converted to core tissue. The mechanisms involved in this transition are only partly known.^{3–7}

During the ischemic event, different lipid pathways are activated by multiple lipids (for example phosphatidylcholine (PC) and Lysophosphatidylcholine (LPC)) and second messenger molecules that can act in a neuroprotective or neurodegenerative way.⁶ Identification of the molecular species in the different regions of the ischemic area, especially in the early stages of infarct development, is crucial to identify the molecular mechanisms that may determine the fate of the penumbra.

Mass Spectrometry Imaging (MSI) is a powerful technique for identifying hundreds of molecules simultaneously within a single tissue section in a histological context.^{8,9} This technique is particularly useful to evaluate lipid distributions on tissue sections for which there are generally no antibodies available. MSI, specifically Matrix-Assisted Laser Desorption Ionization (MALDI), has been applied in neurodegenerative diseases, including research on the distribution of A β peptides co-localizing with different elements,^{10,11} neuropeptides in Parkinson’s disease¹² and global changes in phospholipids in a transgenic mouse model for Alzheimer’s disease.¹³ MSI offers the unique capability to simultaneously detect pathological hallmarks and related biomolecular patterns of neurodegeneration, making it a powerful monitoring tool for disease pathogenesis and prognosis.

Here, we used MSI to assess changes in lipids between the penumbra and infarct core during the early stroke evolution in mice. Until now, studies have focussed on the distribution of phospholipids in the ischemic region compared to the healthy surrounding area of the brain and/or after 24 hours of ischemic stroke induction.^{3–7,14} It was demonstrated that in the ischemic region Na⁺ is elevated with a concomitant reduction of K⁺.^{3,5,6} As large changes in alkali cation concentrations influence the ionization efficiency of phospholipids, matrix effects may have confounded those results. Later studies reduced the matrix effect, by either desalting the tissue sections prior to MALDI or using an internal standard as a normalization factor, and revealed different distributions of phospholipids.^{14,15} An increase of LPC (16:0) was observed in the ischemic region with concomitant membrane degradation of free fatty acids and the degradation of PCs into LPCs.^{4,6}

We assessed MALDI-MSI derived lipid profiles in three regions of interest (core, penumbra and healthy surrounding tissue) at three time points (4, 8 and 24 hours) after transient middle cerebral artery occlusion (MCAO) in mice. Additionally, we included a transgenic mouse model that expresses mutant hyperactive voltage-gated Ca_v2.1 Ca²⁺ channels that cause Familial Hemiplegic Migraine type 1 (FHM1).¹⁶ These mutants are particularly interesting for this study because a higher cerebral blood flow is required for tissue survival after MCAO in these mice. Therefore, infarction already occurs in these mutants with milder ischemia compared to wild-type mice, which may also occur in patients and thereby increasing the translational value of our study. With this approach, we aimed to study in detail the regional lipid distribution in early ischemic stroke development.

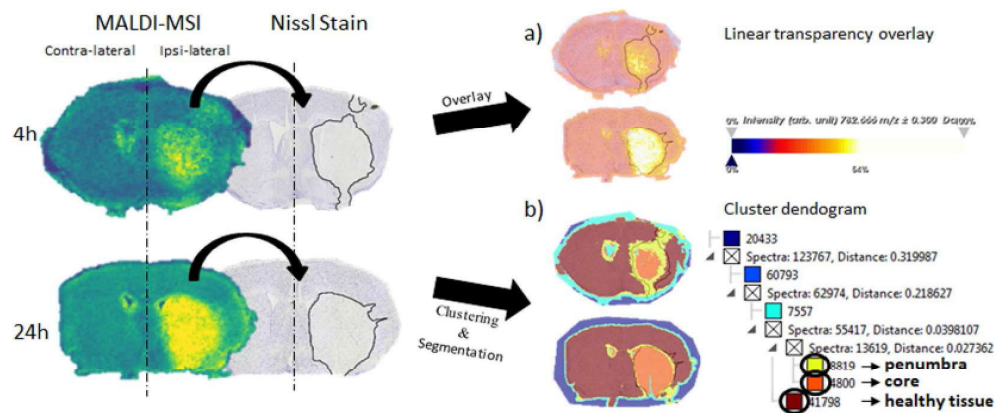


Figure 1. A schematic representation of the manual overlays of MALDI-MSI and Nissl stain with marked necrotic border in SCiLS Lab 2016b. The manual overlay was followed by a linear transparency overlay (A) of a selected m/z value (in the example $m/z = 782.6 \pm 0.3$). The high intensity of the $[PC(34:1) + Na]^+$ perfectly aligns within the necrotic border of the Nissl stain. (B) In the next step, the manual overlay was followed by a spatial segmentation pipeline built in the software. Once the segmentation map was created it detected prominent spatial regions ipsilateral, representing core, penumbra and healthy tissue, here at 4 hours after transient middle cerebral artery occlusion.

MATERIALS AND METHODS

Animal protocol

All animal experiments were approved by the Animal Experiment Ethics Committee of Leiden University Medical Center and all experiments were performed in accordance with relevant guidelines and regulations. Male FHM1 mutant mice and their WT littermates, age 3.5 months $\pm$ 2 weeks, were used. Experimental stroke was induced using the widely used MCAO model.³⁷ Isoflurane (3% induction, 1.5% maintenance) in 70% pressurized air and 30% O_2 was used as anaesthetic. Additional, Carprofen 5 mg/kg, s.c. (AST Farma B.V.) was given before surgery. During surgery the mouse body temperature was maintained at $\sim 37^\circ\text{C}$ using a feedback system. The MCA was occluded using an intraluminal silicone-coated nylon monofilament (Doccol cooperation), for 30 minutes. Thereafter the filament was removed to allow for reperfusion. After surgery, mice were allowed to wake up in a temperature-controlled incubator (Peco Services Ltd) kept at $\sim 33^\circ\text{C}$ for 2 hours with easy access to food and water. On a subset of animals, SHAM surgery was performed using the same protocol, only without insertion of the filament.

Magnetic resonance imaging

A subset of animals were scanned 24 hours after MCAO surgery, to evaluate the infarct region *in vivo*, using a small-animal 7 T MRI system (Bruker BioSpin) under isoflurane anesthesia. A Multi Slice Multi Echo (MSME) sequence protocol was run with TR/TE of 4.000 ms/9 ms, 20 echoes, 2 averages, matrix 128×128 mm, FOV of 2.50 cm, bandwidth 59523.8 Hz, slice thickness of 0.5 mm and 16 slices (no gap). Quantitative T2 maps were calculated using Paravision 5.1 software (Bruker BioSpin).

Sacrifice and histology

Per time point, 4 mice were sacrificed at 4, 8 and 24 hours after MCAO and at 24 hours

after SHAM surgery using the *in situ* funnel-freezing technique³⁸ because this technique limits molecular *post mortem* degradation as much as possible compared to other existing sacrificing techniques. In brief, under deep anaesthesia (3% Isoflurane in 70% pressurized air and 30% O₂), the brains were frozen *in vivo* using liquid nitrogen. Using a funnel placed onto the skull, with the posterior rim at the lambdoidal suture, liquid nitrogen was poured continuously onto the skull for 3 minutes. Thereafter, the brain was dissected onto dry ice to prevent defrosting of the tissue. The excised and frozen brain was stored until further use at -80°C.

Using a cryostat microtome (Leica Microsystems) set at -21°C, 12 µm coronal sections (between -0.10 and +0.40 from Bregma) were cut. Consecutive brain sections (3 per brain) were randomly thaw-mounted (in < 3 seconds) onto indium tin oxide (ITO) coated glass slides (Delta Technologies). In total, 120 sections were analysed with MALDI-MSI. After MALDI-MSI analysis, matrix removal was performed using 70% EtOH where after sections were stained for Nissl substance with Cresyl Violet to enable analysis of the infarct core region.

SIMS-MSI

All TOF-SIMS analyses were performed using a PHI nanoTOF II instrument (Physical Electronics) with a 60 keV Bi₃²⁺ liquid metal ion gun for MS imaging experiments.³⁷ The analytical field-of-view (FOV) was in 400 µm × 400 µm with 512 pixels × 512 pixels, resulting in a 0.8 µm pixel size. The sample dose was in all cases at or below the static limit (1013 ions cm⁻²). The sample bias was set at 3 kV. The TOF-SIMS (MS¹) and tandem MS (MS²) data were collected simultaneously in positive ion polarity as described in Fisher *et al.*³⁹ The tandem MS experiments were performed using 1.5 keV CID with Argon gas estimated to be 1 × 10⁻³ Pa in the collision cell.³⁹ In the course of each acquisition, mass spectral information at each image pixel was collected in the m/z range of 0–3000, and saved into a raw data stream file in both positive and negative polarity.

MALDI-MSI

Prior to MALDI analysis 2,5-dihydroxybenzoic acid (DHB) and Norharmane matrix were applied to sections at 15 mg/mL and 7 mg/mL in 2:1 chloroform:methanol (v/v), respectively with a TM-sprayer (HTX technologies), using 12 passes at 40°C, 0.1 mL/min flow rate, 10 psi spray pressure, at 2-mm spray spacing, 1200 mm/min spray velocity, and with a 40-mm sprayer nozzle distance to sample. Consecutive sections were desalted with 50 mM ammonium acetate prior to applying matrix coating. All data was acquired on a Bruker Rapiflex MALDI Tisuetype™ system operating in reflectron mode (Bruker Daltonik GmbH) with a nominal acceleration potential of ±20 kV. MSI data was acquired using a 50 x 50 µm² raster and a 20 x 20 µm² beam scan area for each polarity with 200 laser shots accumulated at each pixel. The average acquisition rate was 23 pixels/second. Data was acquired in the m/z range of 400–1000 for positive and 600–1600 for negative ion mode. Mass calibration was performed before each analysis using red phosphorous clusters in positive and negative ion mode. The accurate mass and MS/MS profiles from the tissue sections were acquired on the 9.4 T Solarix FT-ICR (Bruker Daltonik GmbH) and the Synapt G2Si (Waters) in sensitivity mode using 30 eV collision energy.

Multivariate data analysis

For batch statistical analysis over SIMS-MSI, data was converted into a Matlab matrix by in-house developed software. Once the mean spectrum files were created they were subjected

to peak picking using the in-house developed Matlab PEAPI compiled program. The peak list was then integrated over the whole images and Matlab format files were created. The ion images were normalized and reconstructed using HisDistGUI software.

The 2D-datasets were processed using Scils (Bruker Daltonik GmbH) software. The data was TIC-normalised using weak de-noising deterministic installation for both polarities. Automatic peak-picking was performed using the default segmentation pipeline. After acquisition each section was imported into SCiLS 2016b. The data was subjected to the segmentation algorithm. Each individual tissue section was annotated and segmented into the three relevant regions of the infarct; core, penumbra and surrounding healthy tissue. The resulting sets of m/z values were used to perform pLSA. Ten components were calculated for the negative and seven for the positive ion mode. The components that were representing the matrix distribution were not considered for further evaluation. The generated components gave distinct distributions within the tissue and thereafter each spectrum was presented as a weighted combination of these components. The resulting components and spectra are shown in Figure 3. Box plots of selected m/z values were created in order to see the intensity variations within the selected ROIs of healthy, core and penumbra tissue ($n = 3$). The mean ratio of signal intensity between ischemic (core, penumbra) and healthy ROIs ($n = 3$) with the same number of spectra at 4 h, 8 h and 24 h was calculated and expressed as fold increase/decrease with 95% confidence intervals of prominent m/z values. After the components were calculated, the second set of analyses was performed in order to elucidate potential molecular differences between ischemia-relevant areas for wild-type and mutant mice separately. The segmentation algorithm differentiated specific ROIs in each data set. The selected data sets were converted from the Scils (.sl) format into Matlab matrix by an in-house programmed Python script. Once the mean spectrum files were created they were subjected to peak picking with the in-house Matlab PEAPI compiled program. The ROIs were created by the in-house Matlab Triftricks 1.52 software. On the whole collection of data sets we performed linear discriminant analysis (LDA) displaying different score histograms (Figure 6) and the corresponding loadings spectra (Supplementary Figures 2 and 3). The histograms are described within the first discriminating function (DF1) revealing the maximum difference within the groups selected. All of the data is described with 80% variance.

RESULTS

Linear transparency overlay, clustering pipeline and spatial segmentation reveal the penumbra and core in ischemic area

We used a histology-directed spatial segmentation approach (Figure 1), employed to segment sections into ipsi- (ischemic) and contra-lateral (healthy) sides. This technique was combined with manual linear transparency overlay. This approach allowed us to successfully co-register the selected m/z values in positive ion mode with the Nissl-stained sections, annotated with the necrotic border. All of the selected ions perfectly align within the necrotic border, separating the ischemic region from the rest of the tissue (Figure 1a). The software-derived spatial segmentation pipeline revealed a further segmentation of the positive ion mode data into ipsi-lateral sub-regions, *i.e.* core, penumbra and surrounding healthy tissue, which cannot be solely observed by histological staining. Prominent spatial regions are visualized already at 4 hours after MCAO, as can be observed in the example of Figure 1b.

Lipid profiles in core and penumbra in the acute phase of ischemic stroke

The segmented regions of the infarct were investigated by first manually drawing ROIs with the same number of pixels (Supplemental Figure 1a). In the positive ion mode mean spectra we observed an increase in relative intensities of $m/z = 756.6$ $[\text{PC}(32:0) + \text{Na}]^+$ and 810.6 $[\text{PC}(36:1) + \text{Na}]^+$, while $m/z = 772.6$ $[\text{PC}(32:0) + \text{K}]^+$ shows a decrease in the core region compared to surrounding penumbra and healthy tissue at 4 hours *post* MCAO. There are no relative intensity changes observed at $m/z = 760.6$ $[\text{PC}(32:0) + \text{H}]^+$ (Supplemental Figure 1b). The same pattern is observed when box plotting intensity variations of these ions ($n = 3$). In Supplemental Figure 1c we show a similar variation in the healthy (ipsi and contra) and penumbra. However, a slight increase of variation in the core region for $m/z = 756.6$ is also shown, while $m/z = 810.6$ (Supplemental Figure 1d) shows a gradual increase from healthy to the core. In contrast, $m/z = 772.6$ shows a larger decrease in the penumbra and core. The variation of signal intensity at $m/z = 760.6$ is constant throughout the ROIs. While investigating the linear transparency overlays with selected lipid signals in negative ion mode, a clear difference was observed between the core and penumbra for $m/z = 965.5$ and $m/z = 1045.5$. The selected molecular species, specifically $m/z = 965.5$, showed an increase in signal intensity (increasing over time), but notably only in the border zone surrounding the core (Figure 2). A decrease in signal intensity was observed in the infarct core at 4 hours that was more pronounced at 8 hours after the stroke induction. MS/MS analysis in negative ion mode identified $m/z = 965.5$ ion as $[\text{PIP}(38:4) - \text{H}]^-$ (phosphatidylinositol 4-phosphate) (Supplemental Figure 3) and $[\text{PIP}_2(38:4) - \text{H}]^-$ phosphatidylinositol 4,5-bisphosphate, ($m/z = 1045.5$) (Supplemental Figure 4). Additional MS/MS of $[\text{PI}(38:4) - \text{H}]^-$ (phosphatidylinositol) was taken for comparison (Supplemental Figure 2). The fragmentation spectra of all three PI and PIP species have been consistent with the previously observed data.^{17,18}

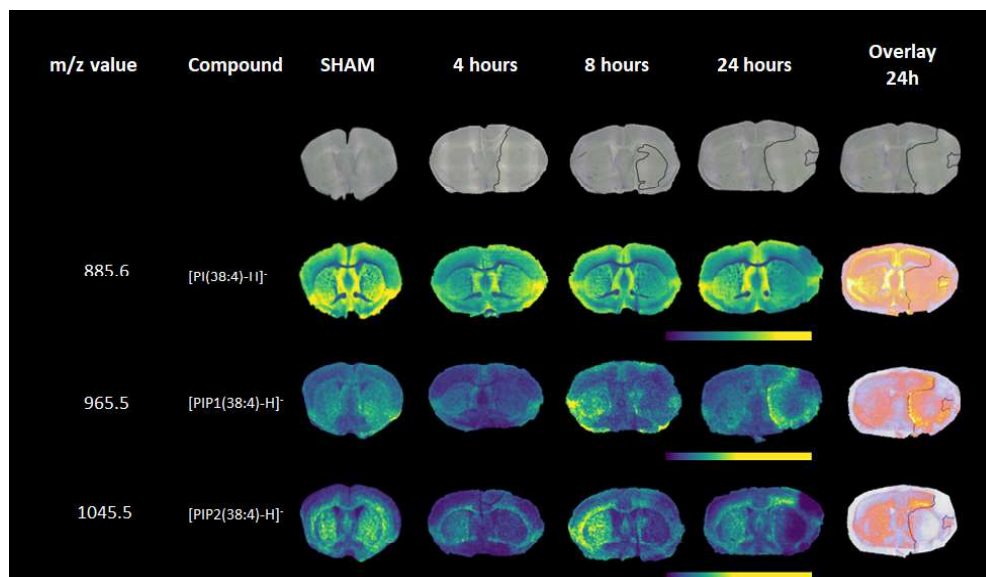


Figure 2. MALDI images showing the distribution of the PIP molecular family and accompanying Nissl stainings. Selected MALDI m/z images acquired at 50- μm spatial resolution showing the distribution of specific lipids $m/z = 885.6$ $[\text{PI}(38:4) - \text{H}]^-$, $m/z = 965.5$ $[\text{PIP}(38:4) - \text{H}]^-$, and $m/z = 1045.5$ $[\text{PIP}_2(38:4) - \text{H}]^-$ in a SHAM WT mouse and at 4, 8 and 24 hours after transient middle cerebral artery occlusion measured in negative ion mode. The last column represents the linear transparency overlays of $m/z = 885.6$ $[\text{PI}(38:4) - \text{H}]^-$, $m/z = 965.5$ $[\text{PIP}(38:4) - \text{H}]^-$, and $m/z = 1045.5$ $[\text{PIP}_2(38:4) - \text{H}]^-$ with Nissl staining at 24 hours after MCAO.

Lipid profiles in ischemic stroke evolution in core and penumbra

The same segmentation pipeline was used to investigate lipid profiles in the spatial regions across the three time points of stroke evolution. Probabilistic latent semantic analysis (pLSA) was used for the statistical analysis of data of the spatial regions (using the described spatial segmentation) (Figure 3). The data obtained for negative and positive ion modes was best described with 7 and 10 components. The components with the clearest difference between the ipsi- and contra-lateral side are shown in Figure 3. Components 4 and 9 exhibited the most marked difference in negative ion mode; components 1, 2 and 7 were most distinctive in positive ion mode. In negative ion mode the largest contributions to component 9 came from the $m/z = 885.6$ $[PI(38:4) - H]^-$, which is known to differentiate grey from white matter. White matter was predominantly characterized by $m/z = 834.6$ $PS(40:6)$ at (component 4). The negative mode ions that attributed most to the border zone of the ischemia are $m/z = 909.6$, 833.6 for component 4 and $m/z = 965.6$ $[PIP_2(38:4) - H]^-$, 1045.5 $[PIP_2(38:4) - H]^-$ for component 9. Changes in these compounds were visible already at 4 hours after MCAO and increased up to 24 hours. The box intensity plots (Supplemental Figure 5) for $[PIP_2(38:4) - H]^-$ show a larger variation of intensity at 4 hours and 8 hours in healthy ipsi- and contralateral tissue and a decrease in the penumbra and core. The biggest variation is observed at 24 hours in the penumbra. For $[PIP_2(38:4) - H]^-$ there is a bigger difference in relative intensities between the healthy tissue and between penumbra and core. At 24 hours the intensity variations in the penumbra become similar to the healthy tissue opposed to the core.

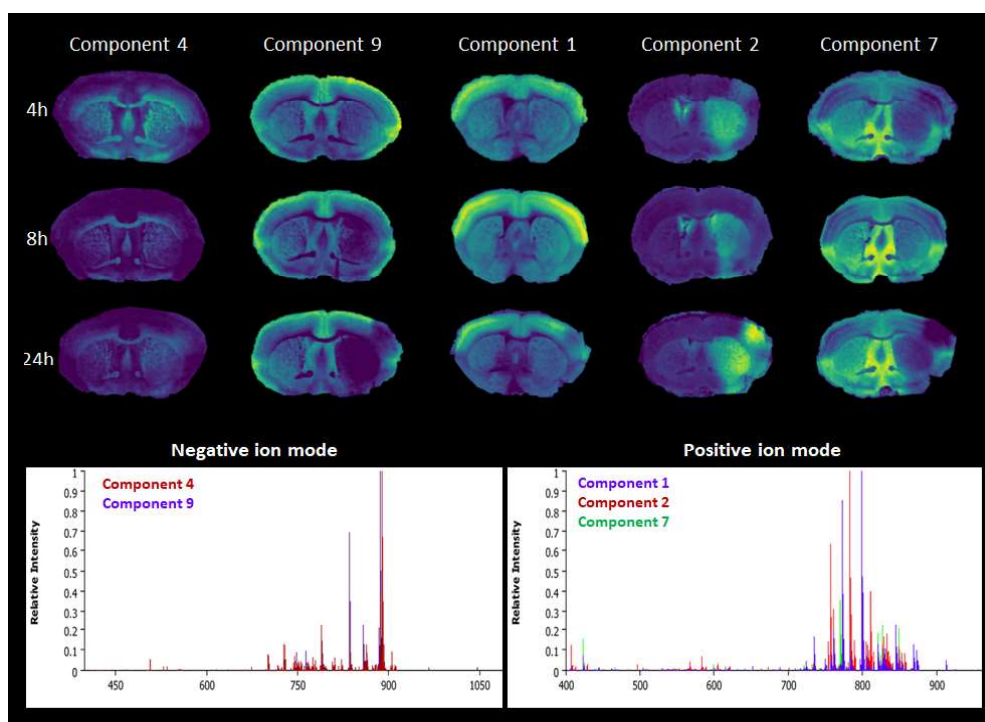


Figure 3. Probabilistic latent semantic analysis (pLSA) loading plots and spectra of resulting components of the WT mice in negative (components 4 and 9) and positive (components 1, 2 and 7) ion mode 24 hours after transient middle cerebral artery occlusion.

We also revealed the presence of cardiolipin (CL) lipids (found in the inner mitochondrial membrane and play a coordinating role in apoptotic cell death¹⁹) and ganglioside (GM) lipids (found in the outer layer of the plasma membrane and important in neuronal function and cell death^{7,20}), although no changes with respect to spatial or temporal distribution relevant to ischemic infarct evolution were observed (Supplemental Table 2).

In positive ion mode, components revealed a clearer differentiation between the ischemic and non-ischemic hemispheres. Specifically, within the ipsi-lateral hemisphere, components 2 and 7 showed molecular profiles that correlated with the penumbra and core of the ischemia. Lipid species, $m/z = 756.6$ [PC (32:0) + Na^+] and $m/z = 782.6$ [PC (34:1) + Na^+] show high abundancy in the ischemic area compared to the lipid profile in the non-ischemic hemisphere. An increase of lyso-lipid intensities in the ischemic area, mainly [LPC (16:0) + H^+] ($m/z = 496.4$) and [LPC (16:0) + Na^+] ($m/z = 518.5$) (Figure 4), can be induced by washing the tissue sections prior to MALDI analysis, which is in line with previous data⁴. The intensities of the LPC species seem to increase in the course of 24 hours (Figure 4). In respect to the images, similar tradelines are observed for box intensity plots ($n = 3$) (Supplemental Figure 5). The variation of intensities increase over the course of 24 hours for [LPC (16:0) + H^+] and [LPC (16:0) + Na^+] in the core. The same pattern is observed for the rest of the selected [PC+ Na^+] species in contrast to [PC (32:0) + K^+]. MALDI images of additional lipid species in positive ion mode (washed and unwashed) at 4, 8 and 24 hours after MCAO in WT mice can be found in Supplemental Table 1.

To quantify the relative changes of prominent lipid species, we calculated fold changes as the mean ratio of signal intensity between ischemic (core, penumbra) and healthy ROIs ($n = 3$)

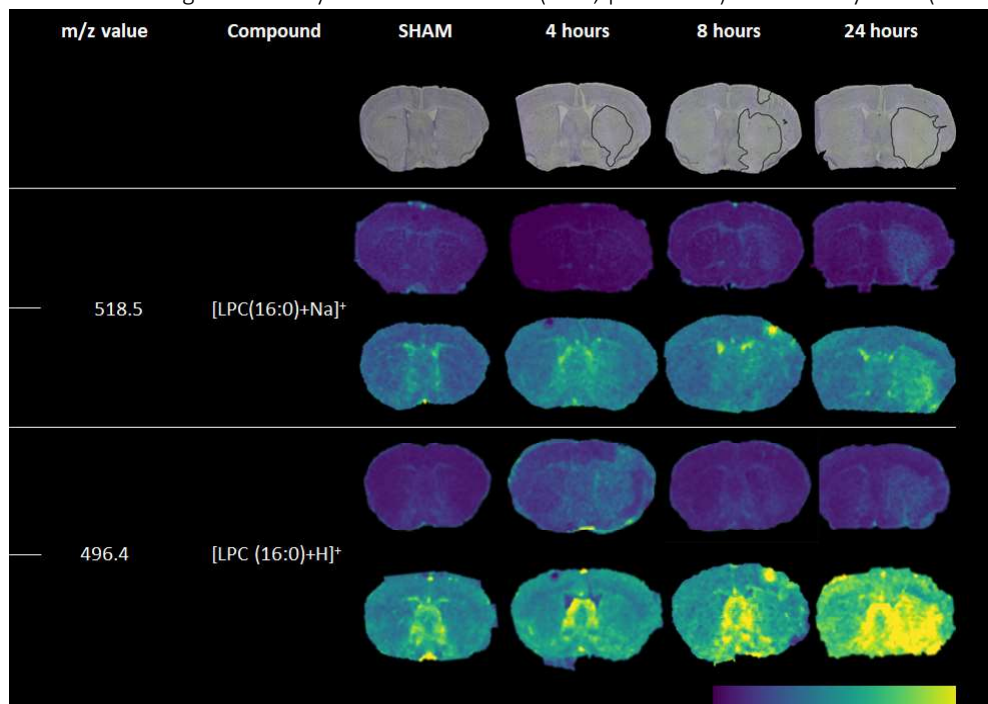


Figure 4. MALDI images showing the distribution of lyso-lipids and accompanying Nissl stainings. Selected MALDI m/z images acquired at 50- μm spatial resolution showing the distribution of lyso-lipids in a SHAM WT mouse and at 4, 8 and 24 hours after transient middle cerebral artery occlusion measured in positive ion mode. Each row of the selected species represents un-washed (top) and washed (bottom) tissue section.

at 4, 8 and 24 hours after MCAO (Supplemental Figure 7) with 95% confidence interval. Fold changes were expressed for 7a) [LPC (16:0) + H]⁺ m/z = 496.4, 7b) [LPC (16:0) + Na]⁺ m/z = 518.5, 7c) [PC(32:0) + Na]⁺ m/z = 756.6, 7d) [PC(32:0) + K]⁺ m/z = 772.6 in positive ion mode and 7e) [PIP (38:4) – H][–] and 7f) [PIP₂ (38:4) – H][–] in negative ion mode. Complementing previously observed data the largest increase of [LPC (16:0) + H]⁺ and [LPC (16:0) + Na]⁺ was observed in the core at 24 hours *post* MCAO. Interestingly, the washed tissue did not show a significant increase at 24 hours *post* MCAO for both selected species, though more pronounced in the MALDI ion images. [PC(32:0) + Na]⁺ and [PC(32:0) + K]⁺ show their respective fold increase/decrease in the core. The biggest difference is observed in the [PIP (38:4) – H][–] ion. At 4 hours and 8 hours there is a fold decrease in the penumbra, while at 24 hours there is a clear increase in the penumbra. Similarly, to the MALDI images there is a significant decrease of the [PIP₂ (38:4) – H][–] in the core and penumbra starting at 8 hours *post* MCAO.

Sodium/potassium ratio's in the early phase of stroke evolution

As the sodiated/potassiated species may change over the course of 24 hours after MCAO, we performed high-resolution Secondary Ion Mass Spectrometry (SIMS) analysis to evaluate the Na⁺/K⁺ ratio. Principal component analysis was performed to determine the distribution of Na⁺ (m/z = 23) and K⁺ (m/z = 39) species and related molecular species. PC4 loadings plot (Figure 5a) described with 0.293% variance reveals the distribution of species related with Na⁺ (positive loadings plot; green) and K⁺ (negative loadings; blue). The distribution of Na⁺ and K⁺ respective related species is shown in the RGB image score overlay of PC + 4 (green), PC – 4 (blue) and PC3 (red) (Figure 5c). SIMS images of specific lipids, such as m/z = 756.6 [PC(32:0)

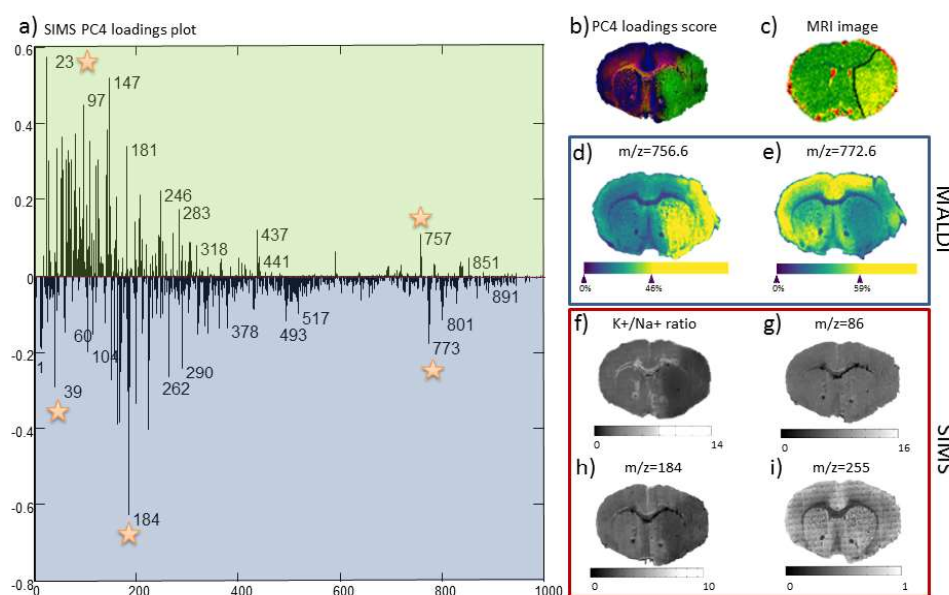


Figure 5. MRI, SIMS and MALDI images revealing the distribution of the infarct core in wild-type mice after transient middle cerebral artery occlusion. (A) PC4 loadings plot revealing the distribution of co-related species with Na⁺ (positive loadings plot-green) and K⁺ (negative loadings-blue), (B) RGB overlay of three PCs (red; PC-3, green; PC4, and blue; PC-4); (C) T2-weighted MRI image with delineated infarct border; MALDI image of (d) m/z = 756.6 [PC(32:0) + Na]⁺ and (e) m/z = 772.6 [PC(32:0) + K]⁺; (F) K⁺/Na⁺ ratio, (G) choline fragment, (H) phosphatidylcholine fragment in positive ion mode and (I) palmitic acid (16:0) m/z = 255, in ischemia 24 hours after transient middle middle cerebral artery occlusion.

+ Na]⁺ and $m/z = 772.6$ [PC(32:0) + K]⁺, show a distribution similar to the acquired MALDI images (Figure 5d and e) of consecutive sections. PC + 4 reveals a significant increase in Na⁺ and related species in the ischemic area opposed to the surrounding healthy tissue (Figure 5c). The distribution of K⁺ and related species shows a slight decrease in the ischemic region (Figure 5b), which is also observed when plotting the ratio between Na⁺ and K⁺ (Figure 5f). The distribution of choline ($m/z = 86$) and phosphatidylcholine fragments ($m/z = 184$) is shown in Figure 5g/h. Even though choline has a very homogeneous distribution across regions, the phosphatidylcholine fragments show a slight depletion in the ischemic core. In negative ion mode, fatty acids palmitic acid (16:0) $m/z = 255$, exhibit a homogeneous distribution across regions (Figure 5i), very similar to the choline fragment.

Lipid composition in a transgenic mouse model with increased stroke vulnerability

Next, we assessed lipid profiles in a transgenic mouse model in which a higher cerebral blood flow is required for tissue survival after MCAO, so the penumbra in these mutant mice is more easily converted to core tissue.²¹ In Figure 6 the histograms of measurements in positive ion mode are shown for the different brain regions of wild-type (WT) and FHM1 mutant mice at 4, 8 and 24 hours after MCAO. In FHM1 mutant mice a more pronounced discrimination between the penumbra and healthy tissue was observed compared to WT mice. Moreover, the largest change in distribution patterns is seen in the penumbra in mutant mice at all time points as well as compared to the WT mice (Figure 6). The scaled loadings (Supplemental Figure 4) show the contribution of each individual molecular mass to the differentiation observed in the histograms between the healthy, penumbra and ischemic regions. The loadings plots indicate that the major difference corresponds mainly to the [M + Na]⁺ and [M + K]⁺ ions of the same lipid species.

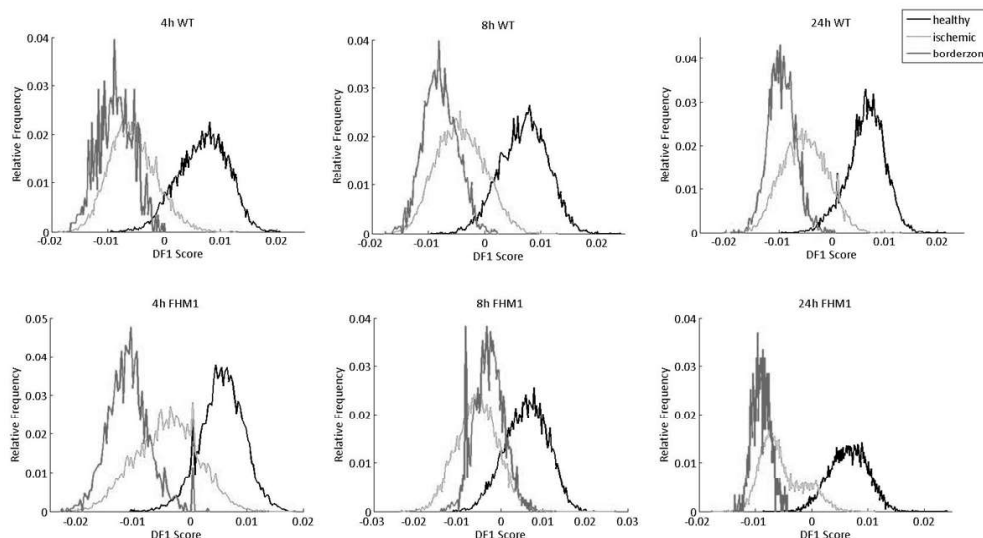


Figure 6. Linear discriminant analysis histograms of wild-type (top row) and transgenic FHM1 mice (bottom row) of segmented ROI areas (healthy (black), ischemic (light grey) and borderzone (dark grey)) at 4, 8 and 24 hours after transient middle cerebral artery occlusion.

DISCUSSION

High-resolution SIMS imaging combined with rapid lipid detection with MALDI-MSI is able to identify molecular profiles that can distinguish the ischemic and non-ischemic (healthy) regions after experimentally induced stroke. Data processing with linear transparency overlays and spatial segmentation made it possible not only to differentiate between ischemic and healthy mouse tissue already 4 hours after stroke induction, but also between infarct core and penumbra, which are not distinguishable at that time point with Nissl-stained images.

The most prominent difference between the ischemic and non-ischemic areas is governed by the Na^+/K^+ availability for the ionization of the molecular species. Lipid profiles revealed an extensive increase in Na^+ species, both from the penumbra to the core and during stroke development. The distribution of Na^+ and K^+ species shows a specific pattern: where Na^+ species are high, species of K^+ are low, and *vice versa*. This suggests that the intensity of sodiated “pseudo-molecular” ions, for example $[\text{M} + \text{Na}]^+$, is dependent on the level of sodium ions present in the tissue, (idem for potassium or other ions). A plausible explanation is the massive failure of sodium/potassium pumps within the ischemic area. This failure results in relative increase in sodium and decrease in potassium concentrations inside the cell due to passive diffusion influx of Na^+ and efflux of K^+ due to intracellular versus extracellular concentration and osmolarity differences.^{4,22} The relative high intracellular levels of Na^+ ions lead to the production of $[\text{M} + \text{Na}]^+$ pseudo-molecular ions during the ionization process. Combined with competition between K^+ and Na^+ ions for the same molecular species during the ionization, this will result in an apparent increase in detected $[\text{M} + \text{Na}]^+$ signal and an apparent reduction in the detected signals of $[\text{M} + \text{K}]^+$. This would imply that lipid species that are related to the Na^+/K^+ ratio should not be considered as reliable biomarkers when studying ischemia.

Overall, $[\text{PC} + \text{Na}]^+$ showed an increase in the ischemic core whereas $[\text{PC} + \text{K}]^+$ showed an overall decrease, a finding which is in line with previous data in rats.⁴ No clear changes in the ischemic or healthy tissue were found with respect to the PC $[\text{M} + \text{H}]^+$ concentration. The same was true for $[\text{LPC} (16:0) + \text{Na}]^+$ ($m/z = 518.5$), which was increased in the ischemic area, starting a few hours after MCAO, and visible as a difference between the various ischemic regions at 8 hours after MCAO (Figure 4 and Supplemental Figures 5 and 7b). These results confirm findings in a study in rat pups where sodium adduct of LPC (16:0) was present in the ischemic area but not in the healthy tissue at 24 and 48 hours after induction of the infarct.⁴ LPCs have a relatively short half-life and are formed, together with fatty acids, as metabolic intermediates, during the breakdown of other lipids. LPCs increase as part of a neuroinflammatory response; an increased activation of PLA_2 eventually results in increased neuronal damage.^{6,22} This could explain the observed decreased intensity of $m/z = 184$ fragment ion in the infarct core.

During neuronal cell death, cell membranes become more permeable, extracellular Na^+ and Ca^{2+} enter the cell and glutamate is released, causing activation of NMDA receptors. This triggers multiple cascades such as peri-infarct depolarizations spreading in the penumbra and activation of multiple phospholipases like PLA_2 resulting in increased amounts of phosphatidylinositol (PI) in the borderzone. Additionally, second messenger lipids are released (1,2-diacylglycerol, (Lyso-)PA, (Lyso-)PC+ Na^+). The latter, on its turn, activates PLA_2 and the destructive circle continues, turning the penumbra further into necrotic core.^{6,22}

A difference between the penumbra and the core region in the negative ion mode MALDI images was observed. Specifically, the distribution of $m/z = 965.5$ ion, identified as $[\text{PIP} (38:4) - \text{H}]^-$ showed an increased signal intensity in the penumbra surrounding the core area 24 hours after MCAO. The more abundant decline of PIP_2 compared to PIP in the infarct core

is in line with data of several animal models for global and focal ischemia.^{23–28} The signal transduction pathway involving hydrolysis of polyphosphoinositides (poly-PI) in the central nervous system is largely responsible for the control of the intracellular calcium homeostasis, as well as receptor activation via neurotransmitters, such as glutamate, acetylcholine, dopamine and serotonin.²⁸ Sustaining receptor-mediated Poly-IP signalling activity is highly energy-consuming, requiring several moles of ATP via PI and PIP kinases.²⁸ With the depletion of ATP during ischemia, PIP₂ high-energy dependent turnover from PIP might be abolished, which could be the reason for the increased PIP concentration compared to PIP₂ in the penumbra at 24 hours. Also, stimulation of the poly-PI pathway in the border zone might be part of an early event resulting in ischemia, which is also suggested by the rapid and transient increase of Ins(1,4,5)P after global cerebral ischemia.²⁸ These results suggest that the change in poly-PI levels is important for the fate of penumbra tissue. PIP and PIP₂ levels, therefore, may serve as a signature biomolecular pattern to identify whether penumbra tissue is about to become necrotic.

The molecular distribution of cardiolipin and ganglioside were also investigated, since they both play an important role in the apoptotic signalling pathway.^{7,19,20} CL has been shown to be affected by post-traumatic injury.^{7,29–31} Catabolism of cardiolipin occurs by the catalysis of phospholipase A₂ (PLA₂). However, CL distribution patterns do not seem to be changed in the ischemic core in the early time course after MCAO. One explanation could be that early after induction of ischemia (minutes-hours), necrosis has the upper hand, whereas apoptosis comes into play at a later stage of infarct development (hours-days).³² This time-dependant activation of different pathways leading to cell death has previous been shown in large detail.³³ GM, as part of the plasma membrane,³⁴ has been shown to ameliorate deficit after stroke.^{35,36} Here we did not find a clear difference in GM-accumulation or degradation in the ischemic core or penumbra compared to the healthy tissue. We speculate that this process is activated at a later stage of infarct evolution.

In ischemic tissue of the transgenic mutant, at 24 hours after MCAO, Na⁺ and K⁺ species seem to contribute more to the difference compared to WT mice. We hypothesize that this difference is due to a higher abundancy of sodium or potassium across the ischemic region in the mutant mice. For all three time points, especially at 24 hours after MCAO, the levels of sodiated species in the border zone are higher in mutant compared to WT mice.

In conclusion, the infarct evolution was clearly shown by 2D-renderings of multiple PC and LPC isoforms. The penumbra became clearly distinguishable from the core at the 8-hour time point after MCAO, as evidenced by differences in the levels of PIP and PIP₂. These lipids, therefore, could serve as early ischemic markers. Further research is needed to investigate whether or not increased levels of PIP and PIP₂ in combination with the ratio between both could indicate if the compromised penumbra will convert into core tissue or not.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

I.A.M., N.O.P., L.A.M.B., A.P. gathered all data, I.A.M. and N.O.P. analyzed all data, I.A.M. and N.O.P. wrote the manuscript, M.J.H.W., R.M.A. and A.M.J.M.M. supervised the project and reviewed the manuscript. All authors reviewed the manuscript

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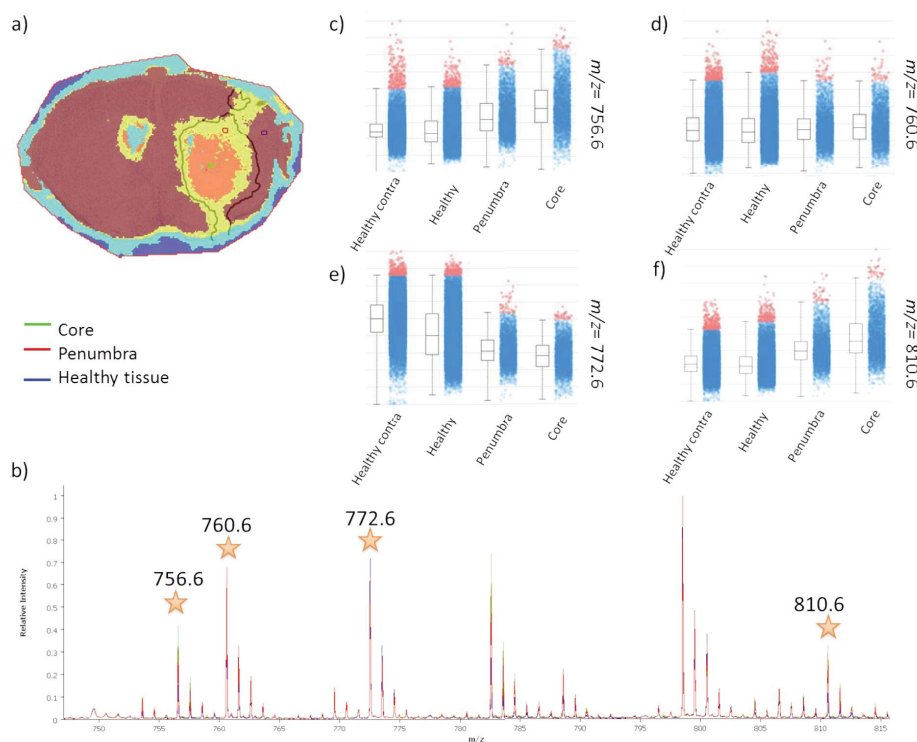
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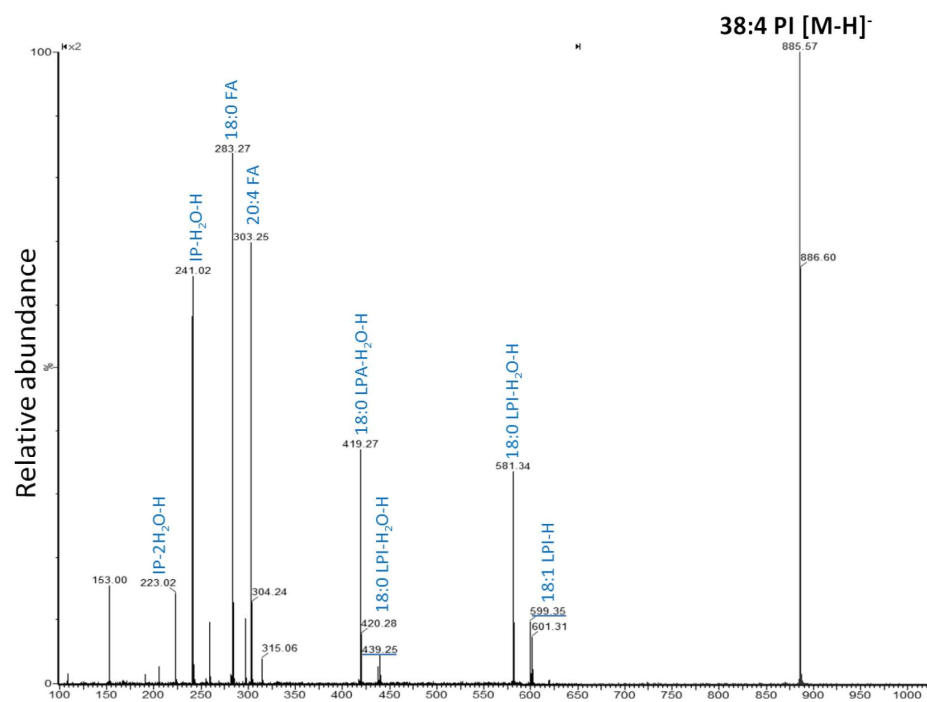
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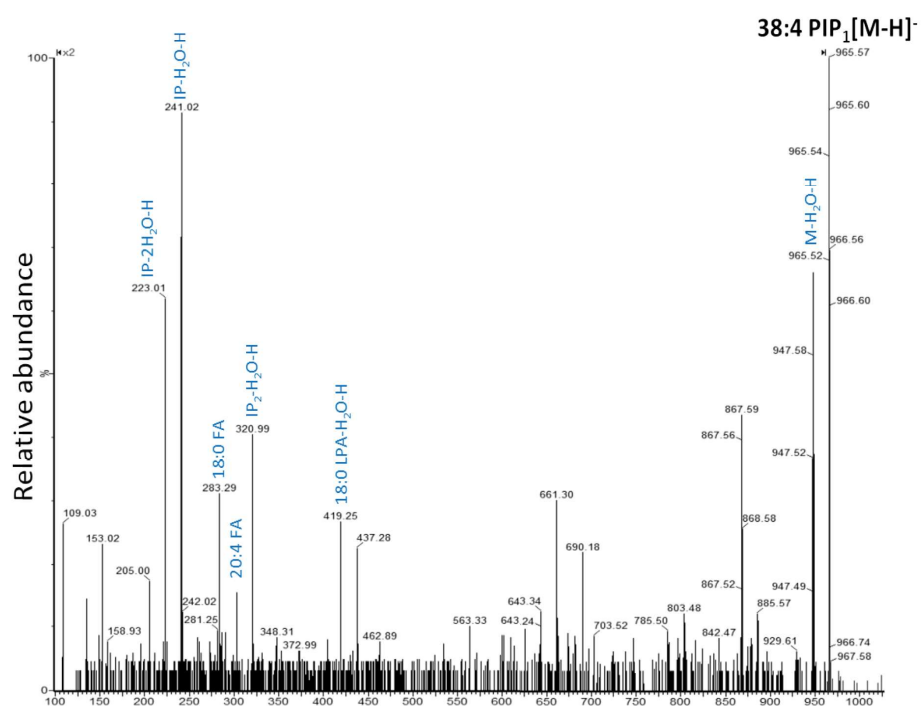
SUPPLEMENTAL MATERIAL



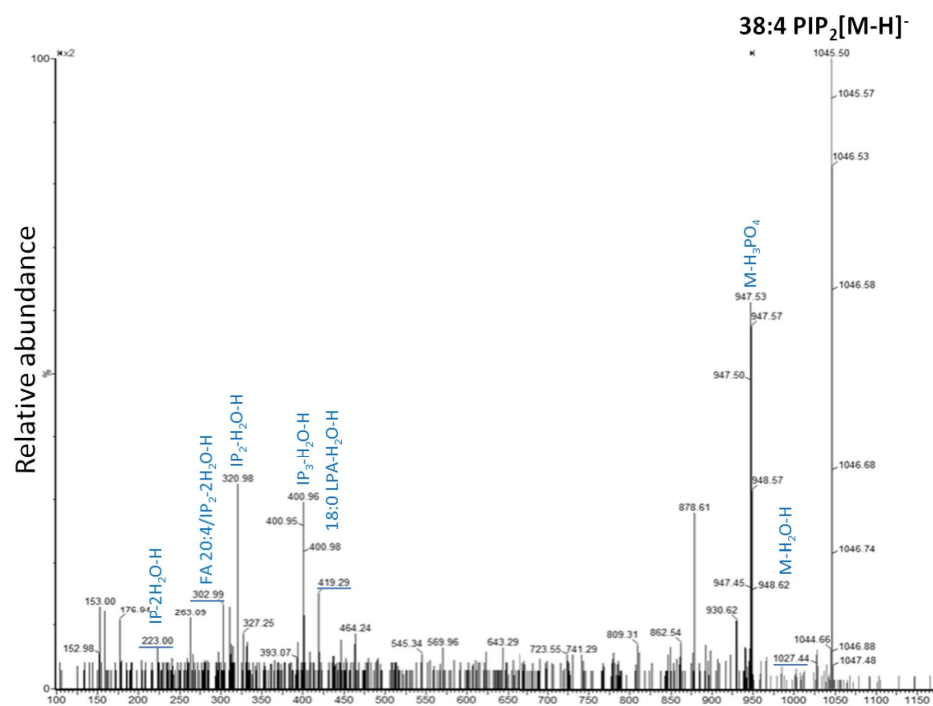
Supplemental Figure 1. Positive ion mode mean spectra of ROI regions selected from the segmented core (green), penumbra (red), and healthy tissue (blue) with the same average number of spectra. The starred $m/z=756.6$ [$\text{PC}(32:0)+\text{Na}$] $^+$, 760.6 [$\text{PC}(32:0)+\text{H}$] $^+$, 772.6 [$\text{PC}(36:0)+\text{K}$] $^+$ and 810.6 [$\text{PC}(36:1)+\text{Na}$] $^+$ show an increase/decrease or no changes in relative intensities in the core region. Their box intensity plots ($n=3$) C) $m/z=756.6$ [$\text{PC}(32:0)+\text{Na}$] $^+$, D) 760.6 [$\text{PC}(32:0)+\text{H}$] $^+$ E) 772.6 [$\text{PC}(36:0)+\text{K}$] $^+$ and F) 810.6 [$\text{PC}(36:1)+\text{Na}$] $^+$ show the intensity variations through the selected tissue areas: healthy contra, healthy, penumbra and core at 4 hours *post* MCAO.



Supplemental Figure 2. MS/MS negative ion mode spectrum of phosphatidylinositol 4-phosphate PI(38:4) [M-H]⁻ with precursor mass m/z=885.5 and associated annotated fragments.



Supplemental Figure 3. MS/MS negative ion mode spectrum spectrum of phosphatidylinositol 4-bisphosphate (PIP₁) (38:4) [M-H]⁻ with precursor mass m/z=965.5 and associated fragments.



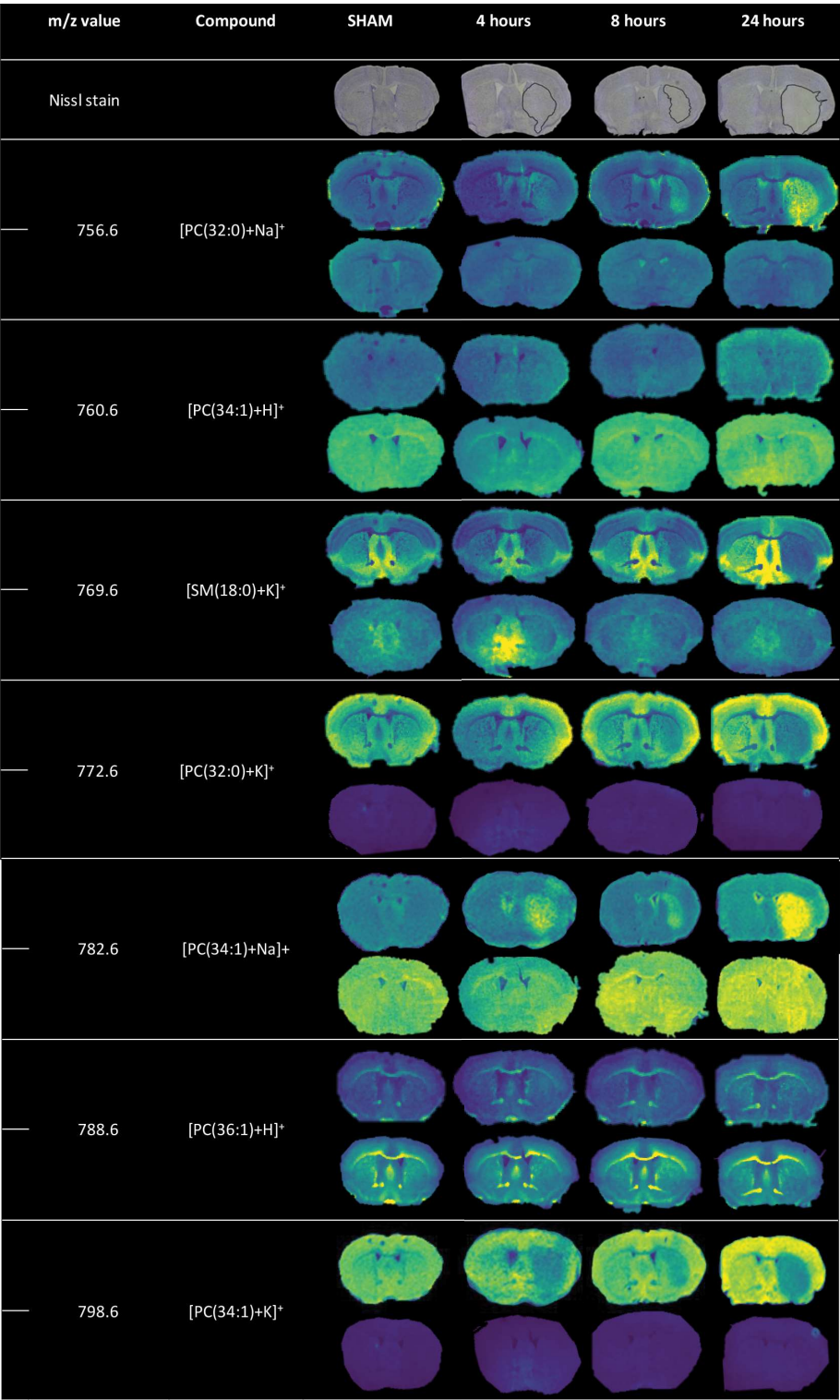
Supplemental Figure 4. MS/MS negative ion mode spectrum of phosphatidylinositol 4-5-bisphosphate (PIP₂) (38:4) [M-H]⁻ with precursor mass m/z=1045.5 and associated fragments.



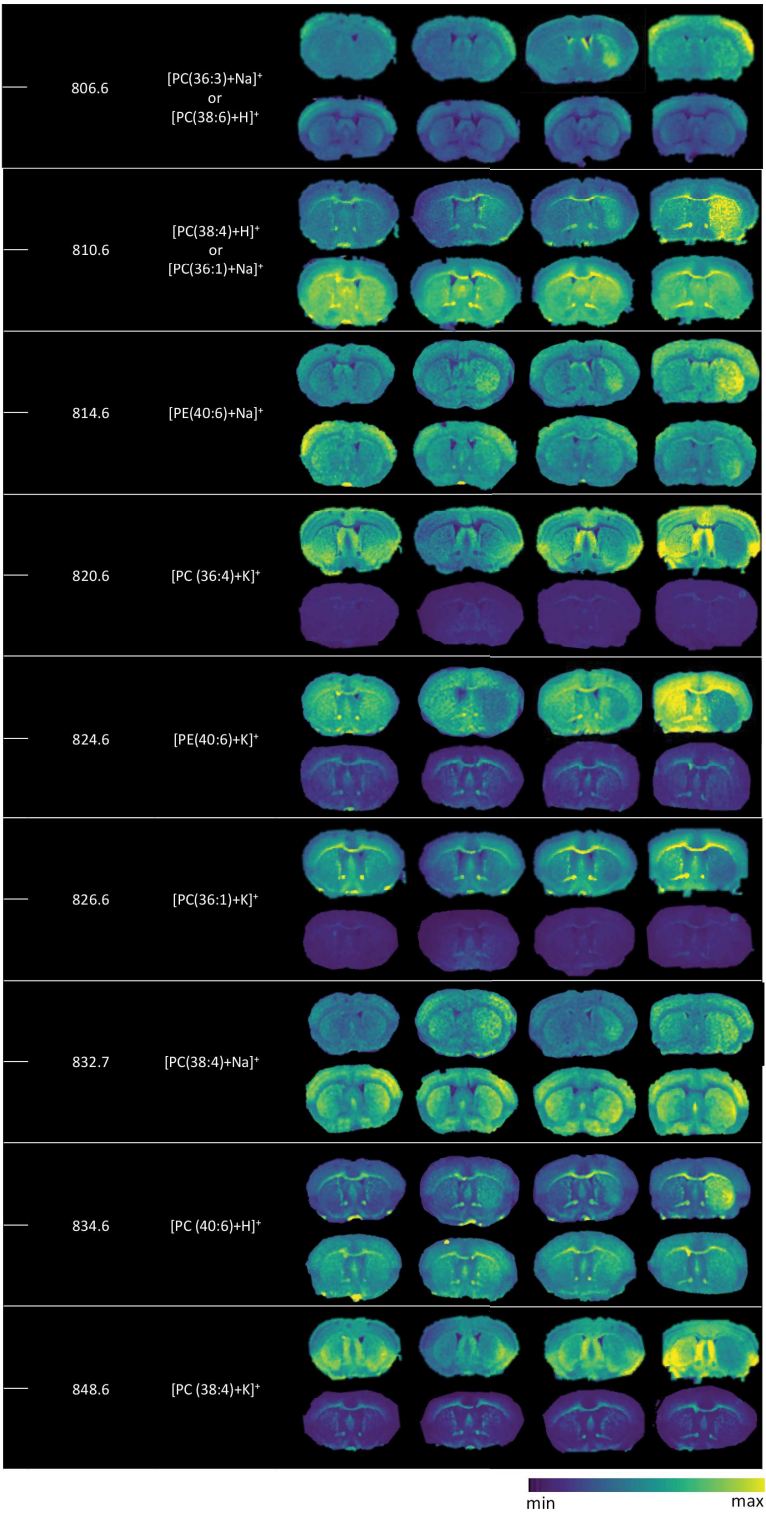
LIPID PROFILES IN CORE AND PENUMBRA



Supplemental Figure 5. Box plots of selected ions (LPC (16:0)+H⁺, LPC (16:0)+Na⁺, PC(32:0)+Na⁺, PC(32:0)+K⁺, PC(34:1)+Na⁺, PC(36:1)+Na⁺) of their intensity variations within the selected ROIs of healthy contra, healthy, core and penumbra tissue at 4 and 8 hours, and including sham at 24 hours in positive ion mode (n=3). Box plots of selected ions (PIP₁ (38:4)-H⁻ and PIP₂ (38:4)-H⁻) of their intensity variations within the selected ROIs of healthy contra, healthy, core and penumbra tissue at 4, 8, 24 hours in negative ion mode (n=2).

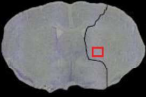
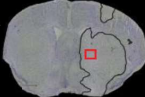
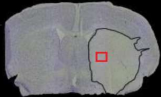
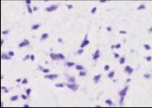
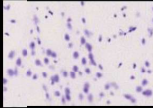
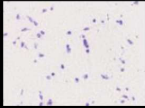
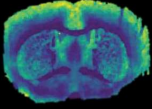
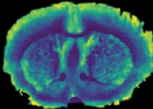
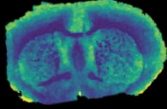
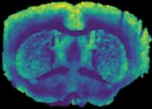
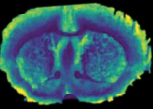
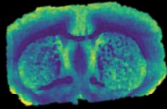
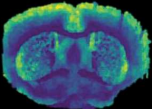
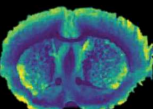
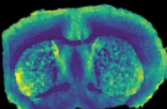
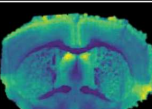
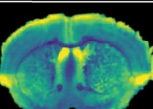
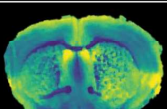


LIPID PROFILES IN CORE AND PENUMBRA

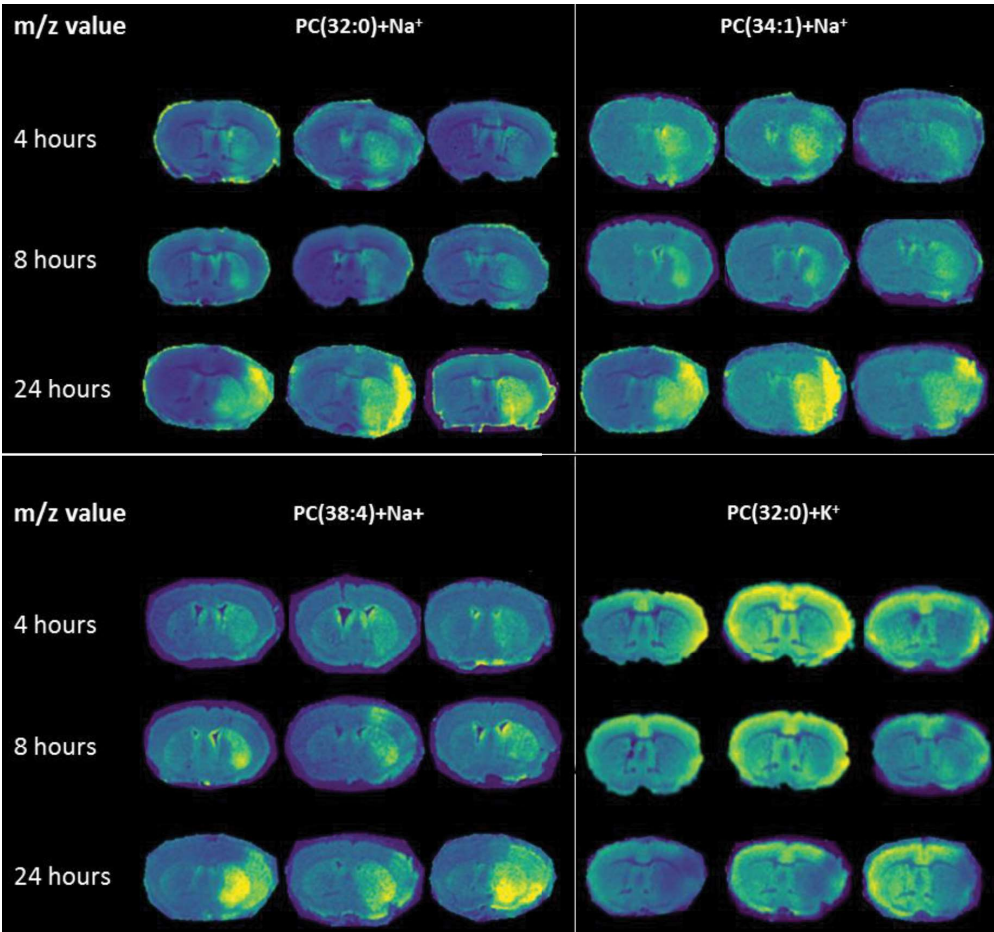


Supplemental Table 1. Washed and unwashed MALDI images in positive ion mode at 4, 8 and 24 hours in wildtype tissue after transient middle cerebral artery occlusion. Selected MALDI m/z images acquired at 50- μ m spatial resolution showing the distribution of selected lipid phosphatidylcholine (PC) species of washed (top row) and unwashed (bottom row) wild-type (WT) tissue sections at 4, 8 and 24 hours after transient middle cerebral artery occlusion in positive ion mode.

LIPID PROFILES IN CORE AND PENUMBRA

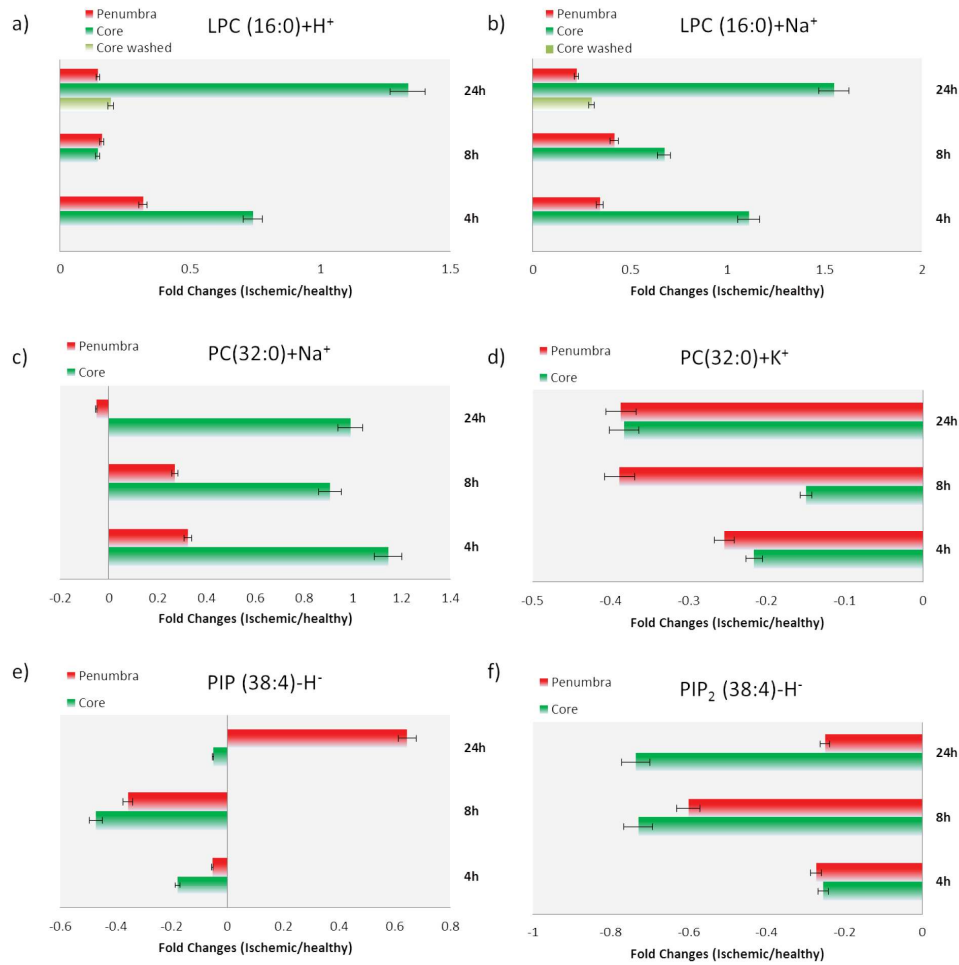
m/z value	Compound	4 hours	8 hours	24 hours
				
				
1452.3	CL(72:6) [M-H] ⁻			
1476.3	CL(74:8) [M-H] ⁻			
1497.3	CL(76:11) [M-H] ⁻			
1544.6	GM1(18:0) [M-H] ⁻			

Supplemental Table 2. Distribution of cardiolipin and ganglioside species in wild-type tissue sections. Selected MALDI m/z images acquired at 50- μ m spatial resolution showing the distribution of cardiolipin (CL) and ganglioside (GM) [M-H]⁻ species in wild-type tissue at 4, 8 and 24 hours after transient middle cerebral artery occlusion in negative ion mode.



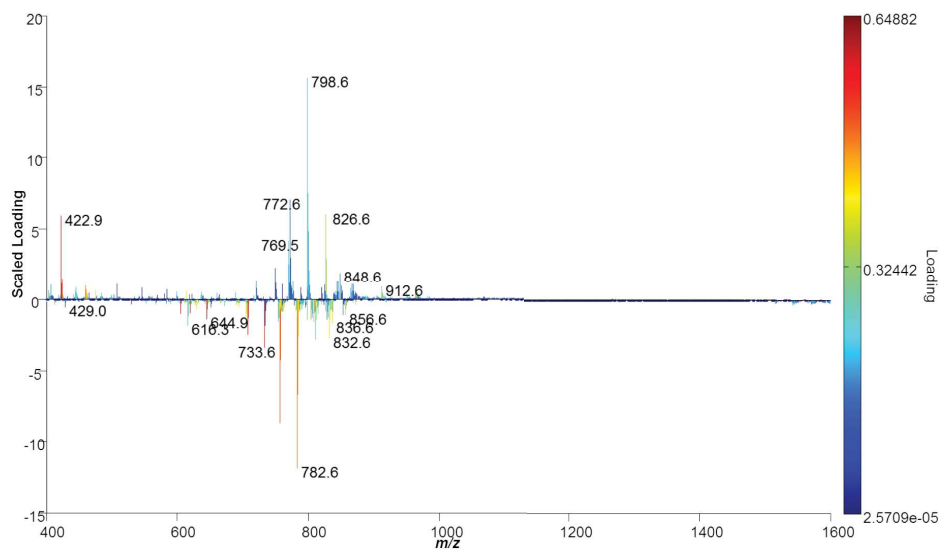
Supplemental Figure 6. Examples of biological replicas (n=3) of PC(32:0)+Na⁺, PC(34:1)+Na⁺, PC(38:0)+Na⁺, PC(32:0)+K⁺ at different timepoints (4, 8 and 24 hours) post MCAO.

LIPID PROFILES IN CORE AND PENUMBRA

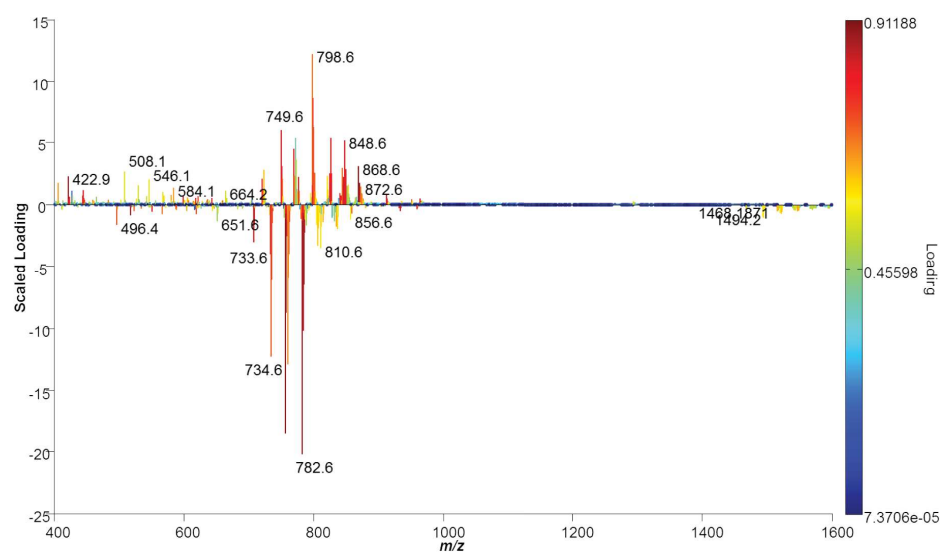


Supplemental Figure 7. Data represents the fold changes expressed as the mean ratio of signal intensity between ischemic (core, penumbra) and healthy ROIs (n=3) at 4, 8 and 24 hours after induced MCAO. Fold changes with 5% error were expressed for the following ions A) LPC (16:0)+H⁺ m/z=496.4, B) LPC (16:0)+Na⁺ m/z= 518.5, C) [PC(32:0)+Na]⁺ m/z= 756.6, D) [PC(32:0)+K]⁺ in positive ion mode and E) PIP₁ (38:4)-H⁻ and F) PIP₂ (38:4)-H⁻ in negative ion mode. All species showed statistical significant p<0.05.

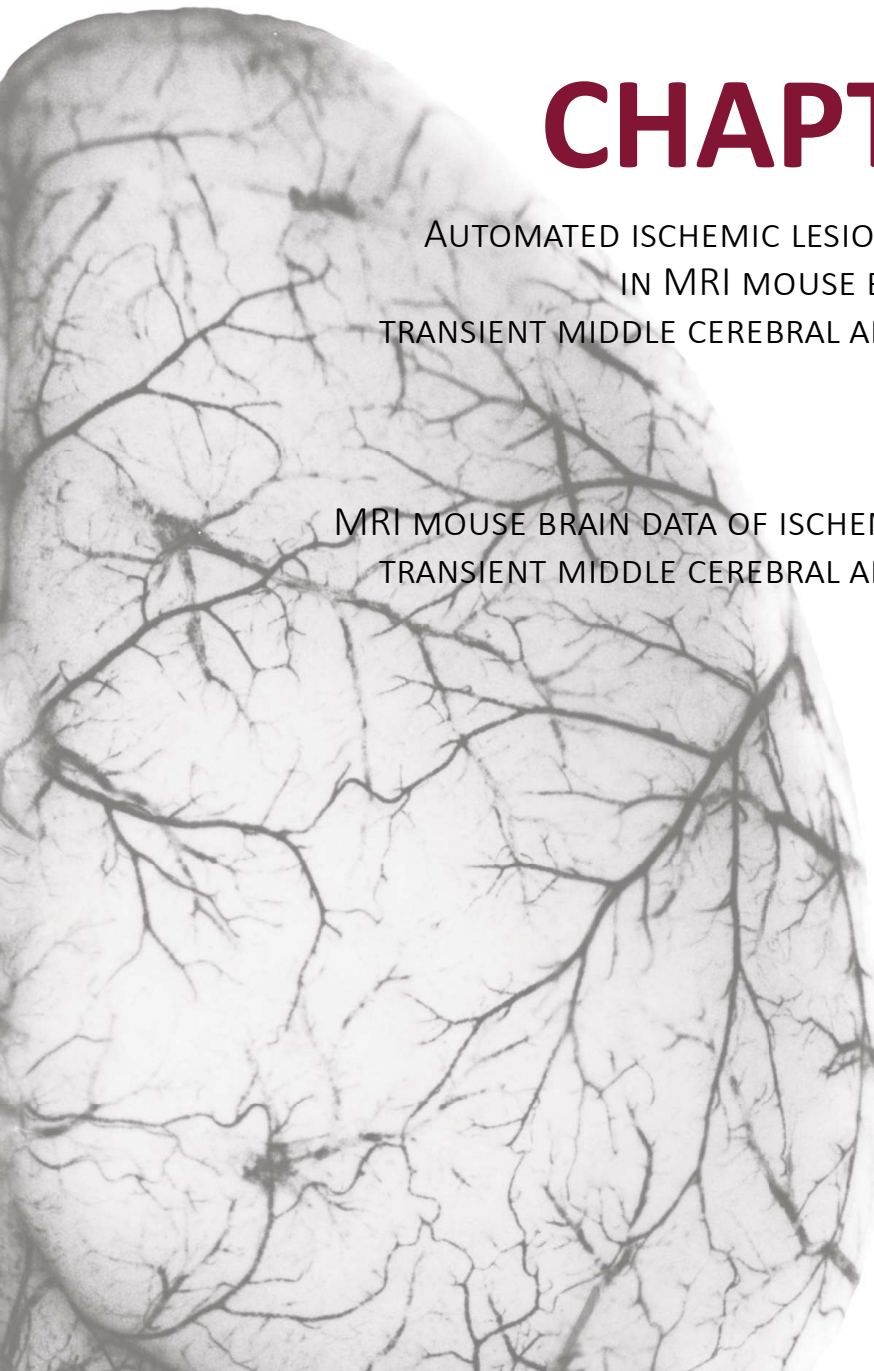
a)



b)



Supplemental Figure 8. Loadings spectra of the histograms at 24 hours of (A) wild-type and (B) FHM1 mutant tissue. The colour bars indicate the contribution of individual masses to the positive or negative loadings. High contribution in red and low contribution in dark blue.



CHAPTER 4

AUTOMATED ISCHEMIC LESION SEGMENTATION
IN MRI MOUSE BRAIN DATA AFTER
TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION

&

MRI MOUSE BRAIN DATA OF ISCHEMIC LESION AFTER
TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION

Mulder IA*, Khmelinskii A*, Dzyubachyk O*, de Jong S, Wermer MJH,
Hoehn M, Lelieveldt BPF and van den Maagdenberg AMJM

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**Authors contributed equally*

ABSTRACT

Magnetic resonance imaging (MRI) has become increasingly important in ischemic stroke experiments in mice, especially because it enables longitudinal studies. Still, quantitative analysis of MRI data remains challenging mainly because segmentation of mouse brain lesions in MRI data heavily relies on time-consuming manual tracing and thresholding techniques. Therefore, in the present study, a fully automated approach was developed to analyze longitudinal MRI data for quantification of ischemic lesion volume progression in the mouse brain. We present an level-set-based lesion segmentation algorithm that is built using a minimal set of assumptions and requires only one MRI sequence (T2) as input. To validate our algorithm we used a heterogeneous data set consisting of 121 mouse brain scans of various age groups and time points after infarct induction and obtained using different MRI hardware and acquisition parameters. We evaluated the volumetric accuracy and regional overlap of ischemic lesions segmented by our automated method against the ground truth obtained in a semi-automated fashion that includes a highly time-consuming manual correction step. Our method shows good agreement with human observations and is accurate on heterogeneous data, whilst requiring much shorter average execution time. The algorithm developed here was compiled into a toolbox and made publicly available, as well as all the data sets.

INTRODUCTION

In pre-clinical research on ischemic stroke, histological evaluation of brain infarct volume is accepted as the gold standard. However, errors introduced due to changes in brain morphology during processing of brain sections (swelling/shrinkage of tissue) combined with its manual-labor-intensive nature make it a suboptimal evaluation method. Moreover, animal sacrifice makes longitudinal studies and multiple readout times impossible, or, alternatively, will considerably increase the number of animals used in case parallel groups are investigated. Hence, magnetic resonance imaging (MRI) has become increasingly important to assess infarct volume development in animal experiments. With MRI, ischemic stroke can be analyzed in the first few days after induction using spin-spin relaxation time contrast T2,¹ which is sensitive to vasogenic edema.² Using MRI, infarct characteristics such as infarct volume and progression can be examined in a longitudinal manner.³⁻⁵ Efficient use of computational image processing methods allows for automated reproducible quantitative analysis of such data.

In clinical research, several algorithms for detection, segmentation and classification of different brain abnormalities (brain tumors, trauma lesions, hematoma, edemas, Alzheimer's disease) in both MRI and CT have been developed.⁶ However, segmentation of brain lesion in MRI animal (especially mouse) imaging data still heavily relies on manual tracing and semi-automated thresholding techniques,⁷⁻¹⁴ making the analysis time-consuming, with low inter- and intra-observer reproducibility.^{10,15,16} Also, there is considerable variability between research centers with respect to the methods used to calculate lesion volume using MRI,¹⁷ which can result in unjustified conclusions.

When it comes to ischemic brain tissue segmentation of pre-clinical data, only a few (semi) automated algorithms have been developed. These algorithms typically use data from multiparametric MR imaging, more specifically by combining the apparent diffusion coefficient (ADC) maps with T1, T2, T1- and T2-weighted images to distinguish and characterize healthy and ischemic brain tissues.^{3,4,18-21} Ghosh *et al.*^{6,15,22} developed an automated method — Hierarchical Region Splitting (HRS) — where adaptive thresholds are automatically selected to detect, quantify and distinguish between core and penumbral tissue regions in T2-weighted MRI data of HII Sprague-Dawley rats. Jacobs *et al.*^{1,23-26} developed an unsupervised segmentation algorithm based on K-means clustering — iterative self-organizing data analysis technique (ISODATA) — for analysis of multiparametric MRI data of focal cerebral ischemia in Wistar rats that was validated with histology data. Ding *et al.*^{27,28} applied a modified version of the same method to analyze embolic stroke rat data. A comprehensive overview of all available image analysis methods for ischemic stroke lesion and a discussion of their pros and cons was provided by Rekik *et al.*¹⁰

Most of the aforementioned methods were developed for rats, are age- and disease-specific and require manual intervention. The only two automated segmentation methods developed for pre-clinical data — HRS^{6,15,22} and ISODATA^{1,23-26} — obtained promising results but specifically focused on neonatal and adult rat and were validated on relatively small data sets. Moreover, current pre-clinical stroke studies are compromised by reproducibility issues.^{29,30} Having a reproducible automated method available might be of particular value when considering published guidelines for reporting animal research studies.³¹ To the best of our knowledge, there are no automated methods that have been developed to segment ischemic lesions in MRI mouse brain data.

Thus, the goal of this study was to develop an automated approach to quantify ischemic lesion volumes in MRI data of mouse brains and to make it publicly available. Our algorithm is built upon existing segmentation paradigm (level sets) with a minimal set of assumptions and

requires only one MRI sequence (T2) as input. We validated our algorithm on heterogeneous data consisting of 121 mouse brain scans, that covered various age groups, time points after infarct induction and were obtained with different MRI hardware and acquisition parameters. Performance of our automated approach was compared against the ground truth obtained in a semi-automated fashion by two observers, evaluating the volumetric accuracy and regional overlap of segmented ischemic lesions. Our approach showed good agreement with results obtained by the human observers and high accuracy on heterogeneous data, whilst having much shorter execution times. Thus, it has considerable potential in replacing the biased manual labor in quantifying ischemic lesion volume in mouse brain MRI data and might be of value for MRI stroke volume analysis in a multicenter setting.

MATERIALS AND METHODS

Animals

Our computational approach was tested on three main groups of male mice that were labeled as “*Leiden-Set*”, “*Cologne-Set-1*” and “*Cologne-Set-2*” depending on the origin of the data and data acquisition protocol.

- *Leiden-Set*: C57BL/6J mice ($n = 65$) were further subdivided into three age groups (3- to 5-, 12- to 14- and 20- to 24-month old). Mice were repeatedly scanned at different time points: 4h, 24h, 48h and 8d after infarct induction (see Section “Experimental Infarct Model”). Histological specimens were obtained on a small subset of animals ($n = 6$) and used for visual validation of the infarct area.
- *Cologne-Set-1*: C57BL/6J mice ($n = 6$; 3-month-old) were scanned at 18h and 4d after infarct induction.
- *Cologne-Set-2*: Transgenic mice expressing luciferase under double cortin control (DCX-Luc³²) ($n = 10$; 2- and 12-month-old) were scanned at 48h after infarct induction.

The group of 3- to 5-month-old mice from the *Leiden-Set*, 24h after stroke induction, was used as a primary validation cohort as it was the largest group (Table 1) and because the

	Data sets													
	Leiden-Set C57BL/6J									Cologne-Set-1 C57BL/6J			Cologne-Set-2 DCX-Luc	
	3-5				12-14			20-24			3		2	12
Age (months)														
Infarct induction	4 h	24 h	48 h	8 d	4 h	24 h	48 h	4 h	24 h	48 h	18 h	4 d	48 h	
N	11	36	8	6	7	9	8	5	7	5	6	3	5	5
MRI ACQUISITION PARAMETERS														
Magnet strength (T)					7						11.7		11.7	
Repetition time (ms)					4000						5000		4500	
Echo time (ms)					9						10.25		10	
Number of echoes					20						16		16	
Number of averages					2						1		1	
Field of view (mm ²)					15 × 15						14 × 14		20 × 20	
Matrix					128 × 128						128 × 128		196 × 196	
Number of slices					16						10		10	12
Slice thickness (mm)					0.50						0.80		0.60	
Voxel size (mm ³)					0.12 × 0.12 × 0.50						0.11 × 0.11 × 0.80		0.10 × 0.10 × 0.60	
Inter-slice gap (mm)					no gap						no gap		no gap	
Bandwidth (Hz)					59,523.8						50,000		75,000	
Acquisition time					12 min 48 s						10 min 40 s		9 min 18 s	

Table 1. Overview of all the used mice and accompanying MRI data sets: origin/acquisition protocol, number, age, timepoints after experimental infarct induction.

24h time point is often chosen in cerebral infarction experiments. For better assessment of the performance of our method on different infarct shapes and volumes, we subdivided this cohort into striatal (Str) and corticostriatal (Ctx+Str) lesions.

Animals were housed with littermates, in a temperature controlled environment, with food and water *ad libitum*. All animal experiments performed at the Leiden University Medical Center (LUMC) were approved by the local committee for animal health, ethics and research of LUMC. All animal experiments conducted at the Max Plank Institute for Metabolism Research in Cologne were performed in accordance with the German Animal Welfare Act and approved by the local authorities (Landesamt für Naturschutz, Umwelt und Verbraucherschutz NRW).

Experimental Infarct Model

Infarcts were induced using a modified transient middle cerebral artery occlusion (MCAO) model first described by Longa *et al.*³³ Mice were anesthetized using isoflurane (3% induction, 1.5% maintenance) in 70% pressurized air and 30% O₂. Painkiller carprofen (5 mg/kg, *s.c.*; Carporal, 50 mg/mL, AST Farma BV, Oudewater, the Netherlands) was given before surgery. During surgery, the mouse body temperature was maintained at 37°C using a rectal probe and feedback system. During the surgical procedure, a silicone-coated nylon monofilament (7017PK5Re, Doccol Company, Redlands, CA, USA) was inserted into the right common carotid artery and advanced via the internal carotid artery and circle of Willis to eventually block the middle cerebral artery (MCA) at its origin (decreasing blood flow substantially in the MCA territory, in the right hemisphere) and the skin was sutured. During the occlusion period, the mouse was allowed to wake up in a temperature-controlled incubator (V1200; Peco Services Ltd, Brough, UK). After 30 min of occlusion, the mouse was re-anesthetized in order to remove the suture and withdraw the monofilament to allow reperfusion. After surgery, the animal was allowed to recover for 2h in the incubator to maintain body temperature at 37°C, with easy access to food and water. At the end of the experiment, brains were fixated by transcardially perfusing the mice using fresh cold 4% buffered PFA (Paraformaldehyde P6148; Sigma-Aldrich Co. LLC, Saint Louis, MO, USA). Brains were collected, processed and sectioned. Sections were stained with Nissl (Cresyl Violet, Merck Millipore, Billerica, MA, USA) using standard protocol.

Magnetic Resonance Imaging

Scans were acquired with small-animal Bruker MRI systems using a Multi-Slice Multi-Echo sequence protocol at different time points after infarct induction. Animals from the Leiden-Set were scanned at 7T (Pharmascan, Bruker BioSpin, Ettlingen, Germany), whilst animals from the Cologne-Sets were scanned at 11.7T (Biospec 11.7T/16, Bruker BioSpin). Quantitative T2 maps were calculated from the multi-echo trains using Paravision 5.1 software (Bruker Pharmascan) for the Leiden-Set and IDL software for the Cologne-Sets. Table 1 shows a complete overview of all 121 scans, together with a summary of the main imaging acquisition parameters.

Ischemic Lesion Segmentation Challenges

The ischemic lesion is characterized by elevated T2 values with respect to the healthy brain tissue. However, it is not possible to segment the ischemic lesion with a simple threshold as several other objects, like ventricles, structures surrounding the brain and other regions in the periventricular zone of the brain, share similar T2 values to that of the infarct lesion. Also, both the ventricles and the stroke regions vary considerably in shape and size between different subjects and different time points after infarct induction, and in many cases the

stroke region engulfs the ventricles making the segmentation task even more difficult. Manual delineation of the ischemic lesion by experts takes into account all this information, together with possible stroke density differences and contiguity of the lesion area throughout the MRI image stack.

Semi-Automated Ischemic Lesion Segmentation

Semi-automated segmentation was performed in a slice-by-slice manner by two trained observers (IM and SdJ) using freely available ImageJ software (<https://imagej.nih.gov/ij/>); see Figure 1.

M1. Threshold determination

The threshold, the same for both observers, was determined as the mean plus two standard deviations of a vector containing average T2 values within a ROI in the contralateral hemisphere for every group of animals (concerning time point, age and data set origin) (Figure 1M1).

M2. Threshold mask

A mask was compiled of all high-intensity pixels above the threshold value (Figure 1M2).

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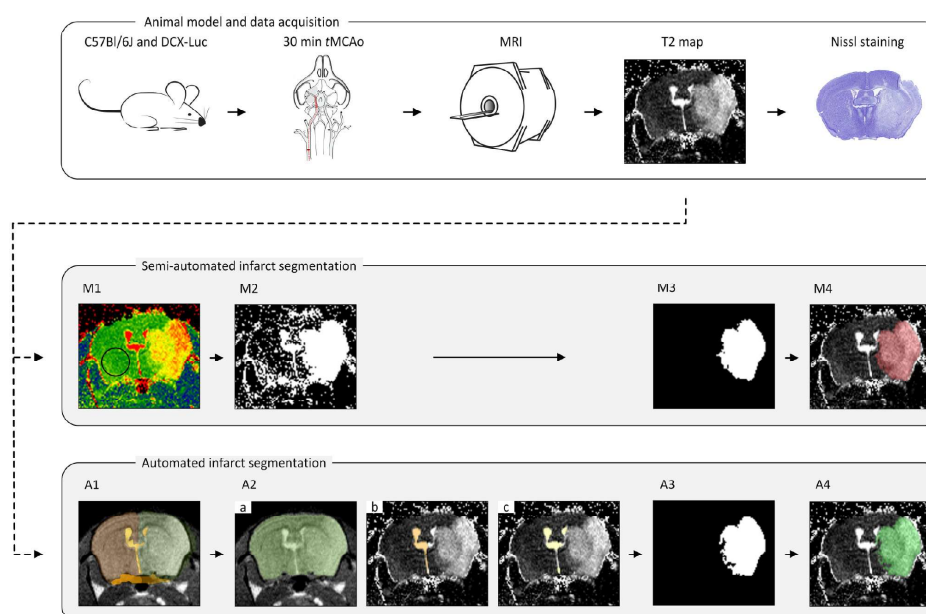


Figure 1. The pipeline for the animal model, data acquisition and lesion segmentation. The transient middle cerebral artery occlusion (MCAO) model was used for infarct induction in mice of different age groups. A Multi-Slice Multi-Echo MRI scan was acquired for determination of quantitative T2 maps at multiple time points ($T = 4h, 18h, 24h, 48h, 4d$ and $8d$) after MCAO. A subset of infarcted brains was used for histological validation. All MRI scans were analyzed in a semi-automated fashion by two observers (IM and SdJ) using threshold determination (M1), threshold mask (M2), manual exclusion of non-infarct-related areas (M3) and lesion volume determination (M4). Automated lesion segmentation was performed using image registration (A1), brain segmentation (A2.a), contralateral ventricle segmentation (A2.b), ventricles segmentation (A2.c), infarct ROI segmentation (A3) and infarct ROI volume quantification (A4).

M3. Infarct ROI segmentation

Confounding regions (ventricles and other non-infarct-related high-intensity areas) were manually removed by both observers individually (thereby highly relying on anatomical knowledge, leftright symmetry, density and experience), resulting in the delineated infarct lesion (Figure 1M3).

M4. Infarct ROI volume quantification

For each observer, the number of voxels located inside the ROIs representing the infarcted area of each MRI slice was multiplied by the voxel size to obtain the total infarct lesion volume (Figure 1M4).

Automated Ischemic Lesion Segmentation

The infarct lesion was segmented on the T2 map based on its elevated T2 values with respect to the healthy brain tissue. However, as mentioned above, several other objects appearing on the T2 map share similar T2 values to that of the infarct lesion. To discriminate the infarcted area from other brain structures and to quantify the infarct lesion volume, we developed a full automated approach that integrates a series of image processing steps depicted in Figure 1. Our approach is based on two general assumptions that: (I) T2 value distribution of the infarct lesion differs from that of the healthy tissue; and (II) only the ipsilateral hemisphere is affected by the infarct, whereas the contralateral hemisphere remains unaffected. Thus, we use the latter to learn the T2 value distributions of the ventricles and of the healthy brain tissue and apply this information to segment the infarcted area. All segmentations were performed on 3D image volumes using a modification of the region-based level sets method of Chan and Vese.³⁴ The parameters of our method (listed in Table 2) were optimized on the *Leiden-Set* and fixed for all three data sets during validation. The remainder of this section provides a detailed description of each particular step.

Image Registration

In this step, each brain scan was registered to a template brain consisting of a number of manually drawn labels: whole-brain (M_{WB}), ipsilateral hemisphere (M_{IBH}), contralateral hemisphere (M_{CBH}), ipsilateral ventricle (M_{IV}), contralateral ventricle (M_{CV}) and periventricular zone (M_{PVZ}); see Figure 1A₁. The template labels were propagated to each subject and used to initialize the subsequent steps. In the following, by referring to a region being occupied by a certain label we mean the result of the label propagation.

	Whole brain	Contralateral ventricle	Ventricles	Stroke
$I(\mathbf{x})$	4th echo	T2 map	T2 map	T2 map
$P(I(\mathbf{x}); \Omega_O)$	Gaussian	Gaussian	Histogram; fixed	Histogram
$P(I(\mathbf{x}); \Omega_B)$	Gaussian	Gaussian	Histogram	Histogram; fixed
Ω	$\mathbb{R}^3$	R_{CBH}	R_{WB}	$R_{IBH} \setminus (R_V \cup M_{PVZ})$
$\phi_0(\mathbf{x})$	M_{WB}	M_{CV}	M_V	R_s^{init}
α	5	0.1	2	0.3
μ	2	0	1.5	0
n_{iter}	80	120	120	80
$g(\mathbf{x})$	—	—	+	—

Table 2. Parameters of the level-set-based segmentation algorithm for different segmentation steps.

For each subject, the sum of all its echo images was used to register the scan of that particular subject to the reference brain scan. Consequently, the template labels were propagated to the individual data sets using the information provided by the deformation field for each subject-to-reference registration. The labels were used to initialize the segmentation of the whole brain and the ventricles as described in the next section. The quality and success of the registration was visually inspected by three independent observers (AK, OD and IM). Registration was performed in a coarse-to-fine fashion. Initially, rigid registration was performed to compensate for translation and rotation. Afterwards, affine registration was conducted to compensate for differences in brain size. Because large deformations occur in stroke brains, a non-rigid B-spline registration was necessary to compensate for the large local changes (especially in the ipsilateral hemisphere and the ventricles region). A Gaussian image pyramid was employed in all registration steps, applying four resolutions for the rigid and two for the affine and B-spline registrations each. Normalized Correlation Coefficient was used as a similarity metric.

Segmentation

For each particular segmentation, we used a level set function $\phi(\mathbf{x})$ to partition the image space Ω into two classes, further referred to as “object” ($\Omega_o = \{\mathbf{x} \in \Omega : \phi(\mathbf{x}) \geq 0\}$) and “background” ($\Omega_b = \{\mathbf{x} \in \Omega : \phi(\mathbf{x}) < 0\}$), respectively. Here $\mathbf{x} \in \Omega \subset \mathbf{R}^3$ are the Cartesian coordinates. The level set function $\phi(\mathbf{x})$ was evolved from its initial state $\phi_0(\mathbf{x})$ for the predefined number of iterations niter or till the stopping criterion was reached. The energy functional $E(\phi)$ contains both image-based and regularization-based terms and, in its most general form, is given by the following equation.³⁵

$$E(\phi) = \alpha \cdot g(\mathbf{x}) \cdot \text{Length}(\partial\Omega_o) + \mu \cdot \text{Area}(\Omega_o) - \log P(I(\mathbf{x}); \Omega_o) - \log P(I(\mathbf{x}); \Omega_b)$$

Here $g(\mathbf{x})$ is the gradient map³⁶ that can be optionally used to drive the segmentation toward the boundaries of the structures of interest, and α and μ are weights. Parameter values for different segmentation steps described in the remainder of this section are summarized in Table 2.

Data preprocessing

After the label propagation was completed, each scan was cropped to the rectangle containing the volume of interest. The cropping rectangle was defined as the smallest rectangle that contains the brain mask M_{wb} obtained as result of the label propagation. The volume intensities were consequently scaled to the [0; 1] range. For the echo images we performed an additional per-slice intensity normalization by mapping the cumulative intensity distribution of each slice to that of the chosen reference slice (the one with the maximum entropy) and rescaling the image intensity accordingly.

Whole brain segmentation

In this step, the segmentation was performed by minimizing the energy functional $E(\phi)$ on the fourth echo image; see Figure 1A2a. The level set function in this case was initialized by the whole-brain mask M_{wb} obtained as result of label propagation. The final result, denoted as $R_{wb'}$ was obtained by: (I) Applying the morphological hole filling and morphological opening

with a disk of radius of two pixels as the structure element on the result of the level set propagation; and (II) Selecting the largest connected component. The parameter values for this step are provided in Table 2.

$$R_s^{init} = P \left(I(\mathbf{x}); R_B \setminus (R_V \cup M_{PVZ}) \right) \\ > P \left(I(\mathbf{x}); R_{CBH} \setminus (R_V \cup M_{PVZ}) \right)$$

Contralateral ventricle segmentation

Contralateral ventricle R_{CV} was segmented from the T2 map by running the level sets starting from the result of the label propagation; see Figure 1A2b. In this case, the level set evolution was restricted to the contralateral hemisphere $R_{CBH} = R_{WB} \setminus M_{IBH}$, the rest of the parameter values are listed in Table 2.

Ventricles segmentation

Ventricles R_V were segmented on the T2 map by evolving the level sets inside R_{WB} ; see Figure 1A2c. The object (ventricle) energy was calculated from the segmented contralateral ventricle and kept fixed. To prevent the segmentation from leaking into the stroke area touching the ventricles, we used the gradient map that was defined as $g(\mathbf{x}) = 1 - e_v^2(\mathbf{x})$, where $e_v(\mathbf{x}) = -\log P_{\text{Gaussian}}(I(\mathbf{x}); R_{CV})$ is the energy (assuming the Gaussian intensity distribution) scaled to the [0; 1] interval, and parameters of the Gaussian distribution were calculated from R_{CV} (the region occupied by the contralateral ventricle).

$$\text{Dice index}(R_1, R_2) = 2 \frac{|R_1 \cap R_2|}{|R_1| + |R_2|}$$

Stroke segmentation

Finally, the stroke area was segmented from the T2 map; see Figure 1A3. To initialize the level set function, we initially calculated the area where the histogram-based energy of the brain region R_{WB} with the ventricles and the periventricular area excluded ($R_{WB} \setminus (R_V \cup M_{PVZ})$) was larger than that of the contralateral hemisphere ($R_{CBH} \setminus (R_V \cup M_{PVZ})$):

Consequently, connected components that did not intersect with dense areas on R_s^{init} were filtered out. The dense areas were defined as binary masks composed of all voxels in R_s^{init} for which at least 75% of their neighbors (the neighborhood was in this case defined as the circle of radius 4 around the voxel of interest) belong to R_s^{init} . In this case, evolution of the level set function was restricted to $R_{IBH} \setminus (R_V \cup M_{PVZ})$ (the ipsilateral hemisphere without the ventricles and the periventricular area). The energy of the background was assumed fixed and equal to that calculated from the contralateral hemisphere R_{CBH} . The rest of the parameters are provided in Table 2.

Parameter selection

The parameters (α, μ, n_{iter}) for the level-set-based segmentation were optimized on the Leiden-Set. Suitable parameter values for segmenting the whole brain region, the contralateral ventricle and both ventricles were determined by trial and error. The parameters for the stroke segmentation were determined by maximizing the Dice index³⁷ via exhaustive search

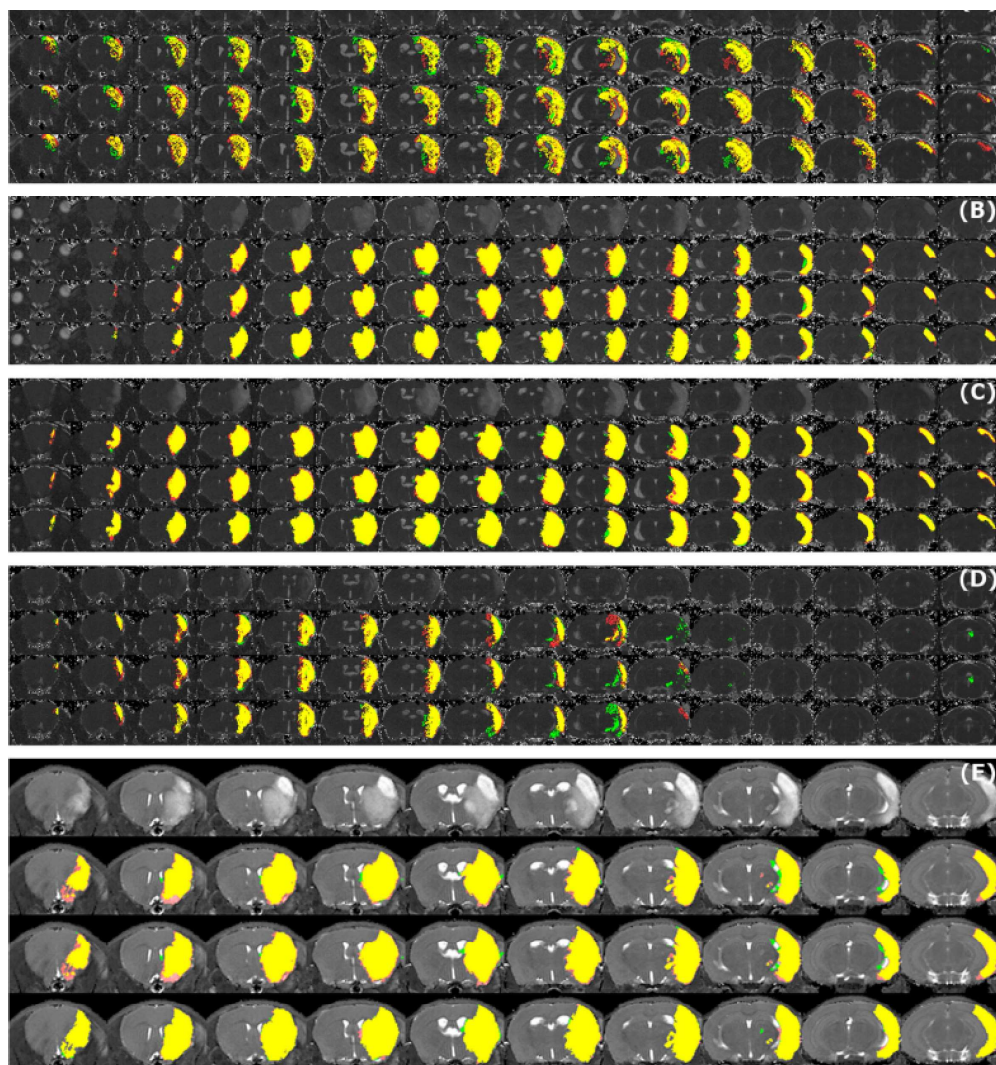


Figure 2. Examples of segmentation results. Datasets of different origin [*Leiden-Set* (A–D), *Cologne-Set* (E)] were acquired at different time points after infarct induction [4h (A), 24h (B), 48h (C,E) and 8d (D)]. For each of the image panels, the first row illustrates reformatted raw image stack and the rest of the rows provide overlaid segmentation results by: automated (green) vs Observer 1 (red), automated (green) vs Observer 2 (red), Observer 1 (green) vs Observer 2 (red), respectively. The regions where two segmentations overlap are colored in yellow. Image intensity was enhanced for visualization purposes.

within an empirically selected range of feasible values for each parameter. The final values used for validation of our method, the same for all three data sets, are reported in Table 2.

Implementation

All the data are publically available and published as a data report.³⁸ The template labels were manually drawn based on the Allen Brain Atlas³⁹ (<http://www.brain-map.org/>) using AMIRA (v5, FEI Software, Hillsboro, OR, USA). Both registration and segmentation were implemented in MATLAB R2012b (The MathWorks, Inc., Natick, MA, USA) and compiled into a toolbox that can be downloaded from the following webpage (www.lkeb.nl). The registration scheme was

implemented using the opensource image registration toolbox elastix (v4.700⁴⁰). Information on the used registration parameters can be found on the elastix website (<http://elastix.bigr.nl/wiki/index.php/Par0038>). Average computational time for executing the entire segmentation routine on one data set on 3.60 GHz Intel(R) Xeon(R) computer with 32 GB RAM was: 4s for segmentation and 309s for prior registration and label propagation.

Performance Measures and Statistical Analysis

Ischemic lesion regions segmented by the observers and the proposed automated approach were compared using two primary measures: total volume of the ischemic lesion and Dice index³⁷ that measures overlap between two regions R_1 and R_2 :

Intraclass Correlation Coefficient (ICC) and the accompanying trend line were also calculated for each group of interest. ICC (2-way mixed with absolute agreement) was determined with SPSS (SPSS Statistics 23; IBM Corp., Armonk, NY, USA) to investigate the correlation between the mean of the two observers and the calculated automated volumes.

RESULTS

Sample segmentation results on the datasets of different origin and acquired at different timepoints after infarct induction are shown in Figure 2. The supplementary material 1 provides corresponding segmentation results on all 121 datasets. Figure 3 illustrates segmented striatal (Str) and corticostriatal (Ctx+Str) lesions, as well as those with fragmented infarct areas and with large edema and morphed ventricles. The results show good agreement between both segmentation methods as well as with the accompanying histological sections.

The results of experiments for all age groups at the various timepoints after stroke induction from *Leiden-Set* and *Cologne-Sets* in terms of the absolute volume difference, ICC and Dice

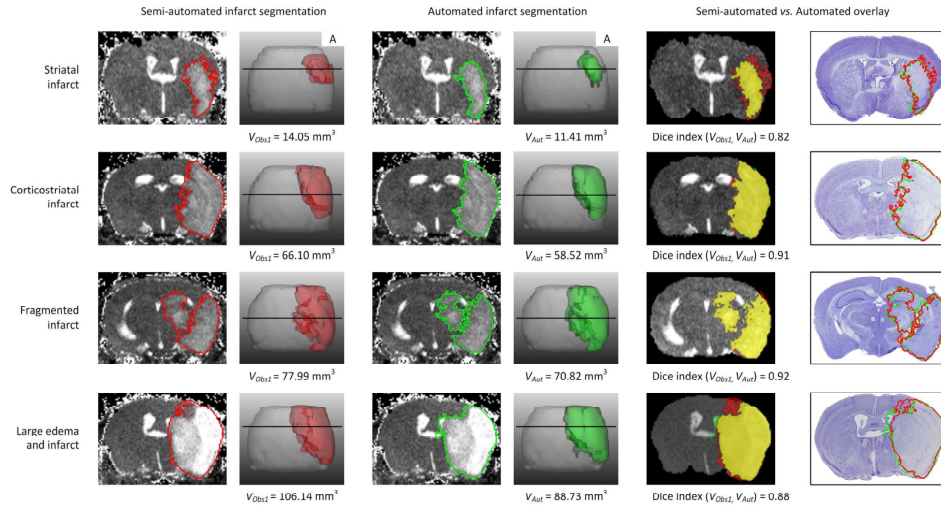


Figure 3. Example traces of different infarct anatomies in a single slice and complete 3D brain. Lesions (striatal, corticostriatal, fragmented and with large edema compressing the ventricle) were segmented using the semi-automated (red delineated area) and automated (green delineated area) approaches. The fifth column shows the overlap between both methods, in yellow. The last column shows the overlay of both approaches on the corresponding Nissl stained histological section. “A” stands for anterior. V_{Obs1} corresponds to the volume obtained by performing semi-automated stroke segmentation by Observer 1 (IM). V_{Aut} corresponds to the volume obtained by the automated method. The Dice index between Observer 1 and Observer 2 for the same data sets was: 0.82 for striatal infarct, 0.91 for corticostriatal infarct, 0.92 for fragmented infarct and 0.88 for the large edema case.

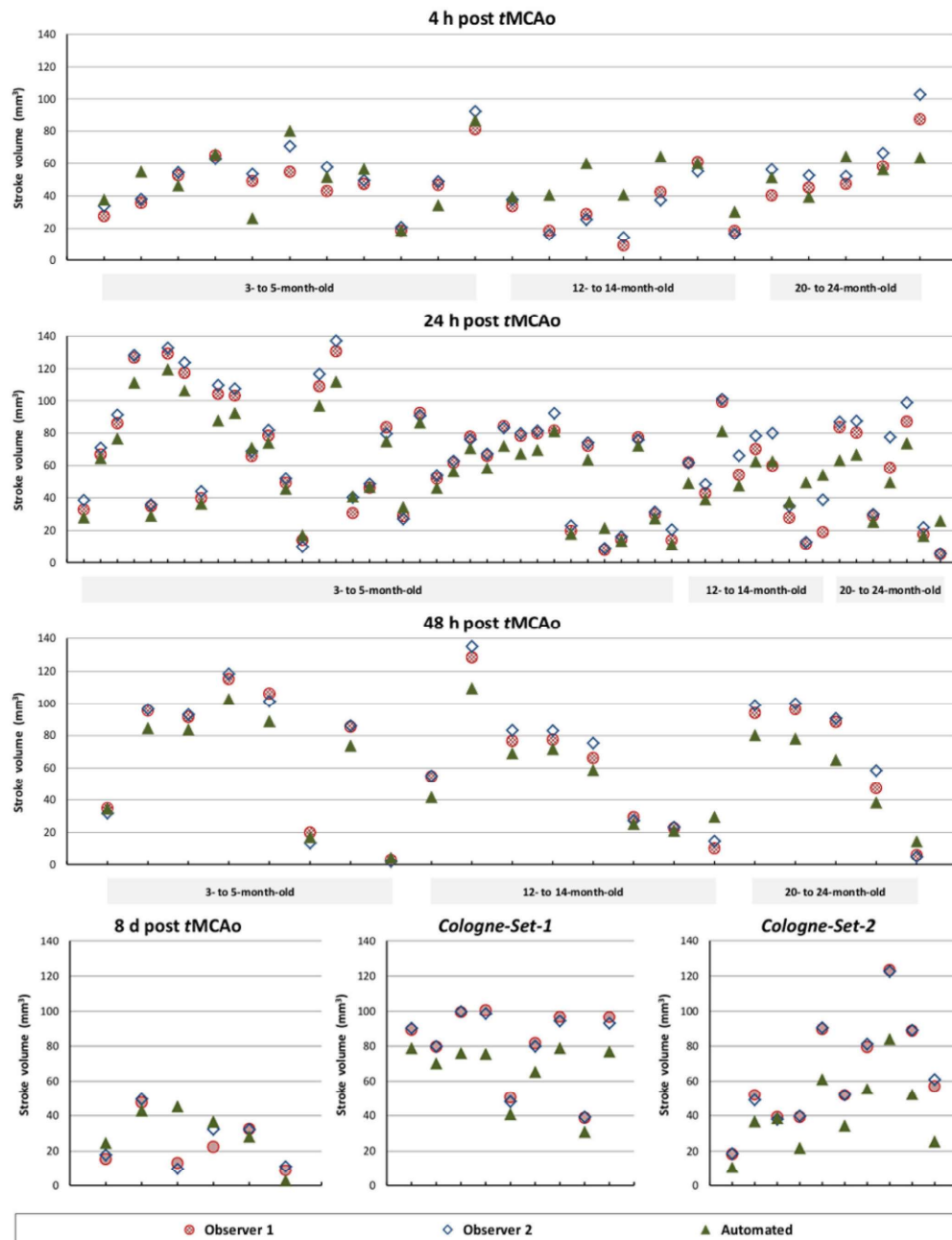


Figure 4. Comparison of total stroke volume between our automated method and human observers, for all test datasets, different times after stroke induction and different mouse ages.

index are reported in Table 3. The mean per-group absolute volume difference in the *Leiden-Set* ranged from 5.9 to 18.4 mm³, for the *Cologne-Set-1* it was 15.3 mm³ (for 18h) and 14.2mm³ (for 4d) and for the *Cologne-Set-2* it was 22.0 mm³. Infarct volumes of all groups, from both observers and by the automated method are presented in Figure 4.

The mean per-group Dice index for Observer 1 vs Observer 2 was between 0.79 and 0.98, whereas for automated vs. observers it was ranging from 0.63 to 0.88 (Observer 1) and from 0.63 to 0.89 (Observer 2).

Figure 5A illustrates the mean infarct volume obtained by the two observers vs the corresponding automated volume for our primary group, for both Str and Ctx+Str lesions. This plot shows very high degree of correlation with an ICC of 0.957 (ICC = 0.966 for Str lesions and ICC = 0.944 for Ctx+Str lesions). Figures 5B–E show the corresponding information for all the other groups. At 24h and 48h, the data match the primary validation group. However, at 4h and 8d after stroke induction the regression line is less perfect: ICC = 0.806 at 4h and ICC = 0.491 at 8d for 3- to 5-month-old mice, ICC = 0.384 at 4h for 12- to 14-month-old mice and ICC = 0.445 at 4h for 20- to 24-month-old mice. Figure 5E shows that our method is also robust with respect to data obtained from different imaging hardware using different acquisition parameters, time after stroke induction and age: ICC = 0.793 at 18h, ICC = 0.964 at 4 days and ICC = 0.552 for 1- to 2-year-old mice scanned at 48h after stroke induction. Figure 6 shows distribution of Dice index for different timepoints. Here, again, the results of the 24h and 48h groups are superior to those of the 4h and 8d groups: mean Dice index of 0.68 ÷ 0.87 at 24h and 48h and of 0.63 ÷ 0.80 for 4h and 8d for all groups, respectively. Finally, Figure 7 provides segmentation results on the datasets that exhibit large disagreement between two observers. These datasets are outliers of the corresponding box-whisker plot on Figure 6.

We have also performed a parameter sensitivity study to assess stability of our algorithm with respect to the parameters. For this, we changed, in turn, the value of each of the parameters listed in Table 2 by ±50% and recalculated the results. Our approach turned out to be highly robust with respect to both validation metrics: maximal change for the mean absolute volume difference was less than 6%, and for the mean Dice index it was less than 1.2%.

DISCUSSION

The major challenges in stroke segmentation in pre-clinical MRI are (I) irregular lesion volume and shape, (II) low resolution compared to clinical imaging data, (III) fluctuating contrast and (IV) considerable brain deformation and asymmetry because of the induced stroke. The presented approach addresses these challenges by estimating intensity distributions per scan from the contra-lateral, unaffected, hemisphere and comparing these with the affected hemisphere. With our approach, an ischemic lesion in a mouse brain can be successfully segmented from T2 MR images¹⁷ in a fully automated manner.

Our approach has a number of advantages over existing methods for stroke segmentation in small animal data:

- 1) A main asset of our automated analysis approach is that it takes only few minutes to analyze an MRI volume (containing 16 slices) and is therefore at least an order of magnitude faster than the semi-automated method (~60min). It is also more robust because of its objective character excluding potential observer bias. Thus, although histology remains the gold standard for assessment of lesion formation in pre-clinical stroke research, its downsides

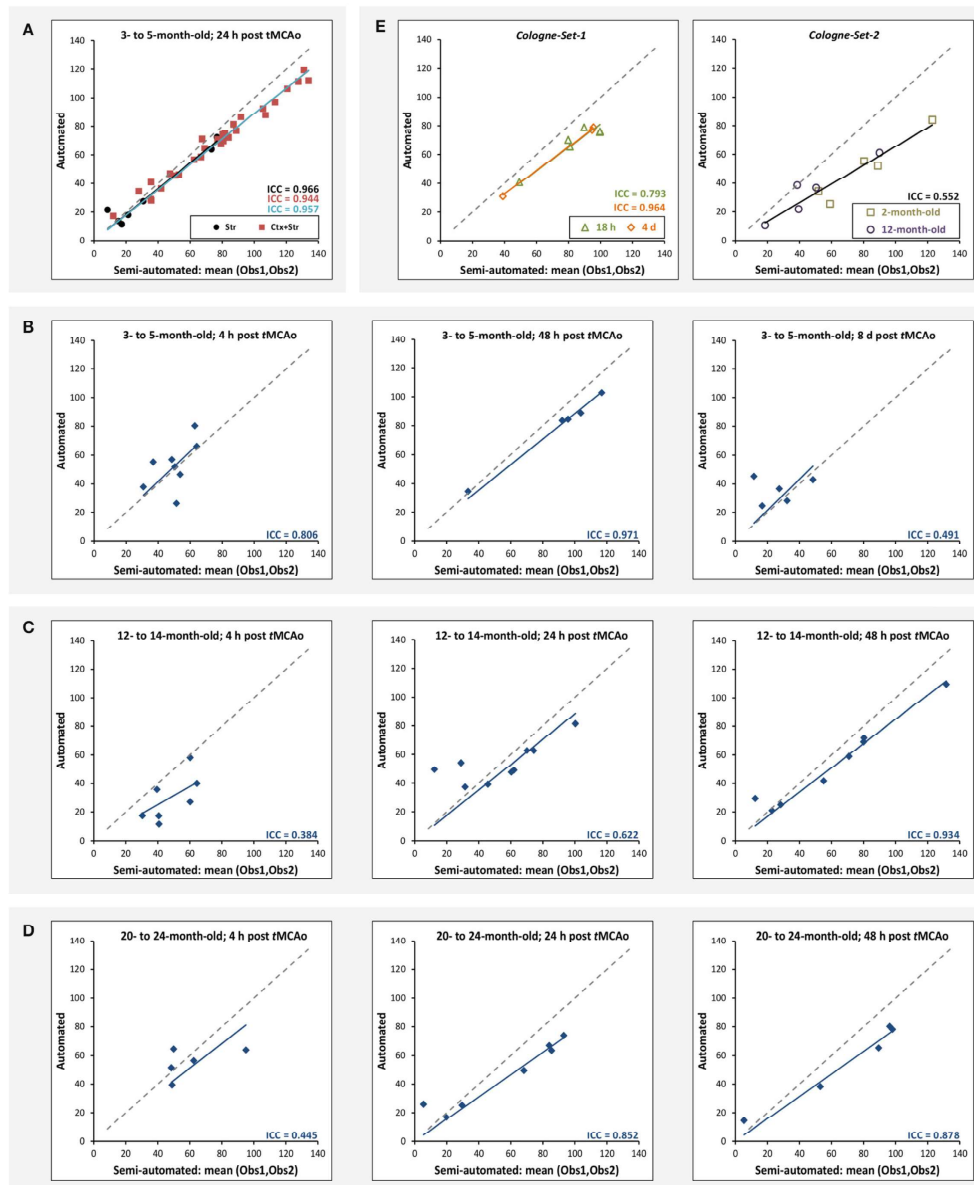


Figure 5. Correlation of lesion volumes calculated in a semi-automated fashion by observers vs. the automated method. (A) 3-to5-month-oldmice, at 24h after MCAO for striatal (Str) and corticostriatal (Ctx+Str) lesions. (B) 3- to 5-month-old mice, MRI at 4h, 48h and 8d after MCAO. (C) 12- to 14-month-old mice, MRI at 4h, 24h and 48h after MCAO. (D) 20- to 24-month-old mice, MRI at 4h, 24h and 48h after MCAO. (E) MRI using different hardware and software at 18h and 4d (*Cologne-Set-1*) and 48h (*Cologne-Set-2*) after MCAO. The solid lines provide the trend of the corresponding data group and the dashed gray line corresponds to equal values on both axes and is shown as a reference.

(that one needs to sacrifice the animal for a given timepoint and the fact that results are confounded by morphological changes due to the processing of fragile tissue) make MRI more desirable when one investigates the development of lesions over time. It is not unexpected that MRI is establishing itself as the main diagnostic modality for such studies, as it allows automated data processing and was shown to correspond well with histology.¹⁷

2) Our method is robust with respect to different infarct shapes and volumes, even for small fragmented lesions or extremely large lesions where ventricles become deformed due to edema formation in and around the lesion. It performed well for a heterogeneous group of 121 brain scans, including different MRI scanners (noise levels, resolution), different lesion anatomies (cortical, cortico-striatal, with large edema, fragmented), over different timepoints after infarct induction (from 4h up to 8d). In particular, it exhibited robust performance on data acquired from different MRI hardware — with differences in slice number and thickness — without the need for modification of the model or registration/segmentation parameter settings. We expect that our method is also suitable for other MRI lesion detection sequences, such as DWI, and perhaps even for different rodent species, with only limited model and parameter adjustments required. It is important to point out that, as we have mentioned in Section “Parameter Selection”, all parameter so four algorithm were optimized with respect to the entire *Leiden-Set*, which means that our method was completely blinded to the properties of each particular dataset. However, in our analysis we subdivided the *Leiden-Set* into smaller groups, based on age and time after infarct induction, and also report results on two unseen sets of images (*Cologne-Sets*). This naturally results in sub-optimal performance on each particular group, confirmed, *e.g.*, by lower ICCs reported in Figure 5 and Table 3. This effect is especially pronounced in relatively small groups as these were under represented at the parameter tuning stage. Optimizing the parameters for each particular group will help improving the performance, which, however, will inevitably be limited by complexity of the data.

3) Unlike numerous published methods in the field,^{1,3,4,18-21,23-26,41,42} our algorithm operates on a single contrast (T2) and does not require a pre-scan.

4) Unlike other methods developed for small animal data (HRS^{6,15,22} and ISODATA^{1,23-26}) the ischemic lesion segmentation approach presented here only makes use of the quantitative T2 maps. Contrary to T2-weighted MRI data, true quantitative MRI maps (T2 or others) are comparable across research centers, whereas parameter-weighted images (T2-weighted or others) are subjective estimates derived for better discrimination of the object of interest from the background and are not comparable across research centers due to arbitrary operator choice of TE and TR values. A combination of complementary quantitative parameters, for instance, T2 and ADC values, on the other hand, can in some cases improve discrimination not only between the different objects present in an image, but also between the different subcategories of the object.^{3,4,43} This improvement, however, comes at the expense of slightly increased scan times and additional data analysis.

5) Our approach is generic as we do not make any specific assumptions or create a general model that would describe all of our data. Instead, the stroke area is segmented on each mouse brain MR volume by using the intensity distributions of the background and the ventricles calculated from the very same image. In all validation experiments, our method was completely blinded to the properties (origin, age, timepoint) of each particular brain volume, meaning that the same parameter settings were used for all datasets (including those acquired with different hardware and software compared to the training set).

The main pitfall of our approach is its multi-step nature. This type of complex algorithm, and our method in particular, is sensitive to propagation of errors made at earlier stages. Our experiments show that success or failure of the registration with label propagation, the first step in our algorithm, has significant impact on subsequent segmentation steps. More precisely, the largest segmentation errors were caused by inability to accurately segment the ventricles, *e.g.*, when they were virtually invisible due to a large stroke area. In particular, inability to achieve high-quality registration due to large difference between the atlas scan and the rest of the data, also explains sub-optimal performance of our method on *Cologne-Sets*.

In the absence of histology, semi-automated segmentation by experts has always been used as the reference standard in stroke quantification, which was also the case for this work. Thus, during development of any automated stroke segmentation method the main goal is to achieve performance comparable to that by experts. This would allow bypassing the manual observer bias and, hence, lead to more objective quantification of the stroke region. In this work, we evaluated the performance of our method by comparing it to two observers (separately and combined) and by analyzing the inter-observer variability.

Our results indicate that quantification of the infarct in its acute phase (4h after induction) remains challenging, both for our automated approach and for the human observers. During this early phase of lesion development, T2 enhancement is still very weak as edema is only slowly evolving, so that in some cases it may barely reach above the normal contra-lateral value. It should be stated that also histological lesion demarcation at this very early timepoint is unreliable, which admittedly complicates infarct detection during the first hours after stroke induction in experimental animal models.

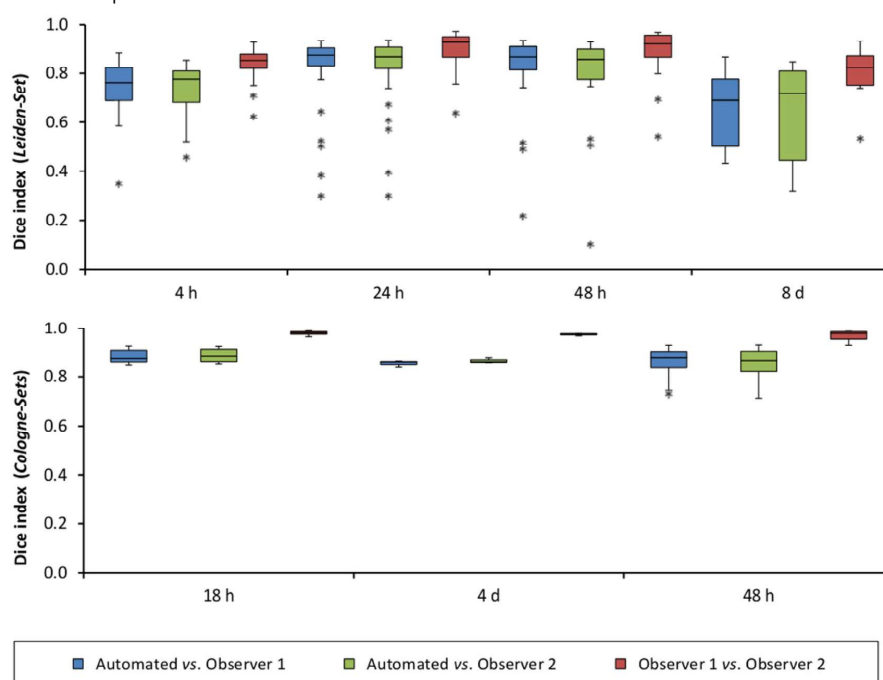


Figure 6. Distribution of Dice index for each of the analyzed time points after stroke induction and for all three datasets: *Leiden-Set*, *Cologne-Set-1* (18h, 4d) and *Cologne-Set-2* (48h).

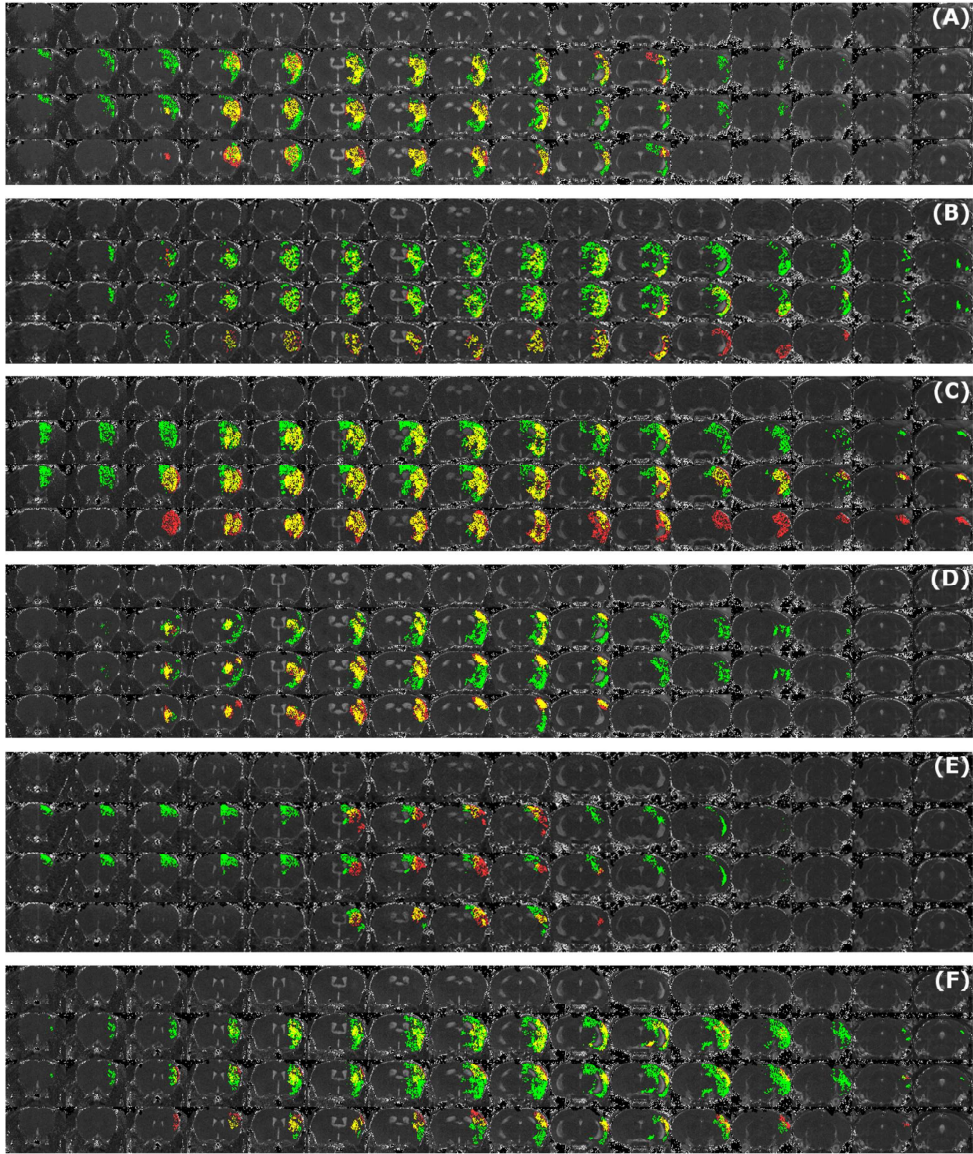


Figure 7. Examples of segmentation results that exhibit large disagreement between two observers. Datasets were acquired at different time points after infarct induction [4h (A,B), 24h (C), 48h (D,E) and 8d (F)]. For each of the image panels, the first row illustrates reformatted raw image stack and the rest of the rows provide overlaid segmentation results by: automated (green) vs Observer 1 (red), automated (green) vs Observer 2 (red), Observer 1 (green) vs Observer 2 (red), respectively. The regions where two segmentations overlap are colored in yellow. Image intensity was enhanced for visualization purposes.

AUTHOR CONTRIBUTIONS

Conceived and designed the study:IM,AK,OD,MH,BL,AvdM. Performed animal experiments, tMCAO surgery and acquired MRI data: IM. Performed histopathology: IM,NR. Performed semi-automated ischemic lesion segmentation: IM, SdJ. Developed the automated ischemic lesion segmentation in MRI mouse brain data after tMCAO occlusion algorithm: AK,OD. Analyzed the data: IM,AK,OD,MH,BL,AvdM. Wrote the manuscript: IM,AK,OD. Discussed the results and commented on the manuscript: IM,AK,OD,MW,MH,BL,AvdM.

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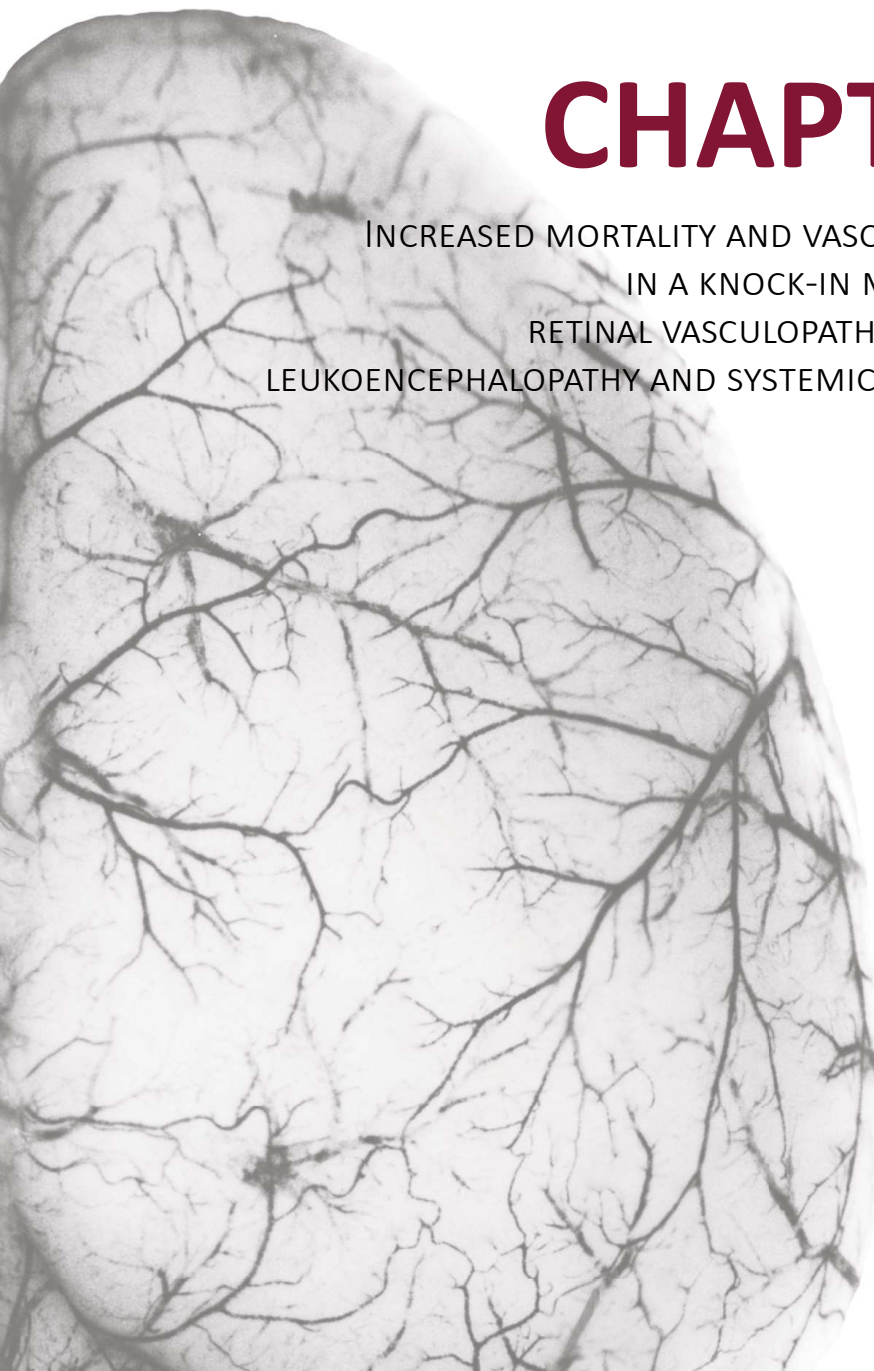
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SUPPLEMENTAL MATERIAL

The supplementary material for this article can be found online at:
<http://journal.frontiersin.org/article/10.3389/fninf.2017.00003/full#supplementary-material>.
 Supplementary Material 1 includes the segmentation results on the entire validation set.



CHAPTER 5

INCREASED MORTALITY AND VASCULAR PHENOTYPE
IN A KNOCK-IN MOUSE MODEL OF
RETINAL VASCULOPATHY WITH CEREBRAL
LEUKOENCEPHALOPATHY AND SYSTEMIC MANIFESTATIONS

Mulder IA, Rubio-Beltran E, Ibrahimi K, Dzyubachyk O, Khmelinskii A,
Hoehn M, Terwindt GM, Wermer MJH*, MaassenVanDenBrink A*, and van
den Maagdenberg AMJM*

**Authors contributed equally*

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ABSTRACT

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant small vessel disease caused by C-terminal frameshift mutations in the *TREX1* gene that encodes the major mammalian 3' to 5' DNA exonuclease. RVCL-S is characterized by vasculopathy, especially in densely vascularized organs, progressive retinopathy, cerebral microvascular disease, white matter lesions, and migraine, but the underlying mechanisms are unknown.

Homozygous transgenic RVCL-S knock-in mice expressing a truncated Trep1 (three prime repair exonuclease 1) protein (similar to what is seen in patients) and wild-type littermates, of various age groups, were subjected to (1) a survival analysis, (2) in vivo postocclusive reactive hyperemia and ex vivo Mulvany myograph studies to characterize the microvascular and macrovascular reactivity, and (3) experimental stroke after transient middle cerebral artery occlusion with neurological deficit assessment.

The mutant mice show increased mortality starting at midlife ($P=0.03$ with hazard ratio, 3.14 [95% CI, 1.05–9.39]). The mutants also show a vascular phenotype as evidenced by attenuated postocclusive reactive hyperemia responses (across all age groups; $F[1, 65]=5.7$, $P=0.02$) and lower acetylcholine-induced relaxations in aortae (in 20- to 24-month-old mice; RVCL-S knock-in: $E_{\max}: 37 \pm 8\%$ vs WT: $E_{\max}: 65 \pm 6\%$, $P=0.01$). A vascular phenotype is also suggested by the increased infarct volume seen in 12- to 14-month-old mutant mice at 24 hours after infarct onset (RVCL-S knock-in: $75.4 \pm 2.7 \text{ mm}^3$ vs WT: $52.9 \pm 5.6 \text{ mm}^3$, $P=0.01$).

Homozygous RVCL-S knock-in mice show increased mortality, signs of abnormal vascular function, and increased sensitivity to experimental stroke and can be instrumental to investigate the pathology seen in patients with RVCL-S.

INTRODUCTION

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant disorder caused by C-terminal frameshift mutations in the *TREX1* gene,¹ which encodes the ubiquitously expressed Trex1 (three prime repair exonuclease 1) protein.^{1,2} The mechanisms underlying the cause of RVCL-S, however, are unknown. RVCL-S is a systemic progressive small vessel disease with middle-age severe symptomatology and decreased life expectancy, characterized by vasculopathy in various organs, most pronounced in retina, kidney, and brain, with white matter lesions and intracerebral mass lesions.³⁻⁶ Patients with RVCL-S report more often migraine (generally without aura) and have increased risk for ischemic stroke, both suggesting vascular involvement. Pathological studies revealed thicker endothelial cells with increased vesicles and coarse cytoplasm, and thicker, multi-laminated, basement membranes of endothelial cells.⁵ Hence it has been hypothesized that the endothelium of small vessels, particularly in highly vascularized organs, is affected in RVCL-S. Endothelial dysfunction (impaired endothelium-dependent vasodilation and endothelial cell damage),⁷ which has been proposed as disease mechanism for the vascular phenotype seen in stroke and migraine, was also shown to underlie RVCL-S in patients.⁴

Although patients with RVCL-S are heterozygous for the *TREX1* mutation, here we investigated transgenic homozygous RVCL-S knock-in (KI) mutant mice that express a truncated Trex1 protein (similar to what is seen in patients with RVCL-S).^{1,5} The mutant mice express a truncated mouse protein (with the endogenous regulation of gene expression still intact), which is slightly different from the existing humanized RVCL-S mouse model in which the human cDNA bearing the same V235fs mutation was introduced in exon 2 of *TREX1*.⁸ Here we assessed whether homozygous RVCL-S KI mice have pathological features seen in patients with RVCL-S, such as a reduced life expectancy and a vascular phenotype (as assessed by functional vascular measurements and induction of experimental stroke).

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Experimental animals

Homozygous transgenic RVCL-S KI and wild-type (WT) littermate mice of different age groups (3–6, 12–14, and 20–24 months) were used. As the heterozygous mutants show a normal survival (Figure 1), we focused on a comparison between the homozygous mutant and WT mice. For the generation of the mutant mice, a targeting construct was used by which a frameshift mutation was introduced at amino acid position 235 of the Trex1 protein, resulting in a truncated protein as observed with the most prevalent RVCL-S mutation in patients (Methods and Supplemental Figure 1).^{1,5} Homozygous mutant and WT mice were generated by interbreeding heterozygous mice. For the stroke experiments, only male mice were used. For the in vivo and in vitro vascular characterization, results of both sexes were pooled because no sex difference was detected. Animals were randomized (flip of a coin) for surgical procedure, and researchers were blinded for genotype during experimental and analyses procedures. Animals were housed in a temperature-controlled environment with food and water ad libitum. All animal experiments were approved by the local committee for animal health, ethics and research of the Leiden University Medical Center.

Survival Analysis

Survival in naive WT and homozygous RVCL-S KI mice was assessed in animals who, at 2 months, were randomly assigned to one of the age groups (3–6, 12–14, and 20–24 months) to be included in the experimental stroke studies (n=97). Mice were censored for analysis when they reached the age of inclusion for the stroke experiment. In addition, data of 51 heterozygous animals, which were not used in experiments, were added to the survival analysis.

In vivo microvascular characterization

Microvascular characteristics were assessed *in vivo* using postocclusive reactive hyperemia (PORH) measurements of the hind leg with laser Doppler flowmetry (PeriFlux System 5000, Perimed, Järfälla-Stockholm, Sweden). Twenty-four hours before dermal blood flow measurements, the hair of the left hind leg was removed with hair removal cream (Veet, ReckittBenckiser, Inc, Berkshire, United Kingdom). On the day of measurement, mice were anesthetized using 4% isoflurane/O₂ ventilation and kept on a heating pad regulated by a rectal thermometer (FHC, Inc, Bowdoin, ME) to maintain body temperature at $36.7 \pm 0.3^{\circ}\text{C}$. After a 5-minute equilibration time period, 10 minutes of baseline perfusion was recorded. Subsequently, the hind leg circulation was occluded for 2 minutes with a tourniquet. After release of the tourniquet, blood flow was monitored until return to baseline blood flow (maximally 10 minutes). The area under the curve and PORH peak were used as readout measures. After the experimental procedure, mice were euthanized using decapitation, where the aorta was collected for *in vitro* measurements.

Transient middle cerebral artery occlusion model

Mice were anesthetized using isoflurane (3% induction, 1.5% maintenance) in 70% pressurized air and 30% O₂. Carprofen 5 mg/kg, *s.c.* (Carporal, 50 mg/mL; AST Farma B.V., Oudewater, the Netherlands) was administered before surgery for pain relief. During surgery, the mouse body temperature was maintained at $36.7 \pm 0.3^{\circ}\text{C}$ as described above. Experimental stroke was induced using the transient middle cerebral artery occlusion (MCAO) model.¹⁰ A silicone-coated nylon monofilament (7017PK5Re; Doccol cooperation, Sharon, MA) was introduced into the internal carotid artery, via an incision in the common carotid artery, to block the middle cerebral artery (MCA) at its origin for 30 minutes. Cerebral blood flow in the MCA territory was measured using laser Doppler flowmetry (PeriFlux System 5000; Perimed) and calculated as %-measure of baseline. In a subset of animals, tcCO₂ was measured (TCM4 CombiM monitor with accompanying software version 3.0; Radiometer Medical ApS, Brønshøj, Denmark) to confirm physiological stability during surgery (data not shown). After surgery, the animal was placed in a temperature-controlled recovery incubator (V1200; Peco Services Ltd, Brough, United Kingdom) maintained at 33°C for 2 hours, with easy access to food and water. Postprocedural observation was performed twice daily.

Magnetic resonance imaging

Animals were scanned using a small animal 7T MRI system (Pharmascan, Bruker BioSpin, Ettlingen, Germany) under isoflurane anesthesia. A Multi Slice Multi Echo sequence protocol was run with repetition time/echo time of 4.000 ms/9 ms, 20 echoes, 2 averages, matrix 128×128, field of view of 2.50 cm, bandwidth 59523.8 Hz, slice thickness of 0.5 mm, and 16 slices (no gap). Quantitative T2 maps were calculated using Paravision 5.1 software (Bruker

BioSpin). Scans were performed at 4, 24, and 48 hours after MCAO surgery. An in-house developed automated software pipeline¹¹ was used to calculate infarct sizes. If automated segmentation showed artifacts, for example, due to wrapping, small manual corrections were made, or scans were excluded due to technical failure.

Behavior analyses

Behavior analyses were performed directly before magnetic resonance imaging measurements, that is, before and after (at 4, 24, 48 hours, and 5 days) MCAO surgery. During behavioral tests, mice were videotaped, and blinded videos were analyzed after completion of the experiments. Neurological deficit scores were measured on a 56-point scale.^{12,13} The score involved general and focal deficits, where 0 reflected no deficits, and 56 reflected the poorest. Categories concerning general appearance and performance included fur (0–2), ears (0–2), eyes (0–4), posture (0–4), spontaneous activity (0–4), and epileptic behavior (0–12). Concerning the focal deficits a score was stated for body asymmetry (0–4), gait (0–4), climbing on a surface inclined at 45° (0–4), circling behavior (0–4), front-limb symmetry (0–4), compulsory circling (0–4), and whisker response to light touch (0–4).

Vessel anatomy

A subset of mice was used for vessel anatomy analysis.^{14,15} In brief, mice were euthanized using CO₂ and transcardially perfused using 2.5 mL PBS with 50 µL heparin (Heparine natrium 5000 I.U./mL; LEO Pharma B.V., Amsterdam, the Netherlands) and 2.5 mL ink mixture (ratio 1:9, Herlitz stempelfarbe ink and Pelikan Scribto ink; Pelikan Vertriebsgesellschaft mbH & Co. KG, Hannover, Germany) at a rate of 3 mL/min, preheated to 37°C. Brains were removed, photographed, and post-fixed in fresh 4% paraformaldehyde. Vessel anatomy was scored as the number of anastomoses between MCA and anterior cerebral artery, distance between midline, and the anastomotic line at 2, 4, and 6 mm from anterior and diameter of the main vessels of the circle of Willis (MCA, anterior cerebral artery, internal carotid artery, posterior cerebral artery, basilar artery, and posterior communicating artery). Pial anastomoses were defined as the narrowest part of the vessel or halfway between the nearest branch points of the anterior and the MCA, localized by tracing the peripheral branches of these major vessels.

Statistical analyses

All statistical analyses were performed in SPSS software (SPSS statistics 23, IBM Corporation, Armonk, NY). Survival analyses were performed using the log-rank (Mantel-Cox) test, and Cox univariable regression analyses were done to calculate the hazard ratio. Animals who reached the inclusion age of the experiment were censored in the analyses. The PORH results were expressed as relative values compared with baseline and included the area under the curve (AUC) and the maximal hyperemia response (E_{max}), expressed as mean $\pm$ SEM. Vascular relaxant responses to acetylcholine and SNP were expressed as the percentage of contraction induced by 10 to 100 nmol/L U46619. Data for the vascular responses to 5-hydroxytryptamine were expressed as percentage of the contractile response to 100 mmol/L KCl. Both the *in vivo* and *in vitro* data were analyzed using univariate ANOVA followed by Bonferroni post hoc tests. Outcome values were expressed as mean $\pm$ SEM. Infarct volume and neurological deficit scores were compared between genotypes using marginal mixed model analyses (data are shown as estimated marginal means $\pm$ SD). Survival analyses post-surgery were performed using the log-rank (Mantel-Cox) test. Group comparison concerning vessel diameter and anastomotic

distance were analyzed using multivariate ANOVA with discriminant function analysis post hoc test. Absence of the posterior communicating artery was analyzed using Pearson χ^2 (2-sided Fisher exact) test, and mean pial number of anastomoses were analyzed with a t test. $P < 0.05$ indicates statistical significance.

RESULTS

Increased mortality in RVCL-S KI mice

Homozygous RVCL-S KI mutant mice showed increased mortality compared with WT controls: 16 (31%) mutant versus 4 (9%) WT mice died during the observation period presurgery ($P=0.03$ with hazard ratio, 3.14 [95% CI, 1.05–9.39]). The increased mortality in the mutant mice compared to WT mice was apparent from 9 months of age (Figure 1). Heterozygous mutant mice did not show a reduced lifespan ($P=0.55$ with hazard ratio, 1.25 [95% CI, 0.60–2.59]; Figure 1). Therefore, only homozygous mutants were investigated in subsequent functional experiments. *Post mortem* examination, although suboptimal because considerable organ deterioration had occurred at the time of tissue examination, did not reveal a macroscopic cause of death (data not shown). *Post mortem* examination also did not show signs of brain atrophy, neither in the heterozygous nor the homozygous mutants, but also here the analysis was not reliable due to organ deterioration (data not shown). Finally, basic histological hematoxylin and eosin staining analyses of various organs (*e.g.*, eye, kidney, skin, liver, brain) of the homozygous and heterozygous mutant mice did not reveal any obvious abnormalities (data not shown). In contrast with patients with RVCL-S,⁵ we observed no structural abnormalities in endothelial cells or the basement membrane of the mutant mice. It remains unclear whether this is because these disease features are not captured in the mouse model or whether these features need longer time to develop than the 2-year maximal age of a mouse.

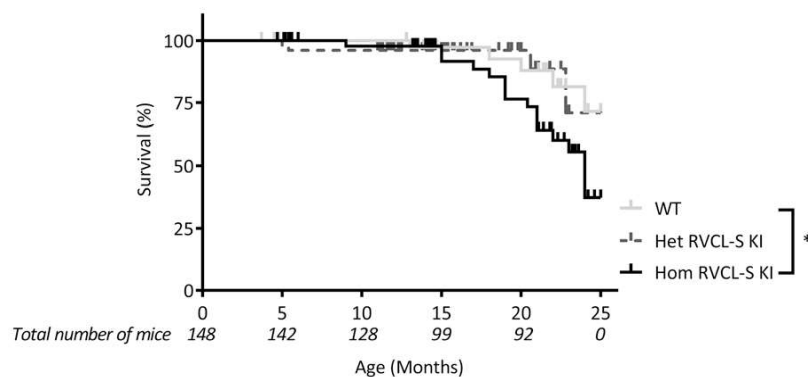


Figure 1. Survival curves from wild-type (WT), heterozygous (Het), and homozygous (Hom) retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) knock-in (KI) male mice. At 2 mo, Hom mutant and WT mice were randomly assigned to one of the 3 age groups for experimental stroke surgery. Mice reaching the age of surgical inclusion were censored from the analysis. WT ($n=45$, gray line), Het RVCL-S KI ($n=51$, black dotted line), and Hom RVCL-S KI mice ($n=52$, black line). Vertical bars along curves represent censored animals. WT and Het survival curves were not significantly different. * $P < 0.05$ indicates statistical significance between WT and Hom RVCL-S KI mice.

Reduced *in vivo* microvascular reactivity in RVCL-S KI mice

Compared with WT, the RVCL-S KI mice showed a significantly lower area under the reactive hyperemia curve across all age groups ($F[1, 60]=5.1$, $P=0.03$; Figure 2A). The maximal reactive hyperemia responses, however, were comparable between genotypes (Figure 2B). Baseline dermal blood flow and time to maximum value were also similar between genotypes (data not shown).

Increased *in vitro* macrovascular reactivity in RVCL-S KI mice

No significantly different contraction reaction to 100 mmol/L KCl was seen between both genotypes. Instead, the concentration-response curve to acetylcholine showed statistically significantly attenuated relaxant responses in 20- to 24-month-old RVCL-S KI mice compared with WT mice ($E_{\max}$: $65 \pm 6\%$ versus $37 \pm 8\%$, respectively, $P=0.01$), but not in mice of other age groups (Figure 2C). No genotypic difference was observed in the response to SNP or 5-hydroxytryptamine (Figure 2C).

Increased Susceptibility to Experimental Stroke With Worse Outcome in RVCL-S KI Mice.

Overall, infarct volume was higher in RVCL-S KI compared with WT mice ($P=0.02$), with a significant effect of age ($P=0.02$) and time after surgery ($P<0.001$). The effects were mainly driven by the increased infarct volume in RVCL-S KI mice at the age of 12 to 14 months (at 4 hours: RVCL-S KI: 60.4 ± 5.4 vs WT: 39.8 ± 4.8 mm³, $P=0.007$; 24 hours: RVCL-S KI: 84.3 ± 9.5 vs WT: 52.9 ± 7.8 mm³, $P=0.01$; 48 hours: RVCL-S KI: 114.0 ± 13.3 vs WT: 58.1 ± 10.7 mm³, $P=0.03$; Figure

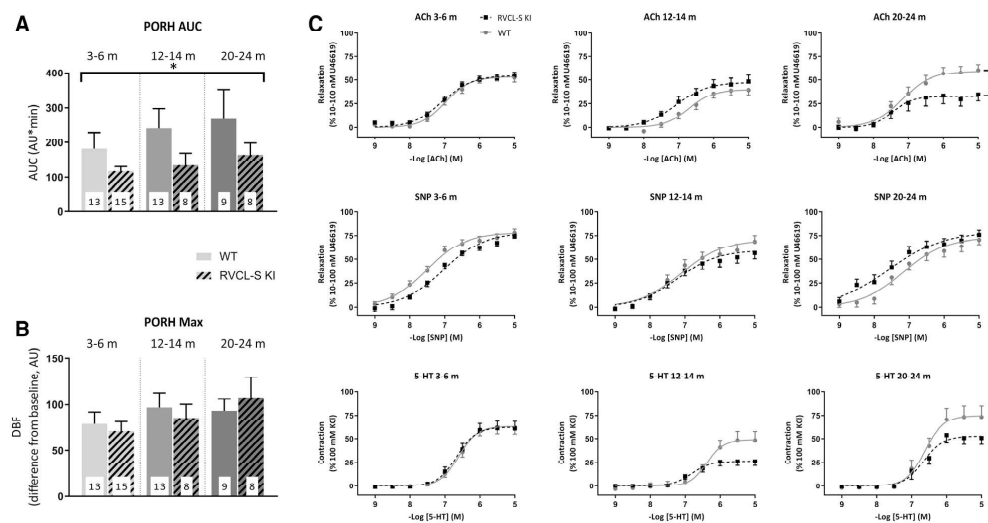


Figure 2. Microvascular and macrovascular characterization of wild-type (WT) and homozygous retinal vasculopathy (RVCL-S) knock-in (KI) mice. In vivo microvascular characterization using postocclusive reactive hyperemia (PORH) measurements of the hind leg with the area under the reactive hyperemia curve (A) and maximal reactive hyperemia (B) responses for WT and RVCL-S KI mice of the 3 age groups (3–6, 12–14, and 20–24 mo). * $p<0.05$ indicates a statistical significant difference between RVCL-S KI and WT mice across all age groups. Number of animals is indicated within each bar. (C) In vitro macrovascular characterization of isolated aortae obtained from WT (gray circles) and homozygous RVCL-S KI (black squares) mice. Panels depict concentration-response curves to acetylcholine (ACh, $n=7-16$), sodium nitroprusside (SNP, $n=7-17$), and serotonin (5-hydroxytryptamine [5-HT], $n=5-15$). * $p<0.05$ indicates statistical significance for attenuated relaxant responses to ACh in 20- to 24-mo-old RVCL-S KI mice compared with WT mice. AU indicates arbitrary units; AUC, area under the curve; DBF, dermal blood flow; M, molar; m, months; and Max, maximal.

3). There was no significant genotypic difference in cerebral vessel anatomy (Supplemental Figure 2 and Table 1). The overall neurological deficit score was worse in RVCL-S KI mice compared to WT for all age groups ($P=0.002$; Figure 4A). This effect was mainly driven by the 12- to 14-month-old group. In that age group neurological deficit scores was worse in RVCL-S KI than WT after surgery for an extended period of time (at 4 hours: RVCL-S KI: 29.3 ± 3.4 vs WT: 11.1 ± 2.9 , $P<0.001$; 24 hours: RVCL-S KI: 24.9 ± 3.4 vs WT: 9.9 ± 2.7 , $P=0.001$; 48 hours: RVCL-S KI: 28.5 ± 3.8 vs WT: 10.0 ± 2.6 , $P<0.001$; 5 days: 24.2 ± 3.7 vs 7.5 ± 2.4 , $P=0.001$), whereas there was no difference before surgery (presurgery: RVCL-S KI: 0.6 ± 0.4 vs WT: 0.5 ± 0.2 , $P=0.20$). Mortality within 5 days after stroke onset was also increased in the 12- to 14-month-old group (RVCL-S KI: 7 [8%] vs WT: 4 [25%], $P=0.04$), an effect also present in the 20- to 24-month-old group (RVCL-S KI: 8 [57%] vs WT: 2 [13%], $P=0.02$); the young age group only showed a non-significant trend (RVCL-S KI: 4 [57%] vs WT: 2 [33%]). There were no differences in basic parameters such as body weight (Supplemental Table 2) or activity levels (determined from presurgery analyses; Figure 4) between mutant and WT mice that could explain the difference in age-related survival.

DISCUSSION

Here, we investigated whether RVCL-S KI mice, which express a truncated mouse Trex1 protein that mimics the truncated protein seen in patients with the RVCL-S V235fs mutation,^{1,5} exhibit features in line with the phenotype of patients with RVCL-S. We demonstrated that homozygous RVCL-S KI mice have (1) a shorter life expectancy, (2) a vascular phenotype, as evidenced by abnormal PORH (microvascular characterization; *in vivo* in hind leg) and

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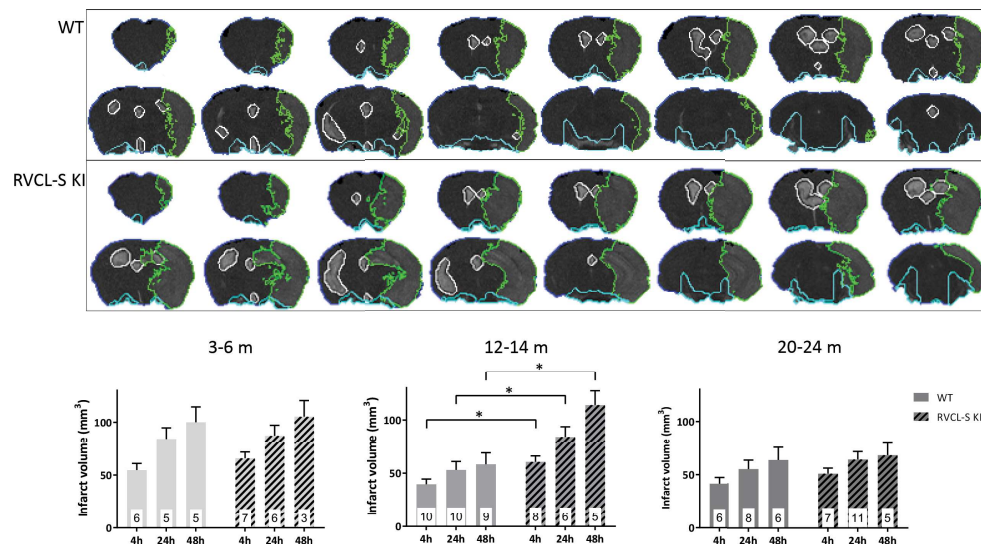


Figure 3. Infarct volume in wild-type (WT) and homozygous retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) knock-in (KI) male mice. A) Example traces of automated stroke segmentation (Green) in T2-weighted magnetic resonance imaging (MRI) images at 24 h after infarct induction in 12- to 14-month-old WT and RVCL-S KI mice. B) Stroke volumes at 4, 24 and 48 h after infarct induction in WT and RVCL-S KI mice of the 3 age groups (3–6, 12–14, and 20–24m; estimated marginal mean $\pm$ SD), * $p<0.05$ indicates a statistically significant difference between 12- to 14-month-old RVCL-S KI and WT mice for each individual time point. Number of animals is indicated within each bar. Numbers can vary due to death of the animal or technical failure of the MRI scan. h indicates hours; and m indicates months.

acetylcholine-induced relaxant (macrovascular characterization; in vitro in aortic segments) responses, and (3) an increased susceptibility to experimental stroke with a worsened outcome. Except for outcome measures of experimental stroke, the other features can be considered spontaneous phenotypes. Except for signs of abnormal microvascular responses that were already observed from a young age (3–6 months), all other abnormalities were only observed in old mice, that is, in mice of 12 to 14 months and 20 to 24 months. Our study in mice is relevant because it is not understood how vasculopathy, at middle-age, is brought about in patients with RVCL-S and how it is related to the lower life expectancy and increased risk for ischemic stroke.^{3–6} Similar to earlier observations in patients with RVCL-S, our data point to decreased functional endothelial responses in mice. In patients, this is exemplified by reduced flow-mediated dilatation responses,⁴ and in mice by reduced blood flow after PORH across all investigated ages and by reduced macrovascular relaxant responses to the endothelium-dependent vasodilator acetylcholine.

RVCL-S KI mice have a vascular phenotype

This study is the first to show a vascular phenotype in RVCL-S KI mice. The results of the *in vivo* PORH experiments point towards a microvascular dysfunction, with a decreased AUC of the PORH but a normal maximal PORH peak. As the effect was seen in all age groups, it shows that a vascular phenotype is developing already before the mortality and increased stroke susceptibility become apparent. The PORH response¹⁶ represents an interplay between multiple vasodilation pathways, such as the endothelium-derived hyperpolarizing factor pathway¹⁷ (which is characterized by the AUC) and the release of neuropeptides from perivascular nerves during the hyperemia peak (which is characterized by the maximal hyperemia response [E_{max}]).¹⁸ Notably, in vitro examinations of macrovasculature showed abnormal endothelial function in response to acetylcholine but only in very old RVCL-S KI mice. The decreased relaxant response to acetylcholine seems because of diminished

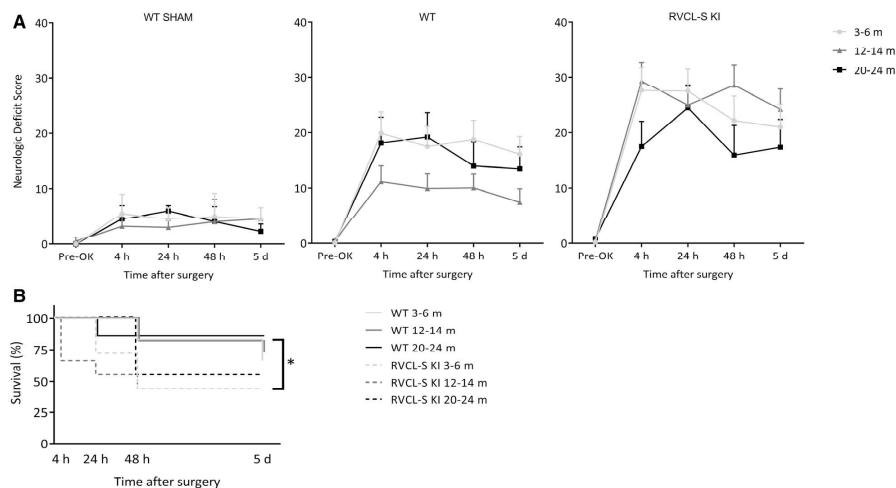


Figure 4. Behavior analysis after infarct induction in wild-type (WT) and homozygous retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) knock-in (KI) male mice. (A) Neurological deficit score (NDS; estimated marginal mean $\pm$ SD) for WT SHAM surgery and WT and RVCL-S KI post-transient middle cerebral artery occlusion (MCAO) and (B) survival curves at 4, 24, 48 h and 5 days after MCAO or SHAM surgery in WT (continuous line) and RVCL-S KI mice (discontinuous line) of the 3 age groups (3–6, 12–14, and 20–24 mo). * $p < 0.05$ indicates a statistically significant difference (A) between 12- to 14-mo-old RVCL-S KI and WT mice for NDS and (B) between RVCL-S KI and WT mice concerning survival post-surgery. d indicates days; h, hours; and m, months.

endothelial function, as responses to the endothelium-independent NO donor SNP were normal in RVCL-S KI mice of the same age category. As mentioned above, the *in vivo* PORH AUC data suggest involvement of an affected endothelium-derived hyperpolarizing factor pathway response. Endothelium-derived hyperpolarizing factor acts as vasorelaxant and is known to act in microvessels rather than in larger vessels.^{19,20} Thus, the results of our *in vitro* macrovascular studies are not in contrast with the microvascular PORH results. Testing the natural ligand 5-hydroxytryptamine²¹ showed no abnormal vasoconstrictive response in RVCL-S KI mice, although concentration-response curves tended to be lower in the 12- to 14- and 20- to 24-month-old groups compared with WT mice.

Increased Ischemic Vulnerability in RVCL-S KI Mice

The vascular dysfunction seen in RVCL-KI mice may be the underlying cause of increased stroke risk in patients with RVCL-S. Therefore, we investigated whether RVCL-S KI mice had a worse stroke outcome. Although the infarct area is still evolving days to weeks after MCAO, we used 48 hours as final magnetic resonance imaging readout point since infarct size on T2-weighted magnetic resonance imaging is maximized at 48 hours post-MCAO.²² An overall increased infarct volume was observed in RVCL-S KI mice, compared with WT, albeit driven by the 12- to 14-month-old group. Given the decreased survival in the very old mutant mice, one can envisage that no genotypic difference in infarct volume was seen in the 20- to 24-month-old group because mutant mice that are most affected had already died before reaching the age of inclusion. Endothelial dysfunction was shown to lead to a pro-coagulatory, pro-inflammatory and pro-liferative state, which predisposes to atherosclerosis and increases stroke risk,⁷ but it remains to be researched whether this mechanism explains the increased infarct size in RVCL-S KI mice. In future experiments, this should be investigated using electron microscopy for in-depth morphological examination to uncover the possible presence of subtle lesions of the brain endothelium. There remains the, in our view unlikely, possibility that thrombus formation in the vessel after MCAO may have influenced infarct outcome, as we have no *a priori* reason to assume that thrombus formation would be different between mutant and WT mice; although this, admittedly, has not been demonstrated, which would require the use of different methodology than used in the present study.

There are other striking differences for the various age groups when considering infarct size and outcome. Not finding a genotypic effect in young mice may be a reflection that the pathology has not advanced enough, although *in vivo* microvascular reactivity was already abnormal at that age. As alternative explanation, a genotypic effect may be masked by the seemingly unusually large stroke volume in the respective WT mice, as volumes in WT mice from that age analyzed in our lab usually are 5% to 20% smaller (previously published data¹¹). It is even more surprising that mutant mice of 20-24 months have a worse outcome after MCAO but no increased infarct size. One plausible explanation may be inclusion bias. Because animals with large infarcts have a higher *a priori* chance of dying within 48 hours from infarct induction, mice that do survive tend to have smaller infarcts. Apparently, the mutant mice that could be included still exhibit mutation-associated pathology, as reflected by the increased mortality. Such underlying pathology may also explain why a total of 50% of the 20- to 24-month-old RVCL-S KI mice (compared with 15% of the WT group; Figure 1) had already died before scheduled surgery. Of relevance, WT aged male mice (and rats) have smaller infarct volumes,²³ but worse functional outcome,^{24,25} when compared with young animals. It remains enigmatic why aged male mice have a decreased infarct size.^{26,27} Aged vessels may have undergone morphological and pathological changes,²⁸ such as structural alterations in

the vessel wall, atherosclerosis and vessel wall calcification,^{29,30} small vessel disease,³¹ and a reduced impairment of poststroke synaptic plasticity.^{27,32}

RVCL-S phenotype in mice *versus* humans

Although the truncated protein product in our KI mouse is very similar to the human canonical *TREX1* frameshift product, one always needs to be cautious when extrapolating data from transgenic mouse models to human disease. Although RVCL-S in humans is an autosomal dominant disorder, the presence of 1 mutated allele causes disease; in our experiments, we focused on homozygotes mice instead to increase chances of capturing disease aspects by doubling the genetic load. It is not uncommon to focus investigations on homozygous mutant mice first as the lifespan in mice (maximal ~2 years) is much shorter than in humans, so a mutation has less time to exert its effect.

In humans, RVCL-S is characterized by various structural abnormalities, foremost the vasculopathy of the retina and other organs, microinfarcts, and white matter lesions.^{5,6} A basic histological analysis of the eye and other organs in our mutant mice did not reveal such abnormalities, even not in mice that were far older than 12 months. This can be regarded as a shortcoming of our model, but this disease feature may not be captured because of the shorter lifespan of a mouse. Not finding histological abnormalities in our mutant mice is in accordance with findings in a previously described RVCL-S mouse model,^{8,33} that also expresses V235fs-truncated, albeit human, *Trex1* protein. In that model, DNase activity, a hallmark of *Trex1*, was maintained in truncated protein.³³ Both heterozygous and homozygous mutants were reported to be grossly normal with no signs of tissue inflammation; the mutant mice did exhibit striking elevations of auto-antibodies against non-nuclear antigens in the serum.³³ Of note, the authors did not report the decreased survival phenotype, likely because they did not investigate very old mice. We would consider both RVCL-S mouse models equally relevant and having multiple transgenic models will allow replication in separate models.

RVCL-S is considered a small vessel disease.⁵ Abnormal functionality of the microvasculature, exemplified by the abnormal PORH response, was observed in mutant mice already of young age. Moreover, in the stroke experiments, outcome (as in human ischemic stroke) is influenced by the status of the microvasculature bed. Both types of experiments show that abnormal functionality of the microvasculature is captured in our mouse model. We recently found that functional endothelial responses were decreased in patients with RVCL-S, while microvascular responses to capsaicin were normal (analyzed using flow-mediated vasodilatation).⁴ This points to endothelial dysfunction in RVCL-S, which is in line with our microvascular and macrovascular observations in the homozygous mutant mice. Although flow-mediated vasodilatation responses are generally considered as a measure for macrovascular function, it should be kept in mind that flow-mediated vasodilatation is a physiological response to the increased shear stress during reactive hyperemia and is thus critically dependent on the reactivity of the downstream microvasculature to transient ischemia.

Limitations

As discussed above, our study has several limitations. First, we used homozygous RVCL-S KI mice, although RVCL-S in humans is caused by the presence of only 1 mutated allele, and therefore, translation of the results should be done with caution. Second, several pathological features seen in RVCL-S in humans (ie, vasculopathy of the retina and other organs, microinfarcts, and white matter lesions) were not seen during basic examination

in our model. In future experiments, a more in-depth analysis, for example, using electron microscopy, should be done to address this point. Furthermore, because of study design, we used surviving older animals, which probably have a less severe aging and disease phenotype. It remains an enigma why experimental stroke outcomes in the oldest animals are less severe. In conclusion, we found that the homozygous RVCL-S KI mice exhibit early mortality in line with the reduced life expectancy seen in (heterozygous) patients with RVCL-S. Moreover, we observed a blunted (cerebral) microvascular response suggestive of endothelial involvement, which seems to occur already in young mutant mice, as well as an abnormal macrovascular response in old mutants. Finally, we observed an increased infarct size and worse outcome in homozygous RVCL-S KI mice in response to experimentally induced stroke, but it is at present unclear how this relates to the phenotype in patients with RVCL-S. Regardless, our RVCL-S KI mouse model seems very promising to help unravel the pathophysiology of RVCL-S.

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SUPPLEMENTAL MATERIAL

METHODS

Generation and characterization of transgenic *TREX1* V235fs knock-in mice.

The mouse *TREX1* gene was modified using a gene targeting approach, in such a manner that the gene product was truncated at amino acid position 235 (which is a Threonine codon in the mouse gene and a Valine codon in the human gene) and contained the same abnormal amino acid sequence until the new stop codon as seen in RVCL-S patients with the V235fs mutation (for details on the sequences, see Supplemental Figure 1D).

Targeting construct for the generation of RVCL-S KI mice (Supplemental Figure 1A).

The targeting construct contained the RVCL-S mutation and a PGK-driven neomycin selection cassette (Neo), flanked by LoxP sites, which had been introduced downstream of *TREX1* and upstream of neighbouring *Scotin*, between the polyA (pA) sequences of both genes.

Targeting ES cells and confirmation of correctly targeted alleles in mice by Southern blot analysis (Supplemental Figure 1B).

E14 ES cells were targeted according to standard procedures, and selected, correctly targeted, ES cells were injected into C57BL/6J blastocysts. Obtained chimeras were bred with C57BL/6J mice and germline transmission of the mutant allele was obtained, thus creating mice with the “RVCL-S + Neo” allele. The selection cassette was then removed by crossing the mutant mice with mice of the EIIA-Cre deleter strain¹ (in which Cre recombinase expression is driven by the EIIA early promoter), which resulted in mice with the RVCL-S KI allele. Heterozygous RVCL-S KI mice were subsequently interbred to provide mice with homozygous mutant and wildtype genotypes. Confirmation of correct targeting was obtained by Southern blotting of EcoRI-digested genomic DNA (from cortex) of mice of the various genotypes (*i.e.* before and after Cre-mediated recombination). Upstream (5') and downstream (3') probes (as depicted in Supplemental Figure 1A) were used for Southern blotting.

Verification of truncated *Trex1* protein by Western blot analysis (Supplemental Figure 1C).

Expression of truncated *Trex1* protein was confirmed by semi-quantitative Western blotting. To this end, cortex tissue of wildtype and homozygous RVCL-S KI mice that had been killed by cervical dislocation was used. Whole cell lysates were homogenized with a potter (ice-cooled), followed by short sonication using RIPA-buffer in the presence of protease inhibitor cocktail (Cat. No. 1836 170, Roche, Mannheim, Germany). Concentrations were measured (Pierce BCA Protein Assay Kit (Thermo, Cat#23225)) and 2 µg was loaded on a 15% PAA gel. After separation, proteins were transferred onto a nitrocellulose membrane. Blots were blocked with PBS/5% low fat milk/0.05% Tween-20 and subsequently incubated for 2 hours at room temperature with primary anti-mouse *Trex1* antibody (BD transduction material number 611986). (1:2,500 diluted in incubation buffer (PBS/0.05% Tween-20)). Secondary peroxidase labelled Rabbit anti-mouse antibody (DAKO, P0260) (1:2,000 diluted in incubation buffer) incubation was performed for 1 hour at room temperature. Primary actin antibody incubation was performed for 2 hours at room temperature (A2066, Sigma, St Louis, MO, USA) (1:2,000 diluted in incubation buffer). Secondary peroxidase-labelled Swine anti-rabbit antibody (DAKO, P0217) (1:2,000 diluted in incubation buffer) incubation was performed for 1 hour at room temperature. Western blotting was done according to the enhanced chemiluminescence ECL

protocols (Amersham). Semi-quantification was based on equal β -actin signal intensity (loading control) and revealed Trex1 protein bands of 26 KDa for the mutant allele (as predicted from the truncation) and 33 KDa for the wildtype gene product.

Direct Sanger sequencing of RT-PCR products verified expression of V235fs mutation containing RNA (Supplemental Figure 1D).

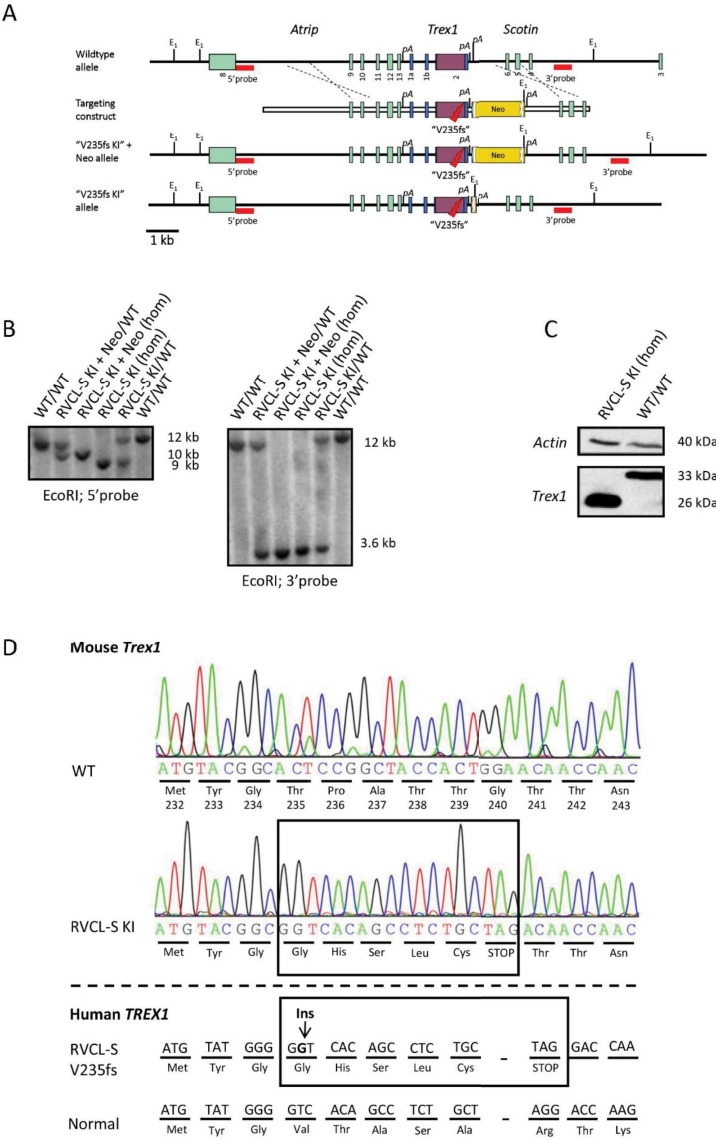
The presence of the truncation mutation at the RNA level was confirmed by direct sequence analysis of RT-PCR product. To this end, RNA was extracted from cortex of wildtype and homozygous RVCL-S KI mice that had been killed by cervical dislocation. Total RNA was isolated using RNA Instapure (Eurogentec, Seraing, Belgium). For RT-PCR, first-strand cDNA was synthesized using random primers, and subsequent PCR was performed using Trex1-specific primers (primer sequences are available from the authors upon request). PCR products were sequenced using standard conditions.

Details on the nature of human and mouse wildtype and mutant *TREX1/TREX1* sequences are provided for comparison. In brief, the relevant part of wildtype mouse *TREX1* sequence starting with the Valine codon at position 235, followed by a sequence of Thr-Ala-Ser-Ala, was changed to a sequence of His-Ser-Leu-Cys, followed by the premature stop codon, in the mutant sequence.

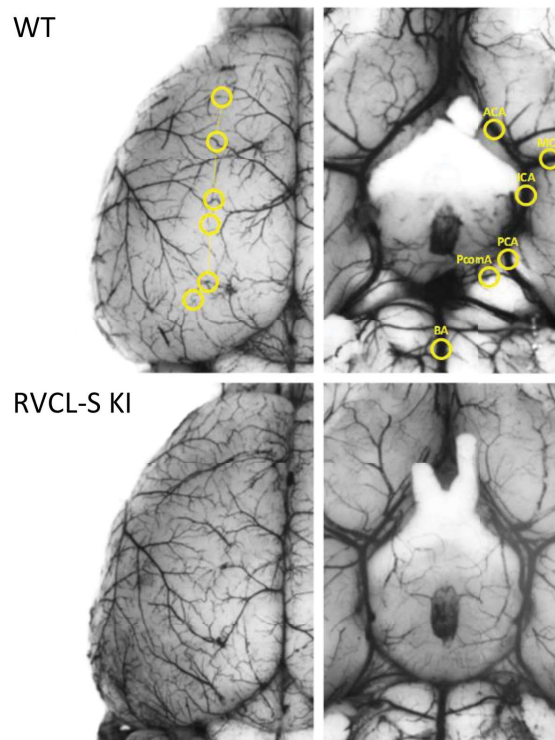
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FIGURES



Supplemental Figure 1. Generation and molecular characterization of RVCL-S KI mice. (A) The relevant part of the genomic structure of the wildtype *TREX1* allele, the targeting vector, and the predicted gene structure after homologous recombination ("V235fs KI + Neo" allele), and after Cre-mediated deletion of the Neo-cassette (Neo) (depicted as a yellow box) ("V235fs KI" allele) are shown. Numbered boxes indicate exons of *Atrip*, *Scotin* and *TREX1* genes. The position of the "V235fs" mutation in *TREX1*'s coding exon 2 (purple box) is indicated. Red horizontal lines indicate probes for Southern blot analysis. EcoRI restriction sites are marked as E1, polyadenylation signal sites are marked as pA. B) Southern blots of EcoRI-digested genomic DNA obtained from mice with the indicated combinations of the RVCL-S KI + Neo, RVCL-S KI, and wildtype alleles. Respective bands obtained with either the 5'- or the 3'-probe are indicated. WT: wildtype; Hom: homozygote. C) Western blot of cortex protein lysate isolated from wildtype (WT/WT) and homozygous (hom) RVCL-S KI mice probed with Trex1 or actin antibody reveals expression of truncated (26 kDa) or normal-sized (33 kDa) Trex1 protein; note that higher levels of Trex1 protein are present in the mutant compared to wildtype mice. D) Electropherograms of relevant parts (black boxes) of *TREX1* obtained from direct sequencing of RT-PCR products of cortex. Total RNA isolated from wildtype (WT) and homozygous RVCL-S KI mice are shown. For comparison, the respective sequences of mutant (RVCL-S) and normal human *TREX1* are shown below the dotted horizontal line.



Supplemental Figure 2. Representative examples of cerebral main vessel anatomy analysis of the Circle of Willis and pial collaterals in wildtype (WT) and homozygous RVCL-S KI mice. Representative ventral and dorsal views of WT and RVCL-S KI brains show the circle of Willis anatomy and pial arterial anastomoses between middle and anterior cerebral arteries. Pictures were taken after transcardiac ink perfusion under deep isoflurane anesthesia. Circles on the dorsal surface indicate examples of analyzed pial anastomoses (number and distance to midline were analyzed), whereas circles on the ventral surface indicate measurement areas for arterial diameters. None of these endpoints significantly differed between WT and RVCL-S KI mice (Supplemental Table 1).

TABLES

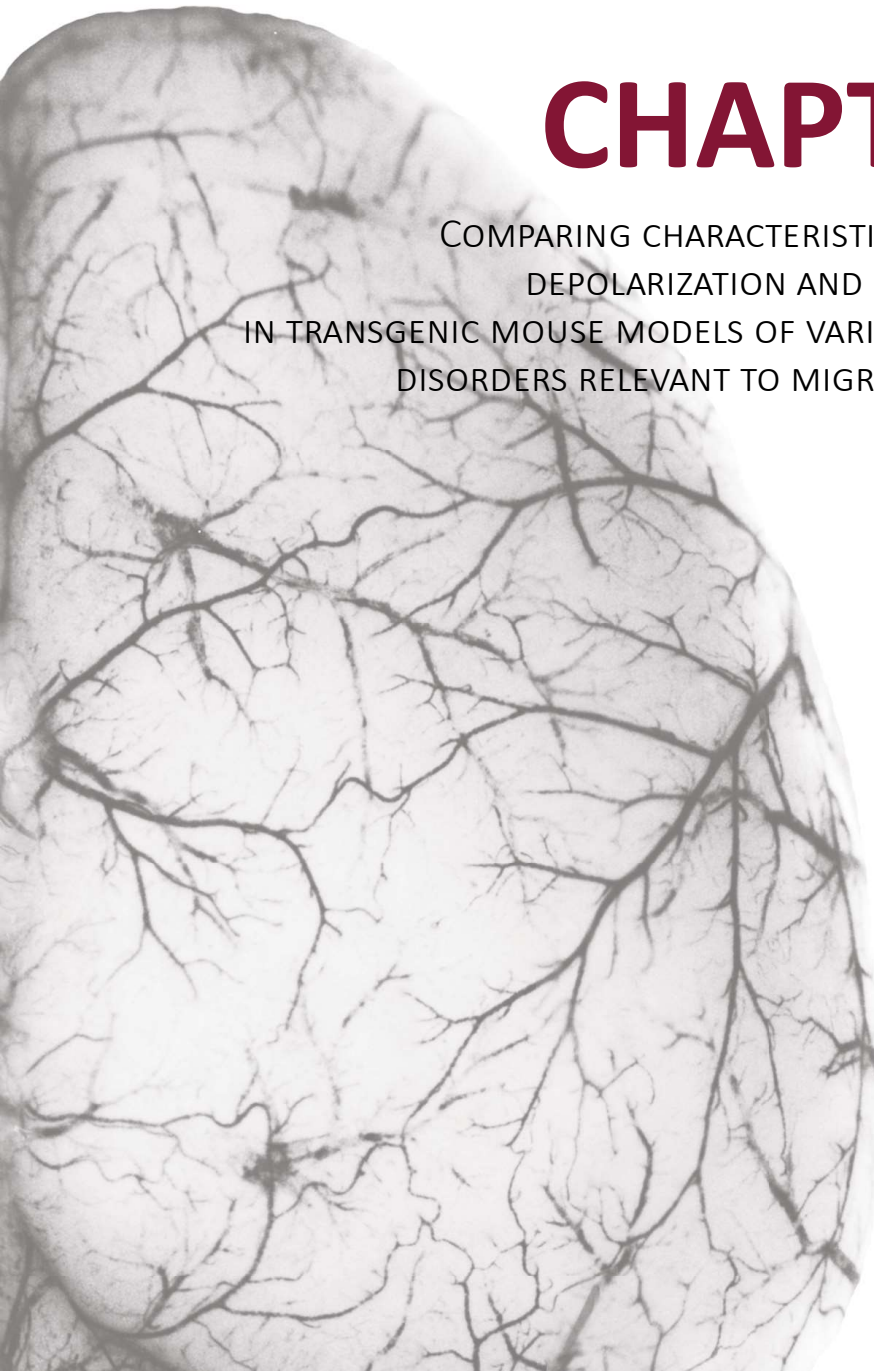
	WT (n=8)	RVCL-S KI (n=9)	p
ACA diameter (μm)	116 $\pm$ 26	114 $\pm$ 18	0.849
BA diameter (μm)	182 $\pm$ 24	168 $\pm$ 19	0.203
ICA diameter (μm)	150 $\pm$ 23	138 $\pm$ 16	0.101
MCA diameter (μm)	123 $\pm$ 24	111 $\pm$ 14	0.254
PCA diameter (μm)	132 $\pm$ 21	119 $\pm$ 15	0.080
PcomA diameter (μm)	31 $\pm$ 15	29 $\pm$ 10	0.559
# mice with unilaterally absent PcomA (n)	2	3	1.000
# pial anastomoses between MCA and ACA (n)	7.3 $\pm$ 1.21	7.6 $\pm$ 0.88	0.686
Anastomoses distance from midline (mm)			
Rostral 1/3 of cortex	1.76 $\pm$ 0.26	1.66 $\pm$ 0.16	0.335
Middle 1/3 of cortex	1.82 $\pm$ 0.31	1.70 $\pm$ 0.13	0.263
Caudal 1/3 of cortex	1.95 $\pm$ 0.29	1.95 $\pm$ 0.21	0.978

Supplemental Table 1. Cerebrovascular anatomy obtained using transcardiac ink perfusion. The representative images and the method of measurements can be found in Supplemental Figure II. ICA – Internal Carotid Artery; MCA – Middle Cerebral Artery; ACA – Anterior Cerebral Artery; PCA – Posterior Cerebral Artery; BA – Basilar Artery; PcomA – Posterior communicating Artery.

MORTALITY AND VASCULAR PHENOTYPE IN RVCL-S

Weight before surgery (mean (g) $\pm$ SD)	3–6 months (n=13)	12–14 months (n=28)	20–24 months (n=31)
WT	33.6 $\pm$ 2.0	39.6 $\pm$ 4.8	36.5 $\pm$ 4.5
RVCL-S KI	32.3 $\pm$ 3.2	38.0 $\pm$ 3.0	35.6 $\pm$ 5.3

Supplemental Table 2. Animal weight (Mean $\pm$ SD) per genotype and age group, measured the day of the surgical procedure (MCAO or SHAM surgery). SD – Standard deviation.



CHAPTER 6

COMPARING CHARACTERISTICS OF SPREADING
DEPOLARIZATION AND ISCHEMIC STROKE
IN TRANSGENIC MOUSE MODELS OF VARIOUS MONOGENIC
DISORDERS RELEVANT TO MIGRAINE AND STROKE

Mulder IA, van Heiningen SH, Broos LAM, Ferrari MD, Tolner EA,
Wermer MJH and van den Maagdenberg AMJM

ABSTRACT

Migraine and stroke have both been linked to monogenetic FHM1, CADASIL and RVCL-S, but it is not understood what mechanisms underlie this relation. Comparing characteristics of migraine and ischemic stroke in transgenic mouse models with pathogenic gene mutations for these diseases can be instrumental to dissect to what extent neuronal (FHM1) and vascular mechanisms (CADASIL, RVCL-S) contribute to disease pathology.

Cortical spreading depolarization (CSD) and transient middle cerebral artery occlusion (MCAO), as experimental surrogates of migraine aura and ischemic stroke, respectively, were induced in young (3- to 6-month-old; both CSD and MCAO) and/or old (12- to 14-month-old; only MCAO) FHM1, CADASIL and RVCL-S mutant mice. As measures of CSD susceptibility, frequency and propagation rate were assessed. Anoxic depolarization latency during MCAO as well as infarct volume and neurologic deficit before and following MCAO were studied to assess susceptibility to stroke. For each strain, mutant mice were compared to the appropriate wild-type controls.

Only young FHM1 mutant mice showed abnormal, increased CSD frequency (18.3 ± 0.6 vs 9.7 ± 0.7 , $p < 0.001$) and propagation rate (5.4 ± 0.7 vs 3.3 ± 0.5 , $p = 0.009$). Anoxic depolarization latency was overall decreased in both the FHM1 ($F(1, 24) = 7.928$, $p = 0.01$) and CADASIL ($F(1, 22) = 16.377$, $p = 0.001$) mutant mice. Infarct volume was increased only in RVCL-S mutant mice ($p = 0.005$ for pooled age groups, but mainly driven by the older mice) and correlated with increased neurologic deficit after infarct ($p = 0.005$).

Both neuronal and vascular mechanisms seem to play a role in the pathophysiology of FHM1, CADASIL and RVCL-S. The interaction between these mechanisms seems to be complex however, as increased CSD susceptibility did not associate well with an increased infarct volume and *vice versa*.

INTRODUCTION

Although migraine and stroke are distinct diseases, there is increasing evidence for comorbidity and overlapping pathology.^{1,2} Epidemiological studies have suggested that the link is more pronounced for migraine with aura,³ a migraine subtype in which the headache is preceded by transient focal neurological symptoms (“aura”).⁴ Human imaging and animal studies have indicated that cortical spreading depolarization (CSD), a wave of massive depolarization of neurons and glial cells that is accompanied by vasodilatation due to neurovascular coupling,¹ is the mechanism underlying the migraine aura.^{5,6} Waves with similar characteristics also occur in ischemic stroke, where they are referred to as peri-infarct depolarizations (PIDs).^{7,8} PIDs circle around the ischemic core, into the border zone (“penumbra”) thereby increasing the ischemic territory with each wave, due to, amongst others, paradoxical vasoconstriction and accompanying supply-demand mismatch.^{7,8} It has therefore been postulated that spreading depolarization (SD), with involvement of neuronal and vascular mechanisms, may explain the link between migraine and stroke.¹

The relation between migraine and stroke is also reflected in the clinical spectrum of various monogenic diseases.⁹ We envisaged that investigating transgenic mouse models for such disorders, and comparing measures of migraine (*i.e.* CSD) and stroke (*i.e.* ischemia after transient middle cerebral artery occlusion (MCAO)) in them, may shed light on the mechanisms relevant to migraine and stroke. Here we used mouse models for three disorders for which the relation between migraine and stroke (in patients and/or in animal studies) is most pronounced: Familial Hemiplegic Migraine type 1 (FHM1),¹⁰ Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL),¹¹ and Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S).¹² FHM1 is a monogenic form of migraine caused by specific missense mutations in CACNA1A that result in attacks of headache accompanied by an aura with hemiparesis.^{4,13} FHM1 exhibits a neuronal phenotype as shown by the enhanced neurotransmission and increased susceptibility to CSD in FHM1 mutant mice.¹⁴⁻¹⁶ FHM1 mice, either with the R192Q or the S218L mutation, were shown to also have an increased vulnerability to ischemic stroke, as evidenced by the increased infarct volume and decreased anoxic depolarization (AD) latency.¹⁷ Of note, a follow-up study only confirmed the infarct volume phenotype in the severer S218L mutant mice.¹⁸

CADASIL is a progressive small vessel disease caused by mutations involving cysteines in *NOTCH3* that result in ischemic strokes and (vascular) dementia.^{19,20} Notably, the presenting symptom of CADASIL in 40% of patients is migraine with aura.^{21,22} CADASIL exhibits a vascular phenotype as evidenced by an accumulation of mutant protein in vascular smooth muscle cells in a transgenic overexpressor mouse model for CADASIL.^{23,24} Of note, another CADASIL mouse model expressing a different NOTCH3 mutation also showed hallmarks of CADASIL, and interestingly, also an increased susceptibility for CSDs.²⁵

Finally, RVCL-S, which is caused by C-terminal truncating mutations in *TREX1* that cause systemic microvascular vasculopathy with white matter lesions.²⁶ Many patients with RVCL-S also have migraine.²⁶ RVCL-S exhibits a vascular (endothelial) phenotype but the exact mechanism that results in pathology is not known. An unpublished knock-in (KI) mouse model that expresses truncated Trex1 protein was generated and analyzed in the present study.

Given that disease onset in patients with CADASIL²⁷⁻²⁹ and with RVCL-S¹² typically occurs at middle age, but at relatively young age in patients with FHM,¹⁰ we compared readouts for migraine and ischemic stroke in young (3- to 6-month-old; both CSD and ischemic stroke) and old (12- to 14-month old; only ischemic stroke) FHM1, CADASIL and RVCL-S mice and the

appropriate wild-type (WT) control animals.

MATERIALS AND METHODS

Mice

Male homozygous transgenic mice of 3- to 6-month-old (both CSD and MCAO groups) and 12- to 14-month-old (only MCAO group) and appropriate WT control animals were used for the experiments. Mutant mice carried either the FHM1 missense mutation R192Q knock-in (KI)¹³ (strain R192Q), the CADASIL missense mutation R182Q³⁰ (overexpressor strain tgN3^{MUT350}) or the RVCL-S truncating mutation at residue 235³¹ (unpublished strain RVCL-S). For the FHM1 and RVCL-S strains non-transgenic littermates were used as controls (from this point on referred to as R192R and WT, respectively). Instead, for the CADASIL strain, mutant mice that carried a human genomic construct with the *NOTCH3* gene and the mutation were compared with transgenic mice that carried a similar construct but without the mutation strain tgN3^{WT}.²⁴ For the experiments, animals were randomized and researchers were blinded for genotype and age. Animals were housed in a controlled environment with food and water *ad libitum*. After the experiments genotypes were confirmed. All experiments were approved by the local committee for animal health, ethics and research of the Leiden University Medical Center.

Experimental Infarct Model

Ischemic stroke was induced using the MCAO model.³² During surgery, isoflurane (3% induction, 1.5% maintenance) in 70% pressurized air and 30% O₂ was used as anesthetic. Before surgery, pain relief medication was given (5 mg/kg, *s.c.*; Carporal, 50 mg/mL, AST Farma BV; Oudewater, the Netherlands). Body temperature of the mice was maintained at 37 ± 0.3°C during surgery using a feedback system including heating pad and rectal probe (FHC Inc.; Bowdoin, ME, USA). To occlude the MCA, a silicone-coated nylon monofilament (7017PK5Re; Doccol Company, Redlands, CA, USA) was inserted via a small incision into the right common carotid artery. The origin of the MCA was blocked for 30 minutes, after which the filament was removed to allow for reperfusion. During surgery, cerebral blood flow (CBF) in the MCA territory was measured using Laser Doppler Flowmetry (PeriFlux System 5000; Perimed Järfälla-Stockholm, Sweden). Anoxic depolarization (AD) was measured using LDF and defined as the time between start of the MCA occlusion until the start of an additional subtle drop in blood flow, which is known to be the result of the first PID spreading through the brain tissue.³³ During a surgical recovery period of 2 hours, the mouse was allowed to wake up in a temperature-controlled incubator (V1200; Peco Services Ltd, Brough, UK). At the end of the experiment, *i.e.* after MRI and behavioral analyses (see below), mice were sacrificed using CO₂ and perfused transcardially with phosphate buffered saline and fresh 4% buffered PFA (Paraformaldehyde P6148; Sigma-Aldrich, St Louis, MO, USA) at 4°C and stored at -80 °C (for parallel histological studies).

MRI

At 4, 24 and 48 hours after MCAO surgery, mice were scanned (under isoflurane anesthesia; 3% induction, 1.5% maintenance in 70% pressurized air and 30% O₂) using a 7T small-animal MRI system (Bruker Pharmascan; Bruker, Ettlingen, Germany). A Multi Slice Multi Echo (MSME) sequence protocol was used with a TR/TE of 4.000 ms/ 9 ms, 20 echoes, 2 averages, matrix

128x128 mm, FOV of 2.50 cm, bandwidth 59523.8Hz, 16 slices with a thickness of 0.5 mm (no gap). Mice were allowed to wake up in a temperature-controlled incubator (Peco Services Ltd) for maximal 1 hour. Using Paravision 5.1 software (Bruker), quantitative T2-maps were calculated. Infarct volume was calculated using an automated lesion volume measurement tool.³⁴ In 5 MRI scans, large errors due to scan artifacts (as a result of movement of wrapping) were manually corrected.

Behavior analysis

Directly before surgery, directly before each MRI session, and 5 days after surgery, behavioral tests were performed and recorded. By analyzing the videos, the neurologic deficit score (NDS) was obtained using a 56-point neurological function scale.^{35,36} The score involved general and focal deficits, where 0 represents 'no deficits' and 56 represents 'poorest performance' in all categories. Categories concerning general appearance and performance include scores regarding fur (0–2), ears (0–2), eyes (0–4), posture (0–4), spontaneous activity (0–4), and epileptic behavior (0–12). Concerning focal deficits scores were stated for body asymmetry (0–4), gait (0–4), climbing on a 45° inclined surface (0–4), circling behavior (0–4), front-limb symmetry (0–4), compulsory circling (0–4), and whisker response to light touch (0–4).

Experimental cortical spreading depolarization

Separate groups of mice were anesthetized using 1.5% isoflurane in 20% O₂ and 80% N₂O. Blood gasses and mean arterial pressure values were monitored via a catheter in the left femoral artery, as described elsewhere.³⁷ In brief, after insertion of the catheter, an endotracheal tube was inserted in the trachea that allowed for artificial ventilation (MiniVent Ventilator, Model 845, Harvard Apparatus, Holliston, MA, USA). Arterial blood gasses (pCO₂, pO₂) and pH were measured at the start and end of the recordings and were maintained within normal limits (adjusting ventilation when needed). For CSD induction, the mouse was placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). Core body temperature was maintained at 37°C ± 0.3°C. After exposure of the skull, three burr holes over the right hemisphere were prepared at the following coordinates: (I) for CSD induction on the occipital cortex (3.5 mm posterior, 2.0 mm lateral from bregma), (II) recording electrode in the frontal cortex (1.0 mm anterior, 2.0 mm lateral from bregma), and (III) recording electrode in the parietal cortex (1.0 mm posterior, 2.0 mm lateral from bregma). At the recording sites, a sharp glass capillary electrode (FHC Inc.) filled with 150 mM NaCl was advanced to a depth of 200–300 µm. DC-potential signals were measured with respect to an Ag/AgCl reference electrode placed subcutaneously in the neck of the animal. A reversible DC-deflection with an amplitude >5 mV was considered positive concerning a CSD event. Data were sampled (200 Hz), amplified (10X) and low-pass filtered at 4 Hz and analyzed off-line using LabChart software (ADInstruments, Colorado Springs, CO, USA). CSD events were induced by placement of a cotton ball soaked in 300 mM KCl on the dura for 30 minutes, with refreshment of the cotton ball every 15 minutes. The total number of CSD events that occurred within 30 minutes was used to calculate the frequency per hour. The time a first CSD event needed to travel from the first (parietal) measurement electrode to the second one (frontal) was used to calculate the propagation rate.

Statistics

Statistical analyses were performed in SPSS (SPSS Statistics 23, IBM Corporation, Armonk, NY,

USA). CSD frequency and propagation rate (mean $\pm$ SEM) were analyzed between mutant and WT mice using a two-tailed Mann-Whitney U test. Time until occurrence of AD during MCAO (mean $\pm$ SEM) for both ages was analyzed per genotype and accompanying WT using univariate ANOVA. Infarct volume and NDS were compared between mutant and WT mice using marginal mixed-models analyses. Data are shown as estimated marginal means $\pm$ SEM. Survival analyses post-OK were performed using the Log-rank (Mantel-Cox) test. P-values <0.05 were considered to indicate statistical significance.

RESULTS

CSD frequency and propagation rate

Young FHM1 mutant mice showed an increased frequency (R192Q (n = 8): 18.3 ± 0.6 vs R192R (n = 7): 9.7 ± 0.7 , $p < 0.001$) and propagation rate (R192Q: 5.4 ± 0.7 vs R192R: 3.3 ± 0.5 , $p = 0.009$) of CSD (Figure 1A and B). For CADASIL mice of the same age group, no genotypic difference was observed for frequency (tgN3^{MUT350} (n = 2): 9.1 ± 3.2 vs tgN3^{WT} (n = 3): 9.9 ± 1.6) or propagation rate (tgN3^{MUT350}: 3.1 ± 0.5 vs tgN3^{WT}: 2.8 ± 0.1) of CSD, but the group sizes were too small to perform statistical analyses (Figure 1A and B). For RVCL-S mice no genotypic difference was observed for frequency (RVCL-S KI (n = 9): 9.1 ± 1.8 vs WT (n = 8): 9.9 ± 1.0) or

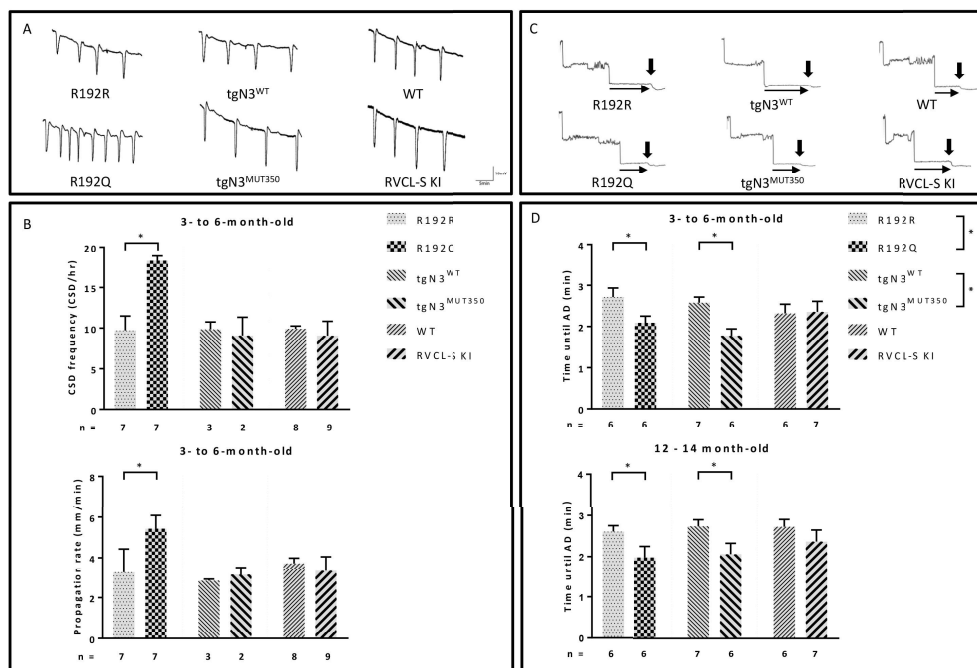


Figure 1. CSD and AD characteristics in FHM1, CADASIL and RVCL-S KI mice. (A) CSD example traces and (B) CSD frequency in WT and mutant FHM1 (R192Q), CADASIL (tgN3^{MUT350}) and RVCL-S KI mice (3- to 6-month-old). Only FHM1 mutant mice display enhanced CSD frequency and propagation rate. (mean $\pm$ SEM, $*p < 0.05$). (C) Laser Doppler example traces of 3- to 6-month-old mice of the various genotypes show cerebral blood flow (CBF) reduction at the time of common carotid artery occlusion that is followed by a transient occlusion of the middle cerebral artery. The third decline in CBF shows the onset of anoxic depolarization (AD) and represents vasoconstriction of ischemic microvasculature due to tissue depolarization. (D) AD latency of 3- to 6-month-old and 12- to 14-month-old mice of the various genotypes show a decreased latency in FHM1 and CADASIL mutant mice, but not in the RVCL-S KI mutant mice. (mean $\pm$ SEM, $*p < 0.05$). SEM = Standard Error of the Mean.

propagation rate (RVCL-S KI: 3.3 ± 0.7 : 3.7 ± 0.7) of CSD (Figure 1A and B).

Latency of anoxic depolarization

FHM1 mutant mice of both young and old age groups showed a decrease in AD latency ($F(1, 24) = 7.93$, $p = 0.010$; young mice: R192Q ($n = 6$): 2.2 ± 0.2 vs R192R ($n = 6$): 2.6 ± 0.3 ; and old mice: R192Q: 2.1 ± 0.4 ($n = 9$) vs R192R: 2.5 ± 0.2 ($n = 7$)) (Figure 1C and D). Also CADASIL mutant mice of both age groups had a decrease in AD latency ($F(1, 22) = 16.38$, $p = 0.001$; young mice: tgN3^{MUT350} ($n = 6$): 2.0 ± 0.2 ($n = 6$) vs tgN3^{WT} ($n = 7$): 2.5 ± 0.2 ; and old mice: tgN3^{MUT350} ($n = 6$) 2.1 ± 0.3 vs tgN3^{WT} ($n = 7$) 2.6 ± 0.2) (Figure 1C and D). However, no genotypic difference was found for AD latency in RVCL-S KI mutant mice (young mice: RVCL-S KI ($n = 7$): 2.3 ± 0.3 vs WT ($n = 6$): 2.3 ± 0.3 ; and old mice: RVCL-S KI ($n = 8$): 2.3 ± 0.5 vs WT ($n = 9$): 2.5 ± 0.3) (Figure 1C and D).

Infarct volume

With respect to infarct volume and evolution of infarct volume over time, no genotypic difference was found for FHM1 and CADASIL, for both young and old mice (see Figure 2). Instead, for RVCL-S KI mice there was an overall genotypic difference ($p = 0.005$) that was mainly driven by the group of older mice in which infarct volume in the mutants was increased at all three time points (4 hours, RVCL-S KI: 60.4 ± 5.3 ($n = 8$) vs WT: 39.8 ± 4.7 ($n = 10$), $p = 0.007$; 24 hours, RVCL-S KI: 82.6 ± 7.8 ($n = 6$) vs WT: 52.9 ± 6.4 ($n = 10$), $p = 0.009$; and 48 hours, RVCL-S KI: 110.7 ± 12.7 ($n = 5$) vs WT: 58.4 ± 10.0 ($n = 9$), $p = 0.004$) (Figure 2).

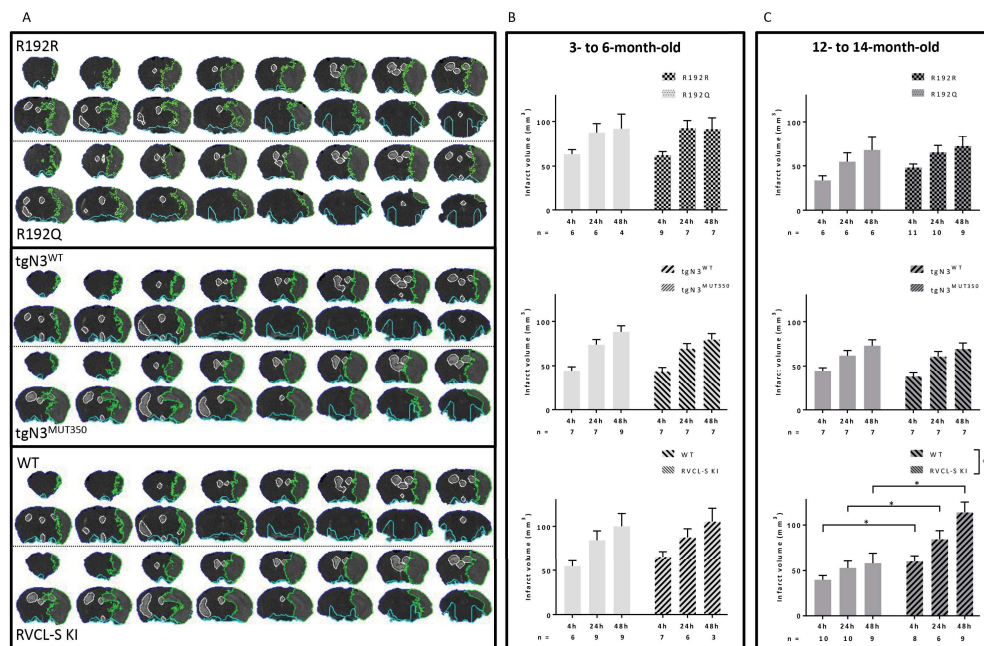


Figure 2. Infarct volume in FHM1, CADASIL and RVCL-S KI mice. (A) MRI example sections at 24 hours post MCAO in WT and mutant mice of the various genotype groups (3- to 6-month-old). (B) Stroke volumes at 4, 24 and 48 hours after MCAO in mutant and WT mice of 3- to 6-month-old and (C) 12- to 14-month-old (mean $\pm$ SEM, * $p < 0.05$) reveal an overall increased infarct volume only for RVCL-S mutant mice, which was mainly driver by the infarct volumes of the older aged mice. SEM = Standard Error of the Mean.

Behavior analysis

Neither for the FHM1 nor the CADASIL mutant mice an overall genotypic difference (across both age groups) was found with respect to the neurologic deficit score (NDS) (Figure 3A). However, a sub-analysis showed that the NDS in mutant CADASIL mice was different for old mice at the two latest time points (24 hours, tgN3^{MUT350}: 23.0 ± 1.8 vs tgN3^{WT}: 16.0 ± 1.8 , $p = 0.01$; and 48 hours, tgN3^{MUT350}: 24.4 ± 2.1 vs tgN3^{WT}: 15.0 ± 2.2 , $p = 0.005$) (Figure 3B). An overall genotypic difference for (across both age groups) was observed for RVCL-S mice ($p = 0.005$). Sub-analysis showed that NDS was increased in old mice at all three time points (4 hours, RVCL-S KI: 31.4 ± 2.6 vs WT: 15.6 ± 2.4 , $p < 0.001$; 24 hours, RVCL-S KI: 26.4 ± 3.1 vs WT: 13.8 ± 2.7 , $p = 0.005$; and 48 hours, RVCL-S KI: 24.6 ± 3.3 vs WT: 13.1 ± 2.4 , $p = 0.012$) (Figure 3C).

Mortality rates were comparable between FHM and CADASIL mutant mice compared to the respective WT control groups for both age groups (Figure 4A and B). Instead, a trend was seen towards increased mortality for RVCL-S KI mice compared to WT control mice for both age groups (Figure 4C).

DISCUSSION

Here we compared characteristics of migraine (by induction of CSD) and ischemic stroke (by induction of an experimental infarct) in three monogenic models in which the relation between migraine and stroke is most prominent.

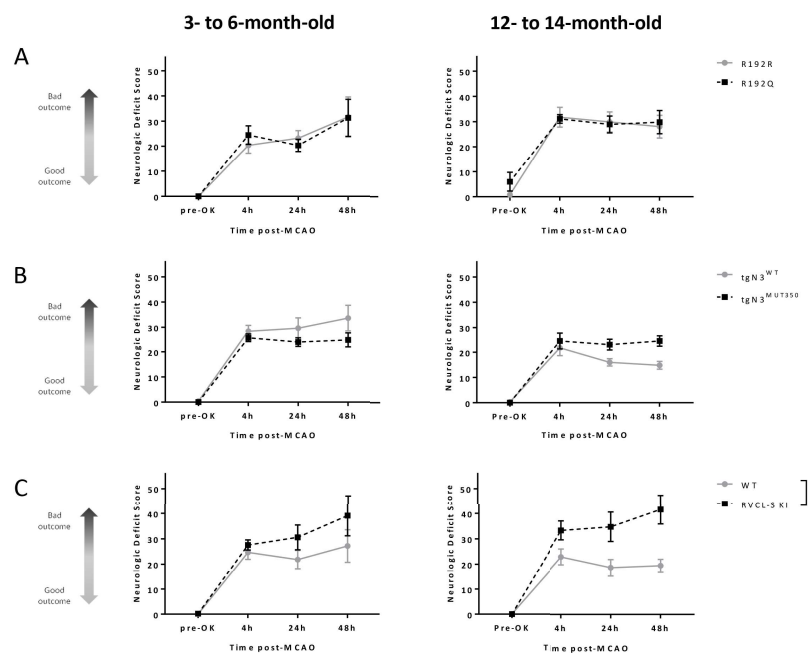


Figure 3. Behaviour analysis of FHM1, CADASIL and RVCL-S KI mice before and after ischemic stroke. Neurologic deficit scores pre-MCAO and at 4, 24 and 48 hours after surgery in the various mutant and WT mice of 3- to 6 and 12- to 14-month-old. (A) FHM1, (B) CADASIL and (C) RVCL-S KI. Figures reveal an overall worsened score only for RVCL-S mutant mice, which was mainly driver by the older aged mice (mean $\pm$ SEM, * $p < 0.05$).

With respect to CSD susceptibility in (male) FHM1 mice, we observed an increased frequency and propagation rate as reported before (although in literature both readouts were not significantly different).³⁸ In fact, the absolute frequency was increased in our study compared to the previous study (~18 vs ~12/hour, respectively), whereas the absolute propagation rate was similar between studies (~5 vs ~4/min, respectively). In WT mice, frequency (~9 vs ~10/hour, respectively), and propagation rate (~2.5 vs ~3/min, respectively) were comparable between both studies. Moreover, whereas the observed decreased AD latency in the FHM1 R192Q mutants in response to MCAO was similar to what was described before (2.6 min vs 2.5 min, respectively),¹⁷ we did not observe the increased infarct volume in the mutant mice. Absolute values for infarct volume in WT mice in our study had a narrower range and were on average higher (~65 mm³ to ~85 mm³), compared to the data of the previous study¹⁷ (~35 mm³ to ~80 mm³).

This could, in hindsight, have led to the incorrect assumption that the infarct volume in the FHM1 mutant mice is increased compared to WT mice. An alternative, but less likely, explanation for the discrepancy between studies could be subtle differences in the MCAO surgery. Whereas we used the CCA as the entrance for the filament, in the previous described study the ECA was used. With both methods, the entrance artery stays occluded after surgery, which in our case could possibly have led to a higher infarct volume already in the WT animals that may have masked a small genotypic difference. Our rationale is supported by the fact that no increase in infarct size in FHM1 R192Q mice was found by the same researchers in a follow-up study.¹⁸ Moreover, the NDS and mortality rate also did not show a genotypic difference.

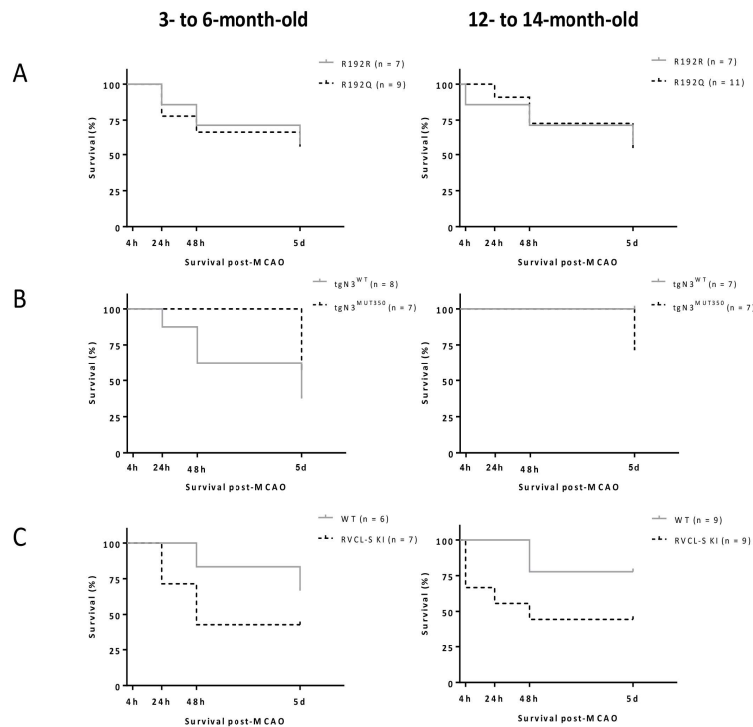


Figure 4. Mortality in FHM1, CADASIL and RVCL-S KI mice after induction of ischemic stroke. Mortality after MCAO surgery during the 5-day survival period for (A) FHM1, (B) CADASIL and (C) RVCL-S KI mutant mice and the respective control mice for both age groups.

With respect to CSD susceptibility in CADASIL mutant mice, we did not observe a genotypic difference for either frequency or propagation rate, unlike what has been reported.²⁵ Absolute values for the current and the previous study for frequency (WT: ~ 10 vs $\pm \sim 9$ /hour and mutant: ~ 9 vs ~ 11 /hour, respectively) and propagation (WT: ~ 2.5 vs ~ 2.5 /min and mutant: ~ 3.0 vs ~ 3.5 , respectively) were similar. This shows that the genotypic difference with respect to CSD characteristics is small and perhaps not relevant to explain disease pathophysiology; admittedly, by increasing the group sizes we may even find the previously observed genotypic difference. In this respect, it is important to point out that different types of CADASIL mutant lines were used in the two studies. Whereas Eikermann-Haerter *et al.*²⁵ used transgenic mice overexpressing human *NOTCH3* cDNA (driven by the SM22a smooth muscle cell promoter) containing CADASIL mutation Arg90Cys (TgNotch3^{R90C}),³⁹ we used transgenic mice in which human *NOTCH3* with the Arg182Cys mutation was overexpressed from a genomic human construct with endogenous regulatory elements present.²² Of note, expression of human mutant NOTCH in the tgN3^{MUT350} CADASIL line we used for our study was shown to be 350% of that of endogenous *NOTCH3* RNA expression,²⁴ whereas *NOTCH3* expression in the TgNotch3^{R90C} CADASIL line was <25% of endogenous expression.³⁹ This study is the first to investigate experimental stroke in CADASIL mutant mice. Given the vasculopathy seen in CADASIL patients and the, albeit, subtle difference in CSD characteristics reported earlier, we *a priori* expected a worsened stroke outcome in the mutant mice. Especially since we observed a decrease in AD latency in the young mutant mice, which would translate to a vasoconstrictive effect on the microvasculature.³³ Surprisingly, we did not find a genotypic difference in infarct volume, neither in young nor old mice. Given that CADASIL usually has a middle-aged onset, with infarct phenotype at an even later stage, it would be interesting to investigate even older (20- to 24-month-old) mice.

In the RVCL-S KI strain, we did not observe a genotypic difference for CSD frequency, propagation rate, nor AD latency, which is perhaps not surprising as these measures reflect mostly neuronal and partially vascular mechanisms³³ whereas RVCL-S is not a neuronal phenotype. Instead, we did observe an increased infarct volume and NDS, which could be the result of endothelial vascular dysfunction. Critical hypoperfusion that results in a lower ischemic threshold could lead to an increased infarct volume and perhaps also the subcortical brain lesions seen on MRI in RVCL-S patients.⁴⁰

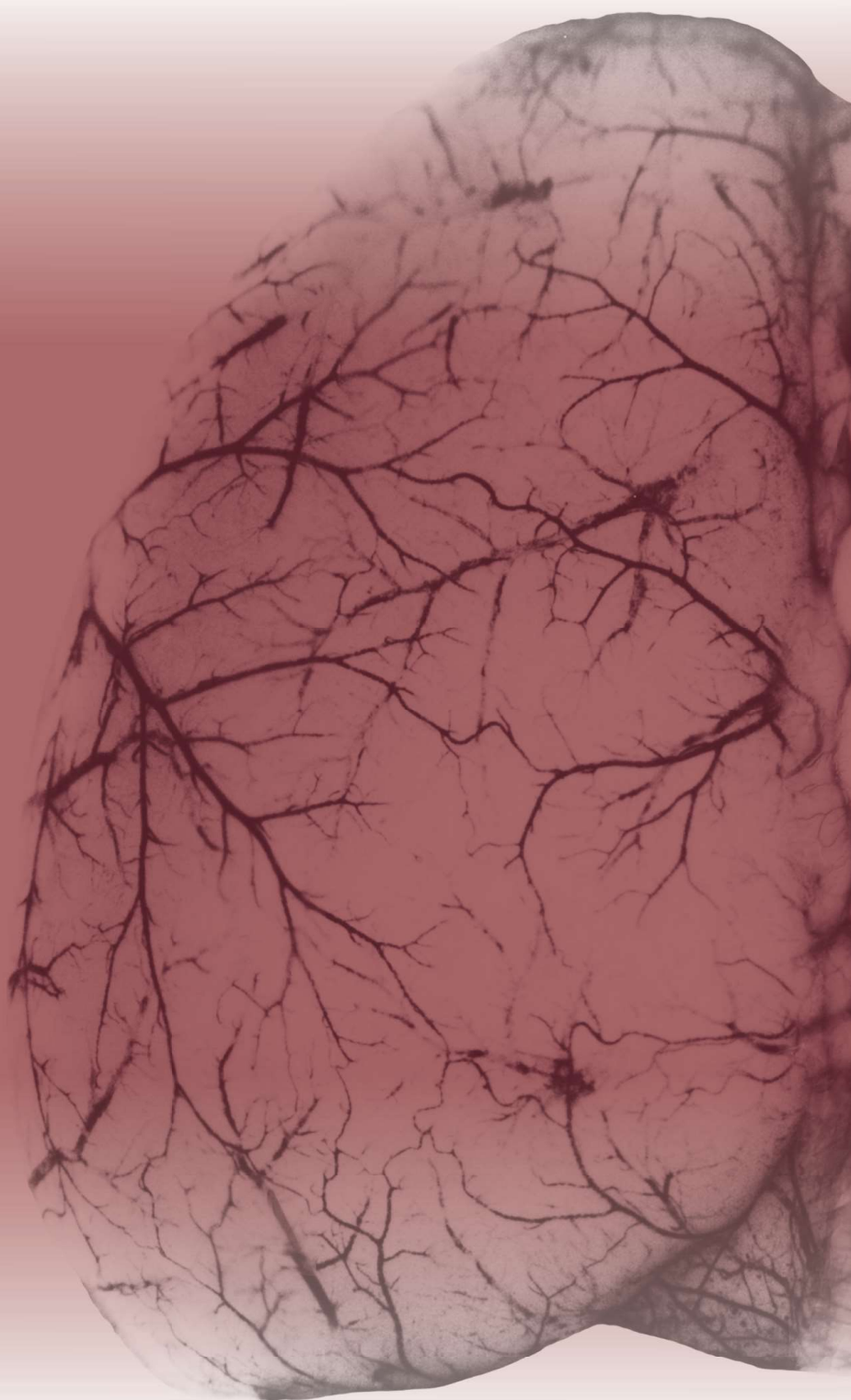
We aimed to unravel molecular and neurobiological mechanisms of stroke and migraine concerning CSD and ischemic infarct characteristics. Both neuronal and vascular mechanisms, and the close interplay between them, are believed to play an important role in monogenic diseases as FHM1, CADASIL and RVCL-S. The relation appears complex because as illustrated by our finding that susceptibility to CSD does not directly correlate with infarct volume. Nevertheless, our finding support the notion that the relationship between migraine and stroke has both neuronal and vascular components.

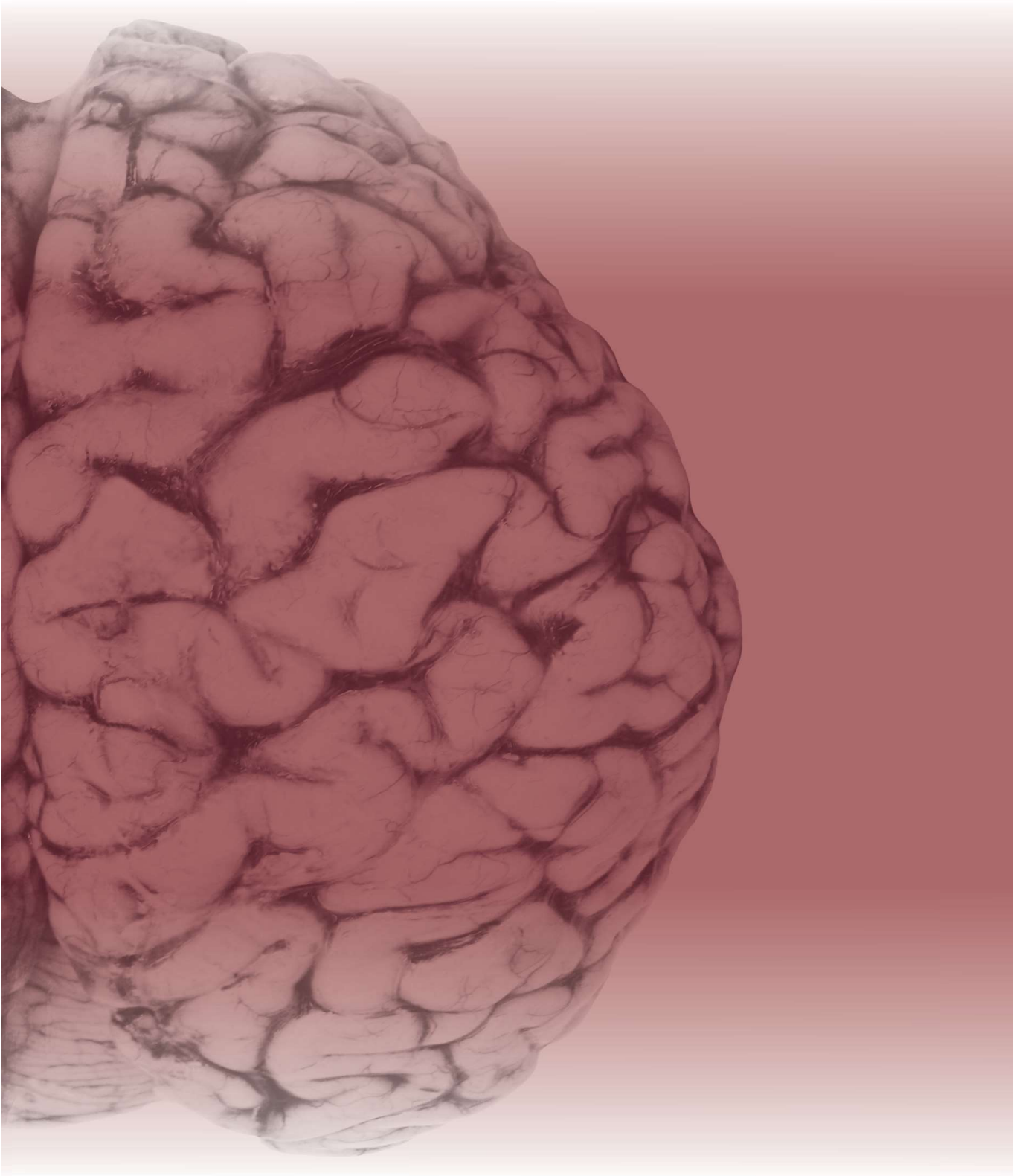
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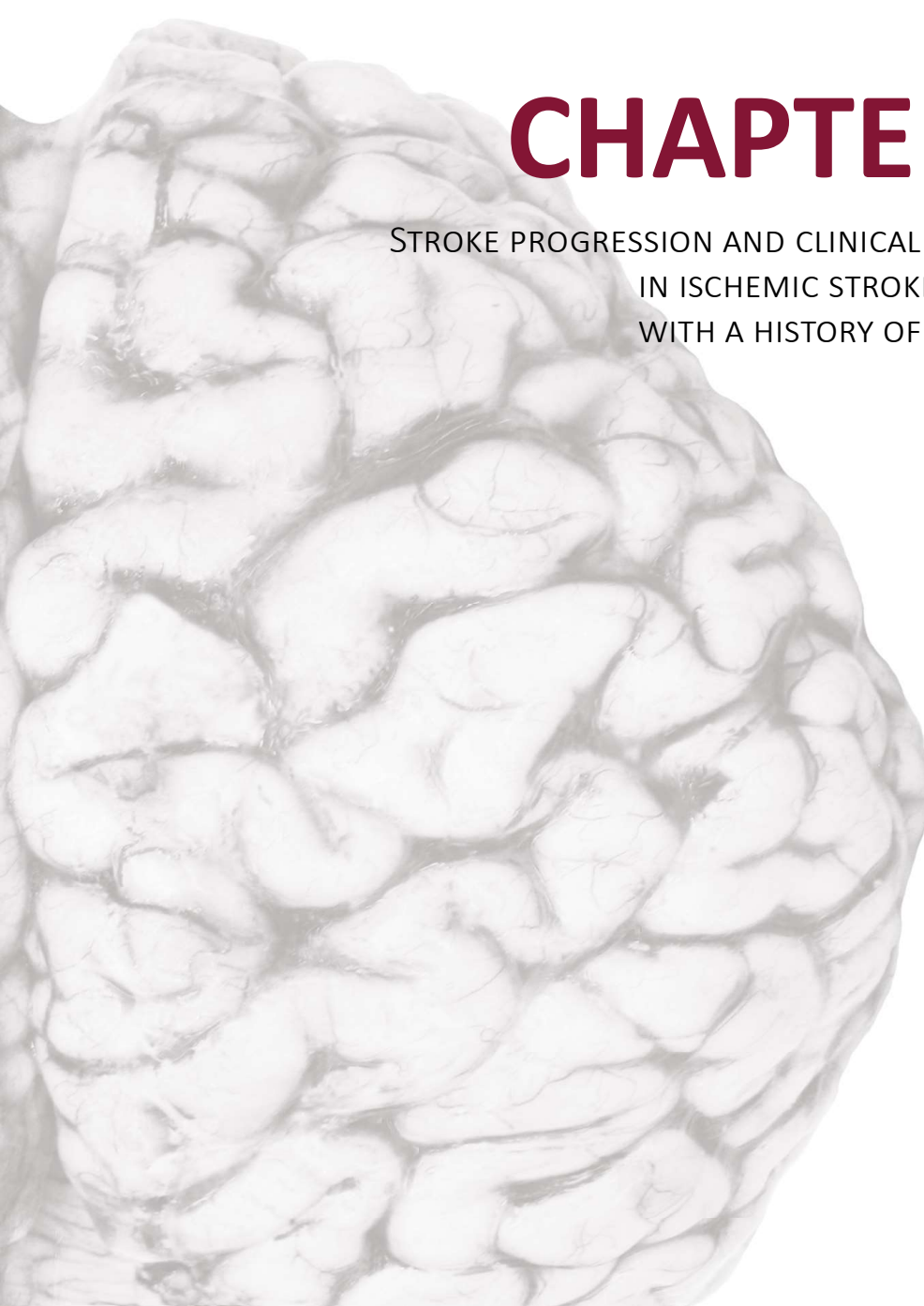
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CHAPTER 7

STROKE PROGRESSION AND CLINICAL OUTCOME
IN ISCHEMIC STROKE PATIENTS
WITH A HISTORY OF MIGRAINE

Mulder IA*, Holswilder G*, van Walderveen MA, van der Schaaf IC, Bennink E,
Horsch AD, Kappelle LJ, Velthuis BK, Dankbaar JW, Terwindt GM, Schonewille WJ,
Visser MC, Ferrari MD, Algra A and Wermer MJH; DUST investigators

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ABSTRACT

Patients with migraine might be more susceptible of spreading depolarizations, which are known to affect vascular and neuronal function and penumbra recovery after stroke. We investigated whether these patients have more severe stroke progression and less favorable outcomes after recanalization therapy.

We included patients from a prospective multicenter ischemic stroke cohort. Lifetime migraine history was based on the International Classification of Headache Disorders II criteria. Patients without confirmed migraine diagnosis were excluded. Patients underwent CT angiography and CT perfusion <9 h of onset and follow-up CT after three days. On admission, presence of a perfusion deficit, infarct core and penumbra volume, and blood brain barrier permeability (BBBP) were assessed. At follow-up we assessed malignant edema, hemorrhagic transformation, and final infarct volume. Outcome at three months was evaluated with the modified Rankin Scale (mRS). We calculated adjusted relative risks (aRR) or difference of means (aB) with regression analyses.

We included 600 patients of whom 43 had migraine. There were no differences between patients with or without migraine in presence of a perfusion deficit on admission (aRR: 0.98, 95%CI: 0.77–1.25), infarct core volume (aB: -10.8, 95%CI: -27.04–5.51), penumbra volume (aB: -11.6, 95%CI: -26.52–3.38), mean blood brain barrier permeability (aB: 0.08, 95%CI: -3.11–2.96), malignant edema (0% vs 5%), hemorrhagic transformation (aRR: 0.26, 95%CI: 0.04–1.73), final infarct volume (aB: -14.8, 95%CI: 29.9–0.2) or outcome after recanalization therapy (mRS>2, aRR: 0.50, 95%CI: 0.21–1.22).

Elderly patients with a history of migraine do not seem to have more severe stroke progression and have similar treatment outcomes compared with patients without migraine.

INTRODUCTION

Migraine patients, especially with aura, have an increased risk of ischemic stroke.¹ The association between ischemic stroke and migraine is complex and probably multifactorial.^{2,3} One interesting mechanism involved in the association is spreading depolarization (SD). SDs are waves of depolarizing neuronal and glia cells, spreading through brain tissue and are the presumed underlying neurovascular mechanism of a migraine aura. SDs have been linked directly to ischemia in pure migrainous infarction. Besides that, it has been shown in mice that micro-emboli can also trigger SDs, with or without causing microinfarctions^{4,5} and increased migraine with aura (MA) and stroke occurrence is reported in patients with patent foramen ovale.⁶ SDs in healthy brain tissue are probably a benign phenomenon but in ischemic tissue they may increase (secondary) tissue changes after stroke.^{2,7,8}

Within minutes after an ischemic event, cells contributing to the infarct core become necrotic with membrane breakdown, dysfunctional cellular metabolism and energy supply, disturbed ion homeostasis, and loss of cell integrity. The tissue surrounding the core (the penumbra) is “struggling to survive” due to collateral blood supply being borderline sufficient.^{9,10} Cells in the penumbra are metabolically still active for some time, until the disruption of the cellular homeostasis in these cells also leads to cell death. Within minutes after vessel occlusion, a first depolarization wave travels through the penumbra, triggered by loss of membrane integrity due to hypoxia and energy depletion. Multiple SDs can follow thereafter, resulting in an additional reduction of blood flow and increased ischemic damage with each wave.^{11–14} These peri-infarct polarizations have been found in patients with malignant middle cerebral artery (MCA) infarctions.¹² Also, SDs possibly affect blood brain barrier (BBB) permeability leading to increased secondary hemorrhagic changes, malignant edema, and faster penumbra turnover into infarct core.^{15–17}

Although SDs also occur in stroke patients without migraine, stroke patients with a history with migraine may be more susceptible for SDs. Mice carrying the familial hemiplegic migraine gene have a decreased cortical SD threshold and increased infarct sizes compared with wild-type mice.⁷ In a recent retrospective MRI study of 72 ischemic stroke patients, patients with migraine had a faster penumbra turnover than patients without migraine.^{18,19} This fast penumbra turnover might restrict the time window for revascularization therapies and decrease the chance of good functional recovery.³

We investigated with modern CT-stroke imaging techniques in a prospective clinical stroke cohort, whether stroke progression is more severe in patients with migraine than in those without and whether migraine patients have poorer outcomes after intravenous thrombolysis or mechanical thrombectomy.

METHODS

Patients

We included patients from a large prospective multicenter cohort study in the Netherlands (Dutch Acute Stroke Trial (DUST)²⁰). The main goal of the DUST study was to analyze the predictive value of computed tomography angiography (CTA) and perfusion (CTP) on clinical outcome in patients with ischemic stroke.

Inclusion criteria were: age ≥ 18 years, stroke symptom onset at time of imaging < 9 h, National Institutes of Health Stroke Scale (NIHSS) ≥ 2 or 1 if intravenous thrombolysis (IVT) with rtPA was indicated, and known absence of renal failure or allergy to contrast agent. Patients with

discharge diagnoses other than ischemic stroke (for example TIA without CT perfusion defect) were excluded from analysis. Non-contrast CT (NCCT), CTP, and CTA were performed on admission, with scan protocols standardized between centers. Study approval was received from the institutional medical ethics committee of all participating hospitals. Ethical approval was obtained from the medical ethics committee of the University Medical Center Utrecht (UMCU), the Netherlands, in addition to local approval from all participating hospitals. Written informed consent was obtained from patients or their legal representative. The medical ethics committee UMCU waived the need for informed consent for patients who died before informed consent could be obtained.

Follow-up NCCT was repeated around three days with (depending on the consent) additional CTP and CTA or MRI if clinically indicated. CT protocols were adjusted within centers according to the hardware used for comparable results, described in detail previously.²⁰

Migraine diagnosis

Migraine (past and present) was diagnosed upon admission with a 5-point questionnaire Migraine Screener for Stroke (MISS)²¹ by trained headache research nurses. Although this questionnaire has a high negative predictive value (0.99), the positive predictive value (0.80 overall and 0.38 for MA) is less accurate. To exclude false positive diagnoses, we approached all patients with a positive migraine screener (at least 1 question answered with “yes”) for an extensive telephone interview to verify the migraine diagnosis. Interview questions were based on International Classification of Headache Disorders II criteria (ICHD-II) migraine and aura criteria in a (semi-) structured manner. In case of ambiguity, a headache neurologist (GMT) was consulted. Patients were excluded when they were unable to answer screener questions due to severe aphasia or decreased cognitive abilities and when migraine diagnosis could not be verified due to loss of follow-up or refusal to participate in the interview.

Clinical characteristics and outcome

We collected data about cardiovascular risk factors, NIHSS, and modified Rankin Scale (mRS) prior to stroke on admission. Stroke-to-imaging time and mode of treatment (intravenous treatment, mechanical thrombectomy or no revascularization therapy) were recorded. Stroke etiology was classified with the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification,²² and infarct localization was scored with the Oxfordshire classification²³ consisting of the subtypes partial anterior circulation infarct (PACI), total anterior circulation infarct (TACI), lacunar infarct (LACI), and posterior circulation infarct (POCI).

After a follow-up period of three months, the mRS was prospectively assessed by a telephone interview performed by a trained research nurse. MRS >2 was considered a poor clinical outcome.

Radiological characteristics

All radiological assessments were performed blinded for clinical diagnosis and migraine diagnosis (except for side of symptoms) by trained observers with at least five years of experience in neurovascular imaging. Upon admission, early ischemic changes were evaluated on NCCT with the Alberta Stroke Program Early CT score (ASPECTS) scale. The ASPECTS is a 10-point (0 = all areas involved, 10 = no areas of the MCA territory involved in the infarct) quantitative topographic CT scan score evaluated on two standardized levels.²⁴ The score was dichotomized ≤ 7 or > 7 which has previously been identified as a useful predictive cutoff point

for clinical functional outcome.²⁵

Clot Burden Score was determined on CTA as the absence of opacification in the anterior circulation on a 10-point scale where a score of 10 indicates the contrast opacification of all vessel segments and 0 lack of opacification of any vessel segment and dichotomized in <7 and ≥7.²⁶ Collaterals were determined on CTA in patients with anterior circulation infarction (ACA and MCA) due to a large vessel occlusion. Subsequently collaterals were defined as poor when collateral filling was ≤50% of the affected area.^{26–28}

The CTP parameters cerebral blood flow (CBF), cerebral blood volume (CBV), mean transit time (MTT), and time to peak (TTP) were automatically calculated (using Extended Brilliance Workstation 4.5; Philips Healthcare software and in-house developed perfusion software applying a bSVD algorithm) and displayed in color coded maps; MTT and CBV thresholds to define infarct core and penumbra were applied as described previously.²⁹ Our primary outcome variable presence of a perfusion deficit (yes or no) at entry was visually assessed using CBF, CBV, MTT, and TTP color coded maps. Infarct core and penumbra area at entry were classified into two levels by applying the ASPECTS to the MTT and CBV thresholds and again dichotomized in <8 and ≥8.³⁰ Volumes of infarct core and penumbra were calculated within the whole scan range using in-house software, with: $T_{max} > 6$ s for total infarct area and a relative CBF threshold of 30% was used to differentiate between core and penumbra.^{31,32}

We calculated the BBB permeability surface area product with CTP data according to the nonlinear regression method described in detail previously.³³ A BBB permeability ratio >1 indicates that the BBB permeability of the ipsilateral hemisphere is higher compared with that in the contralateral hemisphere.

On follow-up we assessed imaging markers of secondary brain damage including presence of malignant edema, defined as a midline shift 5 mm; hemorrhagic transformation, defined using the European Cooperative Acute Stroke Study criteria and included the presence of any subtype;³⁴ and final infarct volume (cc), quantified by manually delineating hypodense territory on NCCT.³⁵ Presence of a persistent occlusion was defined as no recanalization visible on follow-up CTA in the subgroup of patients with vessel occlusion at entry.

Data analysis

The primary outcome variable for our study was considered to be the presence of a perfusion deficit. A post-hoc power analysis was performed for this outcome, resulting in sufficient precision to detect a 15% difference in perfusion deficit presence (in 60% of the patients without migraine vs 75% in those with migraine).

Poisson and linear regression (all univariable and multivariable) analyses with migraine as the determinant were used to calculate (adjusted) risk ratios (RR) or beta-coefficients (B) including 95% confidence intervals (95% CIs). Adjustments were made for age, sex, stroke-to-imaging time (STI), NIHSS on admission, smoking, and a history of hypertension. In addition, for outcome variables at follow-up, an adjustment for IVT and/or mechanical thrombectomy treatment was added. Three subgroup analyses were performed: (1) for patients with MA because patients with MA might be more susceptible for SDs, (2) for patients with large infarcts (based on Oxfordshire classification at discharge: excluding LACI and unknown) because secondary changes are likely to be dependent on infarct size, and (3) for patients with proven vessel occlusions. Missing values were imputed with single imputation for multivariable analyses. Missing values were not imputed when comprising more than 20% of the data (in case of the BBB permeability ratio), when random imputation would lead to improbable results (“infarct locations” and “occlusion”) and when variables were not missing

at random (follow-up imaging variables “malignant edema”, “hemorrhagic transformation”, “final infarct volume”, and “persistent occlusion”).

RESULTS

Patients

We included 600 patients; 43 (7%) with migraine (24 with aura and 19 without aura) and 557 patients without migraine (Figure 1). Baseline characteristics were comparable between patients with and without migraine, except that patients with migraine were in general younger at time of stroke onset, more often female and more often had lacunar or vertebrobasilar infarctions compared with patients without migraine (Table 1).

Stroke on admission and follow-up

No differences were found between patients with and without migraine history in presence of a perfusion defect on admission (adjusted relative risks (aRR): 0.98, 95% CI: 0.77–1.25), ASPECTS infarct core <8 (aRR: 0.95, 95%CI: 0.45–2.00), ASPECTS penumbra <8 (aRR: 0.90, 95%CI: 0.60–1.34), infarct core volume (aB: -10.8, 95%CI: -27.0–5.5), penumbra volume (aB: -11.6, 95%CI: -26.5–3.4), or BBB permeability ratio on CTP (aB: -0.08, 95%CI: -3.11–2.96; Table 2, upper part).

Follow-up imaging on average three days after stroke onset was performed in 522 (87%) patients. In 325 (54%) patients follow-up imaging included CTP. No difference was found between migraine vs non-migraine patients in prevalence of malignant edema (0/30 (0%) 16/327 (5%)) and hemorrhagic transformation (aRR: 0.26, 95%CI: 0.04–1.73) or final infarct volume (aB: -14.8, 95%CI: -29.9–0.2; Table 2, lower part).

Subgroup analysis including only migraine patients with aura or patients with large infarcts

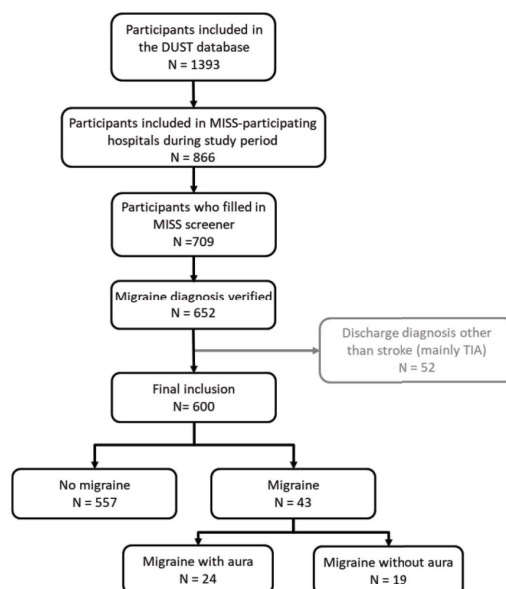


Figure 1. Inclusion flow chart. MISS: Migraine Screener for Stroke.

STROKE PROGRESSION AND OUTCOME IN AIS WITH MIGRAINE

or patients with proven vessel occlusions showed essentially similar results (Supplementary Tables 1 and 2).

N		Migraine (all) 43	Migraine with aura 24	No Migraine 557
Clinical Characteristics				
Women		22 (51%)	9 (38%)	209 (38%)
Age, mean years (SD)		60.0 (11.5)	57.9 (8.5)	67.2 (13.4)
Age < 50		10 (23%)	5 (21%)	70 (13%)
NIHSS (median, IQR)		5 (2 – 11)	5 (2 – 11)	5 (3 – 10)
mRS before stroke >0		9 (21%)	7 (29%)	122 (22%)
STI, min. (median, IQR)		116 (83 – 210)	120 (79 – 225)	113 (72 – 171)
Oxfordshire-classification	TACI	6 (14%)	5 (21%)	82 (15%)
	PACI	11 (26%)	6 (25%)	221 (40%)
	POCI	9 (21%)	7 (29%)	82 (15%)
	LACI	9 (21%)	5 (21%)	70 (13%)
	Unclear	0	0	25 (4%)
	Unknown	8 (19%)	1 (4%)	77 (14%)
TOAST	LVD	12 (28%)	6 (25%)	165 (30%)
	CE source	8 (19%)	3 (13%)	93 (17%)
	SVD	7 (16%)	4 (17%)	75 (13%)
	Other	3 (7%)	1 (4%)	42 (7%)
	Unknown	13 (30%)	10 (42%)	182 (33%)
Treatment	IVT alone	19 (44%)	9 (38%)	344 (62%)
	IAT*	5 (12%)	2 (8%)	32 (6%)
	None	19 (44%)	13 (54%)	181 (32%)
Risk Factors				
Smoking		19 (45%)	12 (50%)	160 (30%)
Hypertension		16 (37%)	7 (29%)	266 (48%)
Diabetes		5 (12%)	3 (13%)	81 (15%)
Myocardial infarction		4 (10%)	3 (13%)	68 (12%)
Previous Stroke/TIA		9 (21%)	6 (25%)	123 (22%)
AF		5 (12%)	2 (8%)	63 (11%)
Hyperlipidemia		18 (42%)	12 (50%)	176 (33%)
Peripheral vessel disease		2 (5%)	2 (8%)	22 (4%)
≥2 Risk Factors		21 (51%)	12 (52%)	271 (50%)

Table 1. Baseline characteristics of patients.

IAT: IA treatment alone or IA as well as IV treatment; SD: standard deviation; smoking: current smoker or quit smoking less than six months before event; TIA: transient ischemic attack; AF: atrial fibrillation; NIHSS: National Institutes of Health Stroke Scale; IQR: interquartile range; mRS: modified Rankin Scale; STI: stroke-to-imaging; TACI: total anterior circulation infarct; PACI: partial anterior circulation infarct; POCI: posterior circulation infarct; LACI: lacunar infarct; TOAST: Trial of Org 10172 in Acute Stroke Treatment; LVD: large vessel disease; CE: cardio-embolism; SVD: small vessel disease; IV: intravenous; IA: intra-arterial. Numbers might not add up due to missing values.

Treatment outcome

Overall, the proportion of patients with a poor outcome (mRS >2) after three months did not differ between migraineurs and non-migraineurs (aRR: 0.60, 95%CI: 0.30–1.20). In the patients who were treated with IVT or mechanical thrombectomy no difference was found in the number of persistent occlusions (aRR: 0.92, 95%CI: 0.41–2.04) or mRS >2 at three months (aRR: 0.50, 95%CI: 0.21–1.22; Table 3).

	Migraine (all)	No Migraine	RR (95%CI)	aRR (95%CI)
N	43	557		
ADMISSION				
Infarct location				
ACA	1/42 (2%)	22/534 (4%)	0.58 (0.08 – 4.18)	
MCA	29/42 (69%)	414/533 (78%)	0.89 (0.72 – 1.09)	
PCA	1/42 (2%)	31/533 (6%)	0.41 (0.06 – 2.93)	
VBA	10/41 (24%)	64/534 (12%)	2.04 (1.13 – 3.66)	
Borderzone	0/41 (0%)	6/533 (1%)	–	
Large vessel occlusion	17/42 (40%)	271/550 (49%)	0.82 (0.56 – 1.20)	
Clot Burden Score < 7	6/43 (14%)	70/557 (13%)	1.11 (0.51 – 2.41)	
Poor collaterals ≤ 50% *	1/13 (8%)	42/228 (18%)	0.42 (0.06 – 2.80)	
Stroke progression on admission				
ASPECTS – NCCT < 8	4/43 (9%)	43/557 (8%)	1.21 (0.45 – 3.20)	1.12 (0.38 – 3.28)
Perfusion defect present	26/43 (61%)	342/557 (61%)	0.99 (0.77 – 1.27)	0.98 (0.77 – 1.25)
ASPECTS Infarct core < 8	6/43 (14%)	89/557 (16%)	0.87 (0.41 – 1.88)	0.95 (0.45 – 2.00)
ASPECTS Penumbra < 8	14/43 (33%)	203/557 (36%)	0.89 (0.57 – 1.39)	0.90 (0.60 – 1.34)
Volume Infarct core (cc) ^a	32.2 (28.9)	43.4 (47.8)	-11.2 (-27.5 – 5.1) [§]	-10.8 (-27.0 – 5.5) [§]
Volume Penumbra (cc) ^a	28.6 (31.7)	40.8 (46.2)	-12.2 (28.0 – 3.7) [§]	-11.6 (-26.5 – 3.4) [§]
Volume Mismatch Ratio ^a	1.64 (1.30 – 2.03)	1.71 (1.32 – 2.58)	-0.11 (-0.77 – 0.54) [§]	-0.13 (-0.77 – 0.52) [§]
BBBP ratio [†]	1.41 (0.89 – 2.56)	1.58 (0.95 – 3.04)	-0.25 (-3.25 – 2.76) [§]	-0.08 (-3.11 – 2.96) [§]
FOLLOW-UP				
Stroke progression on follow-up				
Malignant edema	0/30 (0%)	16/327 (5%)	–	–
Hemorrhagic transformation	1/38 (3%)	45/484 (9%)	0.28 (0.04 – 2.00)	0.26 (0.04 – 1.73)
Final infarct volume (cc) [¶]	11.4 (21.2)	22.7 (51.8)	-11.3 (-28.2 – 5.5) [§]	-14.8 (-29.9 – 0.2) [§]

Table 2. Radiological characteristics upon admission and follow-up.

RR: risk ratio; ACA: anterior cerebral artery; MCA: middle cerebral artery; PCA: posterior cerebral artery; VBA: vertebrobasilar arteries; ASPECTS: Alberta Stroke Program Early CT score; NCCT: non-contrast CT; BBBP ratio: blood-brain-barrier permeability ratio ipsilateral/contralateral; SD: standard deviation. ^aPopulation of patients with occlusion and anterior circulation infarction, N=13 migraine and 228 no migraine patients. ^bWithin scan range; N=34 migraine and 456 no migraine patients. ^cN=30 migraine and 442 no migraine patients. ^dN=37 migraine and 474 no migraine patients. ^eNumbers are displayed as difference of the means (B) or adjusted difference of the means (aB). Missing values were imputed except for variables Infarct location, perfusion defect present, BBBP ratio, and follow-up variables. Adjustments for admission variables were made for age, sex, stroke-to-imaging time, NIHSS on admission, smoking and a history of hypertension. Additional adjustment for treatment was performed concerning the follow-up variables. Volumes (cc) are listed as mean (standard deviation), ratios as median (IQR).

DISCUSSION

Elderly patients with a history of migraine did not have more severe stroke progression or poorer outcome compared with patients without migraine. Also, no difference in outcome after recanalization treatment was present. If anything, brain injury seemed smaller in patients with migraine although these differences were not statistically significant.

Our findings seem to contradict previous findings in animal studies. Mice carrying the familial hemiplegic migraine type 1 (FHM1) mutation developed faster and had a larger infarct volume after MCA occlusion compared with control mice.⁷ This was most likely caused by the lower threshold for occurrence of SDs.^{11,36,37} However, in these animal studies, MCA occlusion was performed in a standardized way which always resulted in an infarct at the

	Migraine	No Migraine	RR (95%CI)	aRR (95%CI)
All	43	557		
Persistent occlusion *	4/12 (33%)	92/175 (53%)	0.63 (0.28 – 1.43)	0.75 (0.32 – 1.72)
FU3 mRS > 2	7/43 (16%)	160/557 (29%)	0.57 (0.28 – 1.13)	0.60 (0.30 – 1.20)
Treated patients	24	376		
Persistent occlusion *	4/11 (36%)	53/125 (42%)	0.86 (0.38 – 1.92)	0.92 (0.41 – 2.04)
FU3 mRS > 2	4/24 (17%)	117/376 (31%)	0.54 (0.22 – 1.33)	0.50 (0.21 – 1.22)

	Migraine with aura	No Migraine	RR (95%CI)	aRR (95%CI)
All	24	557		
Persistent occlusion *	2/8 (25%)	92/175 (53%)	0.48 (0.14 – 1.59)	0.52 (0.15 – 1.85)
FU3 mRS > 2	3/24 (13%)	160/557 (29%)	0.44 (0.15 – 1.26)	0.47 (0.15 – 1.48)
Treated patients	11	376		
Persistent occlusion *	2/7 (29%)	53/125 (42%)	0.67 (0.21 – 2.21)	0.66 (0.20 – 2.17)
FU3 mRS > 2	0/11 (0%)	117/376 (31%)	–	–

<u>Only large infarcts</u>	Migraine	No Migraine	RR (95%CI)	aRR (95%CI)
All	26	385		
Persistent occlusion *	3/11 (27%)	82/156 (53%)	0.52 (0.20 – 1.38)	0.60 (0.22 – 1.64)
FU3 mRS > 2	6/26 (23%)	122/385 (32%)	0.73 (0.36 – 1.49)	0.73 (0.36 – 1.49)
Treated patients	14	273		
Persistent occlusion *	3/10 (30%)	50/113 (44%)	0.68 (0.26 – 1.79)	0.74 (0.28 – 1.94)
FU3 mRS > 2	3/14 (21%)	91/273 (33%)	0.64 (0.23 – 1.78)	0.52 (0.20 – 1.39)

Table 3. Outcome in patients including sub-analyses for patients treated with intravenous thrombolysis or mechanical thrombectomy for patients with or without migraine (top); patients with or without migraine with aura (Middle) and large infarcts, with or without migraine (bottom). FU3: follow-up after three months; mRS: modified Rankin Scale. aPersistent occlusion of total patients with occlusion present at admission and available follow-up imaging. Adjustments were made for age, sex, stroke-to-imaging time, NIHSS on admission, smoking, a history of hypertension, and treatment. In the subanalysis of the treated patients, the same adjustments were made, however without adjusting for treatment.

same location. In our study, reflecting clinical practice, we compared patients with infarcts at very heterogeneous locations and including lacunar infarcts. Unfortunately, we were not able to assess penumbra turnover in our patients because follow-up CTP scans were not available in almost half of the patients and volumes of the perfusion deficits were surrounded by wide CIs. In one retrospective study, a non-mismatch pattern was more often seen in migraineurs with aura within 72 h of stroke onset, based on diffusion and perfusion weighted MRI.¹⁹ Although we could not confirm or refute this finding, we found no differences in the extent of early ischemic changes on NCCT, presence of a perfusion deficit or extent of infarct core, and penumbra on admission (CTP) between patients with and without migraine. A possible explanation for the tendency of even less secondary damage in migraineurs is the hypothesis that migraine attacks could be of homeostatic and neuroprotective response to oxidative stress in the brain.³⁸

Our findings are important for clinical practice, as we found no diminished treatment effect of IVT or mechanical thrombectomy in patients with migraine. Clinical outcome in general was comparable in patients with and without migraine. This finding is in line with the Women's Health study where no difference was found in functional outcome after TIA or stroke in women with a history of migraine, but no treatment effect was assessed.³⁹ Based on our data there seems to be no reason to restrict the time window for revascularization treatment in migraineurs.

Strengths of our study are the large number of included ischemic stroke patients in a prospective manner in combination with the extensive standardized CT imaging in the acute phase of ischemic stroke. All life-time migraine diagnoses were confirmed by telephone interview with questions based on the formerly ICHD-II criteria but for migraine diagnoses comparable with the new ICHD-III criteria, which decreased the chance of misclassification bias.

Our study also has limitations. For the primary analyses, we combined migraine with and without aura, which could have diluted the effect of MA on stroke characteristics. Spreading depolarization is a mechanism that is in particular related to MA although some studies suggest that it also occurs in migraine without aura.⁴⁰ In a secondary analysis, we compared migraine patients with aura with patients without migraine. The effect estimates obtained pointed in the same direction as our main results.

As secondary tissue changes after stroke are dependent on infarct size at admission we also performed an additional analysis including only large infarcts. This analysis again showed comparable results with the main analyses. However, because of the limited group sizes these additional analyses should be interpreted with great caution and replication in future studies with larger number of MA patients is warranted. Our population was in general about 65 years old at time of their stroke. Because migraine is most active at younger age recall bias might have occurred. We also did not take into account the (possible) use of prophylactic migraine medications which may have influenced occurrence of SDs. Unfortunately, we had no data on the date of the last migraine attack or the presence of active migraine. The higher susceptibility for SDs in migraineurs might be associated with current status of migraine activity. In another study of our group on the effect of migraine history on delayed cerebral ischemia (DCI) after subarachnoid hemorrhage (SAH) we found a statistically significant interaction of the effect with age.⁴¹ Only in young SAH patients migraine history was related to DCI.⁴¹ As the proportion of young stroke patients in our present study is low we feel more data are needed before the current reassuring results can be generalized to patients under the age of 50 years.

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SUPPLEMENTAL MATERIAL

A	Migraine with aura	No Migraine	RR (95%CI)	aRR (95%CI)
On admission	24	557		
ASPECTS – NCCT < 8	2/24 (8%)	43/557 (8%)	1.08 (0.28 – 4.20)	0.91 (0.19 – 4.33)
Perfusion defect present	15/24 (63%)	342/557 (61%)	1.02 (0.74 – 1.40)	1.00 (0.72 – 1.40)
ASPECTS Infarct core < 8	4/24 (17%)	89/557 (16%)	1.04 (0.42 – 2.60)	0.99 (0.43 – 2.29)
ASPECTS Penumbra < 8	7/24 (29%)	203/557 (36%)	0.80 (0.43 – 1.51)	0.75 (0.42 – 1.36)
Infarct core volume (cc) ^Δ	24.9 (17.2)	43.4 (47.8)	-13.42 (-40.6 – 3.8) [§]	-19.36 (-41.5 – 2.7) [§]
Penumbra volume (cc) ^Δ	23.5 (29.2)	40.8 (46.2)	-17.30 (-38.9 – 4.3) [§]	-21.64 (-42.0 – -1.3) [§]
Volume Mismatch Ratio ^Δ	1.69 (1.31 – 1.97)	1.71 (1.32 – 2.58)	-0.37 (-1.25 – 0.51) [§]	-0.52 (-1.39 – 0.34) [§]
BBBP ratio [‡] (Median, IQR)	1.41 (0.87 – 2.30)	1.58 (0.95 – 3.04)	-1.83 (-6.14 – 2.48) [§]	-1.40 (-5.75 – 2.96) [§]
At follow-up				
Malignant edema	0/18 (0%)	16/327 (5%)	–	–
Hemorrhagic transformation	1/23 (4%)	45/484 (9%)	0.47 (0.07 – 3.24)	0.47 (0.07 – 3.02)
Final infarct volume (cc) [¶]	12.8 (24.5)	22.7 (51.8)	-9.9 (-31.3 – 11.4) [§]	-14.9 (-33.9 – 4.1) [§]
B	Migraine	No Migraine	RR (95%CI)	aRR (95%CI)
On admission	26	385		
ASPECTS – NCCT < 8	3/26 (12%)	38/385 (10%)	1.17 (0.39 – 3.53)	0.98 (0.29 – 3.39)
Perfusion defect present	18/26 (69%)	271/385 (70%)	0.98 (0.76 – 1.28)	0.96 (0.74 – 1.25)
ASPECTS Infarct core < 8	6/26 (23%)	79/385 (21%)	1.13 (0.54 – 2.33)	1.12 (0.54 – 2.30)
ASPECTS Penumbra < 8	12/26 (46%)	175/385 (46%)	1.02 (0.66 – 1.56)	0.97 (0.66 – 1.44)
Infarct core volume (cc) ^Δ	23.7 (15.9)	38.3 (37.4)	-14.54 (-30.71 – 1.63) [§]	-16.52 (-32.26 – -0.78) [§]
Penumbra volume (cc) ^Δ	31.4 (35.4)	41.9 (43.6)	-10.48 (-29.64 – 8.68) [§]	-12.43 (-30.50 – 5.64) [§]
Volume Mismatch Ratio ^Δ	1.78 (1.30 – 2.20)	1.81 (1.36 – 2.90)	0.01 (-0.96 – 0.98) [§]	-0.01 (-0.96 – 0.95) [§]
BBBP ratio [‡] (Median, IQR)	1.72 (1.18 – 3.03)	1.80 (1.06 – 4.05)	-0.58 (-5.43 – 4.26) [§]	0.36 (-5.26 – 4.54) [§]
At follow-up				
Malignant edema	0/21 (0%)	15/254 (6%)	–	–
Hemorrhagic transformation	1/26 (4%)	38/339 (11%)	0.34 (0.05 – 2.40)	0.34 (0.05 – 2.31)
Final infarct volume (cc) [¶]	15.2 (24.3)	28.7 (57.3)	-13.6 (-35.9 – 8.7) [§]	-17.8 (-37.8 – 2.1) [§]

Supplemental Table 1. Radiological features of stroke progression upon admission and follow-up in migraine patients with aura and patients without migraine (A); the subpopulation with large infarcts (B) (Oxfordshire classification TACI, PACI or POCI).

^Δ (A) N = 18 migraine with aura and 456 no migraine patients; (B) N = 21 migraine and 300 no migraine patients.

[‡] (A) N = 14 migraine with aura and 442 no migraine patients; (B) N = 16 migraine and 290 no migraine patients.

[¶] (A) N = 23 migraine with aura and 474 no migraine patients; (B) N = 26 migraine and 332 no migraine patients.

[§] Numbers are displayed as difference of the means (B) or adjusted difference of the means (aB). Adjustments for admission variables were made for age, sex, stroke-to-imaging time, NIHSS on admission, smoking and a history of hypertension. Additional adjustment for treatment was performed concerning the follow-up variables. Volumes (cc) are listed as Mean (SD), ratio's as median (IQR). (a)RR – (adjusted) Risk Ratio; ASPECTS – Alberta Stroke Program Early CT score; NCCT – Non-Contrast CT; BBBP ratio – Blood-Brain-Barrier Permeability ratio ipsilateral/contralateral; SD – Standard Deviation; (a)B – (adjusted) Beta.

	Migraine	No Migraine	RR (95%CI)	aRR (95%CI)
On admission	17	271		
ASPECTS – NCCT < 8	3/17 (18%)	42/271 (16%)	1.14 (0.39 – 3.30)	1.16 (0.39 – 3.49)
Perfusion defect present	15/17 (88%)	250/271 (92%)	0.96 (0.80 – 1.14)	0.94 (0.79 – 1.13)
ASPECTS Infarct core < 8	6/17 (35%)	83/271 (31%)	1.15 (0.59 – 2.25)	1.11 (0.58 – 2.13)
ASPECTS Penumbra < 8	12/17 (71%)	189/271 (70%)	1.01 (0.74 – 1.39)	0.97 (0.73 – 1.30)
Infarct core volume (cc) ^Δ	35.5 (22.6)	47.6 (47.5)	-12.12 (-36.51 – 12.27) [§]	-14.32 (-38.51 – 9.87) [§]
Penumbra volume (cc) ^Δ	48.3 (37.5)	58.8 (49.8)	-10.56 (-36.40 – 15.27) [§]	-14.07 (-38.81 – 10.67) [§]
Volume Mismatch Ratio ^Δ	1.94 (1.64 – 2.36)	2.17 (1.57 – 3.40)	0.07 (-1.21 – 1.34) [§]	0.02 (-1.24 – 1.28) [§]
BBBP ratio [‡] (Median, IQR)	2.28 (1.03 – 3.03)	2.11 (1.16 – 3.63)	0.03 (-5.96 – 6.03) [§]	0.32 (-5.70 – 6.34) [§]
At follow-up				
Malignant edema	0/16 (0%)	14/219 (6%)	–	–
Hemorrhagic transformation	1/17 (6%)	38/236 (16%)	0.37 (0.05 – 2.50)	0.38 (0.06 – 2.46)
Final infarct volume (cc) [¶]	22.3 (27.6)	41.8 (65.9)	-19.4 (-51.2 – 12.3) [§]	-24.9 (-54.0 – 4.2) [§]

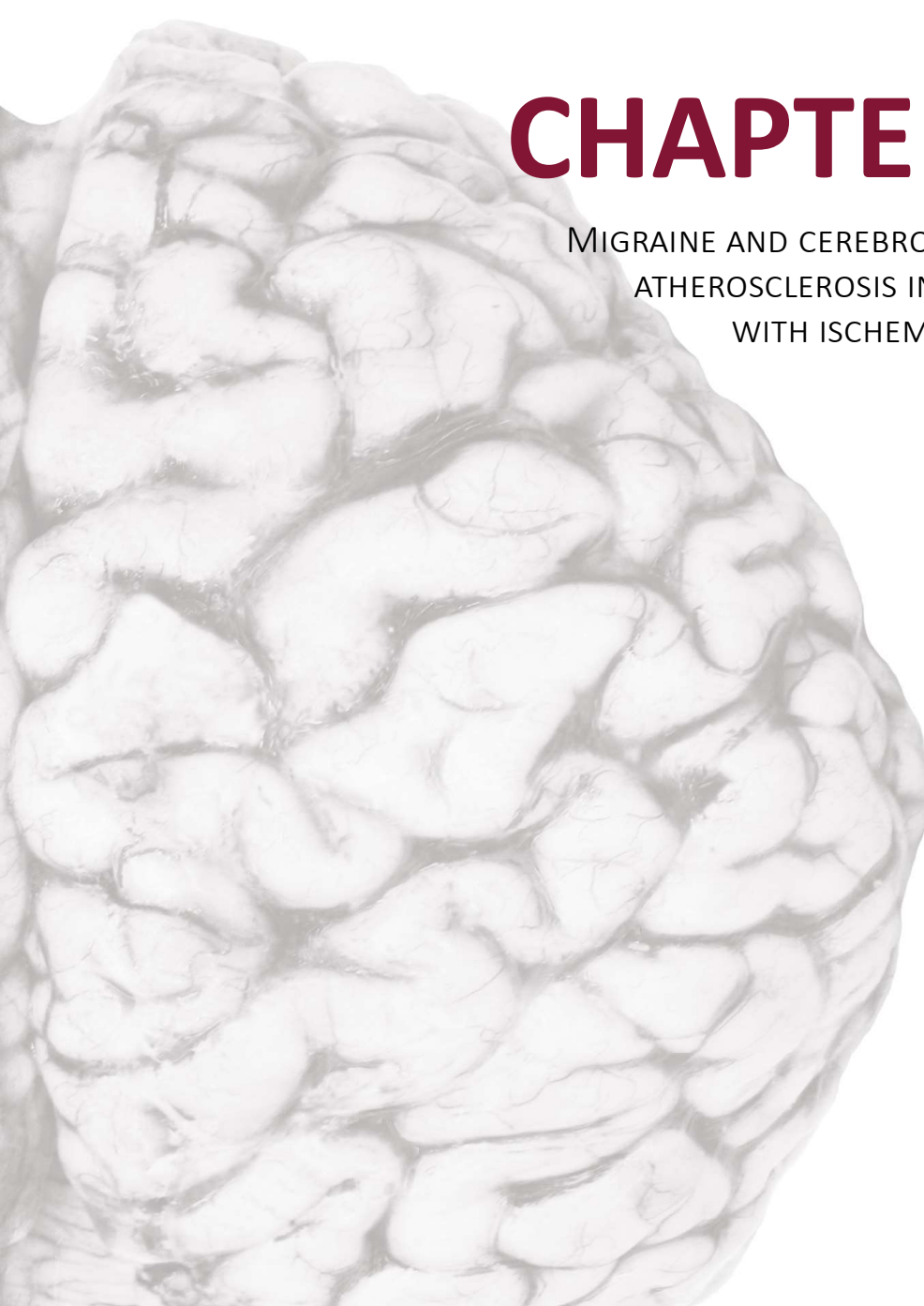
Supplemental Table 2. Radiological features of stroke progression upon admission and follow-up in migraine patients and patients without migraine in a subpopulation of patients with proven occlusion at day 0.

^Δ N = 15 migraine with aura and 219 no migraine patients.

[‡] N = 12 migraine and 212 no migraine patients.

[¶] N = 17 migraine and 233 no migraine patients.

[§] Numbers are displayed as difference of the means (B) or adjusted difference of the means (aB). Adjustments for admission variables were made for age, sex, stroke-to-imaging time, NIHSS on admission, smoking and a history of hypertension. Additional adjustment for treatment was performed concerning the follow-up variables. Volumes (cc) are listed as Mean (SD), ratio's as median (IQR). (a) RR – (adjusted) Risk Ratio; ASPECTS – Alberta Stroke Program Early CT score; NCCT – Non-Contrast CT; BBBP ratio – Blood-Brain-Barrier Permeability ratio ipsilateral/contralateral; SD – Standard Deviation; (a)B – (adjusted) Beta.



CHAPTER 8

MIGRAINE AND CEREBROVASCULAR
ATHEROSCLEROSIS IN PATIENTS
WITH ISCHEMIC STROKE

van Os HJA, Mulder IA, Broersen A, Algra A, van der Schaaf IC, Kappelle LJ, Velthuis BK, Terwindt GM, Schonewille WJ, Visser MC, Ferrari MD, van Walderveen MAA and Wermer MJH; DUST Investigators

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ABSTRACT

Migraine is a well-established risk factor for ischemic stroke, but migraine is also related to other vascular diseases. This study aims to investigate the association between migraine and cerebrovascular atherosclerosis in patients with acute ischemic stroke.

We retrieved data on patients with ischemic stroke from the DUST (Dutch Acute Stroke Study). Migraine history was assessed with a migraine screener and confirmed by telephone interview based on the ICHD criteria (International Classification of Headache Disorders). We assessed intra- and extracranial atherosclerotic changes and quantified intracranial internal carotid artery calcifications as measure of atherosclerotic burden on noncontrast computed tomography and computed tomographic angiography. We calculated risk ratios with adjustments for possible confounders with multivariable Poisson regression analyses.

We included 656 patients, aged 18 to 99 years, of whom 53 had a history of migraine (29 with aura). Patients with migraine did not have more frequent atherosclerotic changes in intracranial (51% vs 74%; adjusted risk ratio, 0.82; 95% confidence interval, 0.64–1.05) or extracranial vessels (62% vs 79%; adjusted risk ratio, 0.93; 95% confidence interval, 0.77–1.12) than patients without migraine and had comparable internal carotid artery calcification volumes (largest vs medium and smallest volume tertile, 23% versus 35%; adjusted risk ratio, 0.93; 95% confidence interval, 0.57–1.52).

Migraine is not associated with excess atherosclerosis in large vessels in patients with acute ischemic stroke. Our findings suggest that the biological mechanisms by which migraine results in ischemic stroke are not related to macrovascular cerebral atherosclerosis.

INTRODUCTION

Migraine, especially with aura, is a risk factor for ischemic stroke.¹ Migraine patients also have an increased risk for cardiovascular disease in the systemic circulation, such as myocardial infarction and peripheral artery disease.² The connection between migraine and cardiovascular disease is complex and probably multifactorial. One of the possible mediating mechanisms is enhanced atherosclerosis.

The aim of our study was to investigate the association between migraine and cerebrovascular atherosclerosis in a large cohort of patients with acute ischemic stroke.

METHODS

We included patients from the DUST (Dutch Acute Stroke Study), a large prospective multicenter cohort study performed between May 2009 and August 2013.³ Inclusion criteria for DUST were age ≥ 18 years, onset of stroke symptoms < 9 hours, and National Institutes of Health Stroke Scale score of ≥ 2 or ≥ 1 if intravenous thrombolysis with rtPA (recombinant tissue plasminogen activator) was indicated.

Exclusion criteria were known renal failure and contrast agent allergy.³ DUST was approved by the Medical Ethical Committee of the participating hospitals. Informed consent was obtained from all patients for use of the data.

All patients underwent noncontrast computed tomography, computed tomographic angiography, and computed tomography perfusion on admission with standardized scan protocols (Methods in the Supplemental Material). Radiological parameters were assessed by trained neuroradiologists with good interobserver variability.³

At baseline, we collected data on cardiovascular risk factors and medical history. Stroke subtype was classified according to the TOAST criteria (Trial of ORG 10172 in Acute Stroke Treatment). The DUST research nurses recorded the Migraine in Stroke Screener, a 5-item

Characteristics	Migraine (n=53)	No Migraine (n=603)
Demographics		
Age, mean y ($\pm$ SD)	59.9 $\pm$ 11.0	67.0 $\pm$ 13.4
Age < 50 , n (%)	10 (19)	76 (13)
Women, n (%)	29 (55)	223 (37)
History, n (%)		
Hypertension	21 (40)	289 (49)
Diabetes mellitus	7 (13)	92 (15)
Hyperlipidemia	21 (40)	200 (34)
Previous stroke or TIA	14 (26)	139 (23)
Myocardial infarction	5 (10)	77 (13)
Atrial fibrillation	6 (11)	70 (12)
Peripheral artery disease	3 (6)	24 (4)
Smoking: current*	23 (44)	170 (30)
Smoking: lifetime*	34 (65)	372 (65)
Alcohol use	27 (73)	267 (62)
Baseline NIHSS, median	5	5

Table 1. Clinical Characteristics of the Participants. NIHSS indicates National Institutes of Health Stroke Scale; and TIA, transient ischemic attack. *Current smokers and smokers who stopped smoking > 6 months ago.

migraine screener that retrospectively assesses migraine history and was validated previously in a stroke cohort. Migraine in Stroke Screener data were obtained when the patient entered the DUST study.

The Migraine in Stroke Screener has a very high negative predictive value (0.99), but a moderate positive predictive value especially for aura symptoms.⁴ In case of ≥ 1 positive answers to the screener, the participants were contacted by telephone by a migraine research nurse. This semi-structured telephone interview consisted of detailed questions on headache and aura characteristics, including ICHD-II (International Classification of Headache Disorders) migraine and aura criteria. Patients were excluded when there was suspicion of migraine based on the screener, but the migraine diagnosis could not be confirmed by telephone because patients were lost to follow-up or refused participation.

We assessed patients with any sign of atherosclerosis in intra- and extracranial vessels of the anterior and posterior circulation on computed tomographic angiography. We measured intracranial internal carotid artery calcification volume, using calcium as a measure for atherosclerosis. Calcium volumes were measured from the petrous part to the top of the intracranial carotid arteries on noncontrast computed tomography using dedicated software (Methods in the Supplemental Material).

We performed multivariable Poisson regression analyses (Methods in the Supplemental Material). Risk ratios and adjusted risk ratio with 95% confidence intervals were calculated.

RESULTS

In total, 707 DUST participants (82%) filled in the screener. Fifty-one patients were lost to follow-up or refused to participate in the telephone interview and were excluded. Therefore,

Atherosclerotic Changes	Migraine (n=53)	No Migraine (n=603)	RR (95% CI)	aRR (95% CI)*
Intracranial circulation				
Any sign of atherosclerosis†	27 (51%)	445 (74%)	0.69 (0.53 – 0.90)	0.82 (0.64 – 1.05)
Any sign of stenosis	4 (8%)	80 (13%)	0.57 (0.22 – 1.49)	0.77 (0.29 – 2.02)
Tertile largest ICA calcification volume‡	12 (23%)	211 (35%)	0.65 (0.39 – 1.08)	0.93 (0.57 – 1.52)
Extracranial circulation				
Any sign of atherosclerosis†	33 (62%)	476 (79%)	0.79 (0.64 – 0.98)	0.93 (0.77 – 1.12)
Atherosclerosis anterior circulation	32 (60%)	465 (77%)	0.78 (0.63 – 0.98)	0.92 (0.76 – 1.13)
Atherosclerosis posterior circulation	12 (23%)	225 (37%)	0.61 (0.36 – 1.01)	0.86 (0.54 – 1.37)
Any sign of stenosis	18 (34%)	260 (43%)	0.79 (0.53 – 1.16)	0.97 (0.67 – 1.41)
Stenosis $\geq 70\%$	10 (19%)	139 (23%)	0.82 (0.46 – 1.46)	0.91 (0.51 – 1.62)

Table 2. Prevalence of atherosclerotic changes according to presence or absence of migraine. aRR indicates adjusted risk ratio; CI, confidence interval; ICA, internal carotid artery; and RR, risk ratio. * Age and sex adjusted. †Anterior and posterior circulation combined. ‡ Tertile largest vs tertiles medium and smallest volume of internal carotid artery calcifications.

656 patients were included in this study of whom 53 had a confirmed migraine diagnosis (29 with aura) by telephone interview, and 603 had no history of migraine. The median of time since the last attack was 1 year (n=47), and 38% of patients reported to have active migraine. Median attack frequency was 2x per month (n=22). The baseline characteristics are shown in Table 1.

Atherosclerosis in intracranial vessel segments was as frequent in migraine patients as in patients without migraine (Table 2). This was the same for extracranial vessels and was also true for both the anterior and posterior circulation. High intracranial internal carotid artery calcification volumes were as frequent in migraine patients as in patients without migraine. We found no differences in atherosclerotic changes in migraine patients with and without aura, although group sizes were small. Our results remained consistent after stratification for age and stroke cause (Tables 1 and 2 in the Supplemental Material).

DISCUSSION

Our findings argue against the hypothesis that migraine patients are at higher risk for ischemic stroke because of higher atherosclerotic load in the cerebral vasculature. If anything, our data suggest that the prevalence of atherosclerotic changes was lower in stroke patients with migraine. This confirms previous findings in the literature where the risk for ischemic stroke was apparent for migraine patients without vascular risk factors (except for use of oral contraceptives and smoking) and low Framingham risk scores.⁵

Strong points of our study include the large number of participants and the state-of-the-art imaging methods enabling detailed assessment of the radiological characteristics of atherosclerosis. All migraine diagnoses were confirmed by an extensive telephone interview according to the ICHD-II criteria which are comparable with the recent updated ICHD-III criteria.

Our study also has limitations. First, the study is performed in a stroke population with highly prevalent traditional risk factors, such as older age, history of hypertension, diabetes mellitus, and hyperlipidemia. Compared with these traditional risk factors, the contribution of the possible migrainerelated atherosclerosis may be too small to be detected. Second, our study did not include a control group without stroke. One could hypothesize that migraine patients might show enhanced atherosclerosis at younger ages resulting in earlier strokes but with comparable atherosclerotic changes than patients without migraine at the time of the stroke. However, although migraine patients were indeed younger at time of their stroke, our results were consistent in different age categories. Third, not all patients filled in the Migraine in Stroke Screener and not all screen positives could be confirmed by telephone interview. Patients with possible migraine but without confirmation were excluded from the study to avoid misclassification bias. Therefore, the exact prevalence of migraine in our stroke population cannot be derived from our study. Also, patients who were moribund or severely aphasic were less likely to have filled in the screener. We cannot rule out that this affected the generalizability or the internal validity of the results.

Our study does not provide information on other possible mechanisms underlying the increased ischemic stroke risk in migraine patients. Endothelial dysfunction has been related to early development of atherosclerosis but also to activation of the coagulation pathway, enhanced inflammatory responses, and impaired vascular reactivity.⁶ Although we found no excess atherosclerosis in migraine patients, future studies should investigate the possible

impact of endothelial dysfunction on stroke risk via other mechanisms.

AUTHORS CONTRIBUTION

H.J.A. van Os contributed to analysis and interpretation, critical revision of the article, study concept design, and is a lead author; I.A. Mulder contributed to data acquisition, analysis and interpretation, and critical revision of the article; Drs van der Schaaf, Kappelle, Velthuis, Visser, and Schonewille contributed to data acquisition and critical revision of the article; Dr Broersen contributed to critical revision of the article and provided and supported use of software for data analysis; Dr Algra contributed to analysis and interpretation and critical revision of the article; Drs Terwindt and Ferrari contributed to critical revision of the article and study concept design; Dr van Walderveen contributed to data acquisition, analysis and interpretation, critical revision of the article, and study concept design; and Dr Wermer contributed to data acquisition, analysis and interpretation, critical revision of the article, study concept design, and acts as a supervisor of lead author.

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SUPPLEMENTAL MATERIAL**Methods***Standardized scan protocol*

All patients underwent non-contrast CT (NCCT), CTA and CTP on admission with standardized scan protocols. Scan parameters of the NCCT were: 120 kV, 300-375 mAs and 1 mm reconstructed slice thickness. For CTA 50–70 mL of contrast agent (300 mg I/mL) was injected into the antecubital vein (18-gauge needle) at a rate of 6 mL/s followed by a 40-mL saline flush at a rate of 6 mL/s. The scan parameters for the CTA were: 120 kV, 150 mAs and 5 mm reconstructed slice thickness. Radiologic parameters were assessed by trained neuroradiologists with good inter observer variability.¹

MISS screener

The MISS consisted of five items: 1. Did you ever or do you still have migraine attacks? 2. Were you ever diagnosed with migraine by a physician? 3. Did you ever suffer from attacks of severe headache that lasted for several hours to days, with concomitant nausea and possible vomiting? 4. Did you ever suffer from attacks of severe headache that lasted several hours to days during which you had very low tolerance of light and noise? 5. Did you ever experience attacks that lasted between 5 to 60 minutes during which your sight was diminished or blurry at one side with possible flashes or glitters in the visual field, followed by headache?²

Radiological assessment of atherosclerosis

We assessed patients with any sign of atherosclerosis in intra- and extracranial vessels of the anterior and posterior circulation on CTA. Signs of atherosclerosis in extracranial vessels were defined as presence of soft plaque, calcified changes or mixed plaque. Intracranial vessels were divided into segments: the anterior cerebral (A1, A2), middle cerebral (M1, M2), posterior cerebral (P1, P2), vertebral (V1, V2), the internal carotid arteries up to and past the clinoid process and the basilar artery. Extracranial vessels were divided into the posterior (vertebral arteries) and anterior circulation (internal carotid arteries). Stenosis was classified in intracranial vessel segments as any sign of stenosis and additionally in extracranial vessels as stenosis $\geq 70\%$.³

We also assessed atherosclerotic changes by measuring intracranial internal carotid artery (ICA) calcification volume, using calcium as a measure for atherosclerosis since these two parameters are highly correlated.⁴ Calcium volumes were measured from the petrous part to the top of the intracranial carotid arteries on NCCT using dedicated software (customized research version of CalcScore V11.1 by Medis Specials bv, The Netherlands). Regions of interest were drawn to discern intracranial ICA calcifications from the skull base using a threshold. A small pilot study was performed to find the optimal threshold for visually discerning ICA calcifications from the skull base. Since DUST was a multicenter study with CT data from different vendors, we tested several thresholds on 10 randomly selected data sets per center. We found the optimal threshold to be 160 Hounsfield units, which resulted in a spread of ICA calcification volume data that did not notably vary between centers. Continuous intracranial ICA calcification volume data were subsequently divided into tertiles (small, medium and large ICA calcification volumes).

Statistical analysis

We performed multivariable Poisson regression analyses to identify possible relationships between history of migraine and radiological characteristics of atherosclerosis. Adjustments were made for age and sex. Adjustments for hypertension and smoking had only a minimal effect on the results and were therefore not performed in the final analyses. Additionally, we stratified for age and stroke etiology according to the TOAST criteria. Risk ratios (RR) and adjusted RR (aRR) with 95% confidence intervals (CI) were calculated.

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TABLES

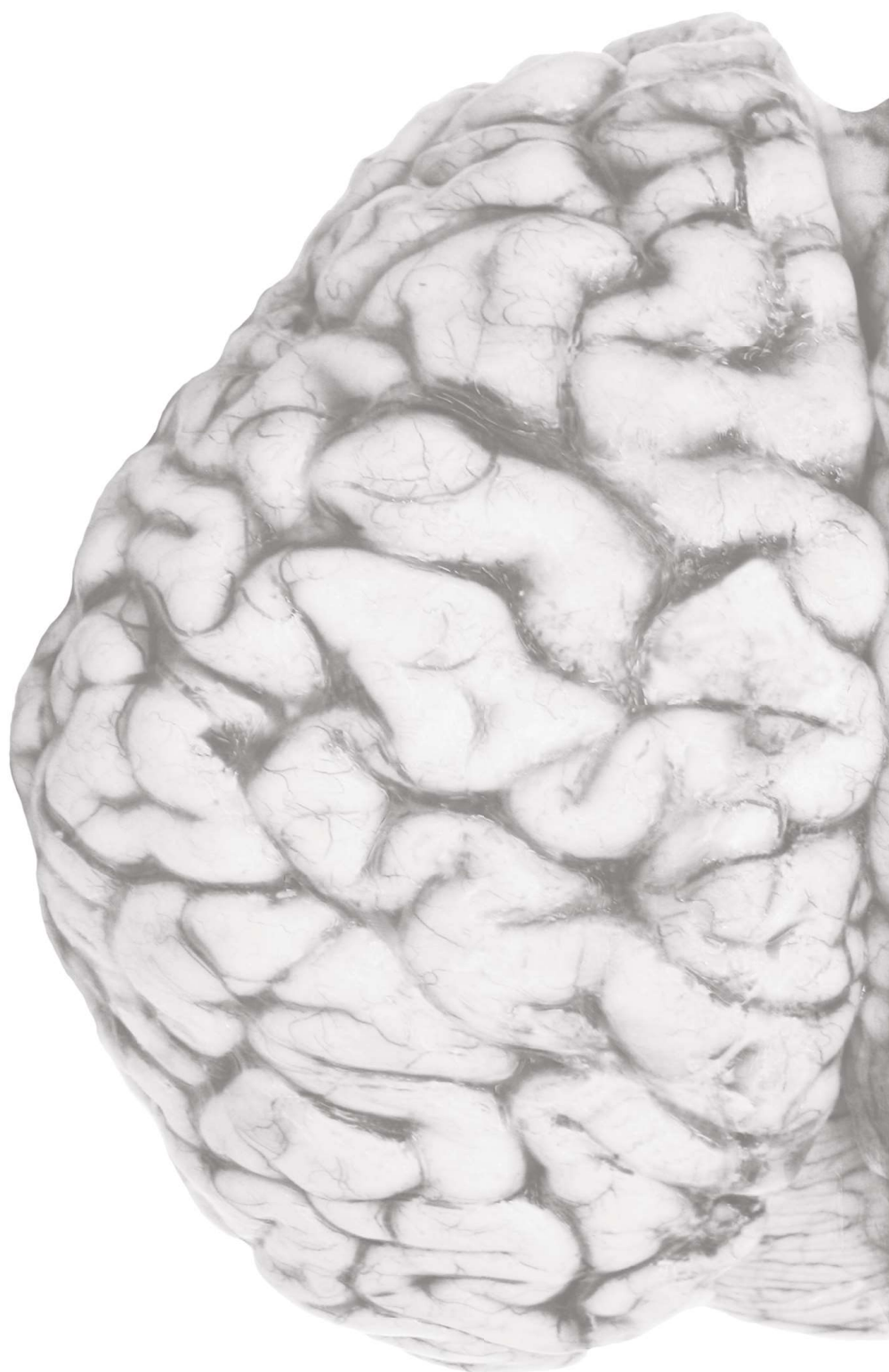
	MA (n=29)	MO (n=24)	RR (95% CI)	aRR (95% CI)†
Intracranial circulation				
Any sign of atherosclerosis*	14 (48%)	13 (54%)	0.89 (0.53 – 1.51)	1.41 (0.80 – 2.45)
Any sign of stenosis	2 (7%)	2 (8%)	0.83 (0.13 – 5.45)	1.05 (0.36 – 3.04)
Tertile largest ICA calcification volume**	6 (21%)	6 (25%)	0.83 (0.31 – 2.24)	1.11 (0.28 – 4.44)
Extracranial circulation				
Any sign of atherosclerosis*	19 (66%)	14 (58%)	1.12 (0.73 – 1.73)	1.40 (0.87 – 2.25)
Any sign of stenosis	9 (31%)	9 (38%)	0.83 (0.39 – 1.75)	0.86 (0.41 – 1.83)
Stenosis ≥70%	7 (24%)	3 (13%)	1.93 (0.56 – 6.67)	1.84 (0.54 – 6.26)

Supplemental Table 1. Prevalence of atherosclerotic changes in the 53 migraine patients according to presence or absence of aura. *Anterior and posterior circulation combined. **Tertile largest volume versus tertiles medium and smallest volume of internal carotid artery (ICA) calcifications. † Adjusted for age and sex

ATHEROSCLEROSIS IN AIS WITH MIGRAINE

Stratification factor	Any sign of atherosclerosis	Migraine	No migraine	RR (95% CI)	aRR (95% CI)†
Age group, n (%)					
<50 (86, 14%)	Intracranial	0 (0%)	18 (24%)	–	–
	Extracranial	0 (0%)	25 (33%)	–	–
50-69 (294, 45%)	Intracranial	19 (56%)	190 (73%)	0.77 (0.56 – 1.04)	0.77 (0.56 – 1.05)
	Extracranial	25 (74%)	210 (81%)	0.91 (0.74 – 1.12)	0.94 (0.77 – 1.16)
≥70 (276, 42%)	Intracranial	8 (89%)	237 (89%)	1.00 (0.79 – 1.27)	1.00 (0.79 – 1.28)
	Extracranial	8 (89%)	241 (90%)	0.99 (0.78 – 1.25)	0.99 (0.79 – 1.24)
Stroke etiology group, n (%)*					
Large vessel disease (184, 43%)	Intracranial	8 (62%)	145 (85%)	0.73 (0.47 – 1.12)	0.85 (0.55 – 1.33)
	Extracranial	10 (77%)	150 (88%)	0.88 (0.65 – 1.19)	0.96 (0.72 – 1.29)
Cardioembolism (105, 25%)	Intracranial	7 (88%)	70 (72%)	1.21 (0.91 – 1.62)	1.27 (0.95 – 1.69)
	Extracranial	6 (75%)	77 (79%)	0.95 (0.63 – 1.43)	0.98 (0.69 – 1.40)
Small vessel disease (88, 21%)	Intracranial	3 (43%)	58 (72%)	0.60 (0.25 – 1.42)	0.66 (0.27 – 1.61)
	Extracranial	5 (71%)	60 (74%)	0.96 (0.59 – 1.57)	1.06 (0.76 – 1.47)

Supplemental Table 2. Any sign of atherosclerotic changes according to presence or absence of migraine, stratified for age or stroke etiology. † Adjusted for age and sex. * Stroke etiology according to the TOAST criteria.





CHAPTER 9

GENERAL DISCUSSION



DISCUSSION

The work described in this thesis aims to unravel the connection between stroke and migraine in a translational manner, that is by investigating the molecular and functional aspects of this connection in relevant mouse models (Part I) and the clinical and radiological characteristics in stroke patients with or without migraine (Part II).

Part I describes the optimization of experimental methodology by minimizing *post mortem* degradation of compounds in (stroke) brains and by developing a more efficient, automated analysis tool for stroke volume segmentation of mouse MRI data. Subsequently, this methodology was used in studies investigating characteristics of migraine and stroke in various transgenic mouse models. Part II describes the use of neuroimaging CT techniques (CT angiography (CTA) and whole brain CT perfusion (CTP)), in addition to the conventional non-contrast CT (NCCT), to analyse brain damage after stroke upon hospitalization as well as at follow-up, in patients with or without migraine.

Minimizing post-mortem degradation during the harvesting of mouse brains

Matrix-Assisted-Laser-Desorption / Ionization Mass Spectrometry Imaging (MALDI MSI) can be used to simultaneously record the distribution of hundreds of molecules directly from a tissue sample, so within its histological context. When analysing the presence of compounds in stroke tissue with a technique such as MALDI MSI, it is important to minimize post-mortem degradation of molecules. An often used method to harvest a mouse brain involves decapitation and freezing of the head (or removing the brain first from the skull) in liquid nitrogen, which may take minutes in which considerable *post mortem* degradation of molecules can occur. *Ex vivo* heat-stabilization, which should prevent degradation by inactivating responsible enzymes with heat, is also often used.¹⁻⁴ For molecules with a slow turnover time, such as many proteins, the short time that degradation can occur may not affect the interpretation of results, but this is very different for molecules with a very fast turnover time as their degradation will be noticeable already seconds after death.³ The latter is the case for molecules such as ATP/ADP/AMP, which are instrumental for analysing the ischemic infarct territory and pinpointing biological mechanisms that occur in the core and penumbra.¹ Therefore, in stroke research MALDI MSI can only be properly exploited when adequate sacrificing and tissue processing protocols are used. A promising method is the *in situ* funnel-freezing technique.³⁻⁵ With this technique, liquid nitrogen is poured into a plastic funnel that is placed directly on the intact skull while the animal is under anaesthesia. The benefit of the method is that blood circulation remains essentially intact until the tissue is frozen, giving *post mortem* degradation very little chance. In **Chapter 2**, *in situ* funnel-freezing was shown to be superior to *ex vivo* heat-stabilization when it comes to *post mortem* degradation, especially when combined with fast thaw-mounting of tissue sections onto glass slides where after sections can be analysed using MSI. An alternative to *in situ* funnel-freezing is the use of microwave radiation of the anesthetized animal,² which also is efficient in minimizing *post mortem* degradation. However, serious down-sides of this technique are the obvious ethical concern of having to sacrifice an animal in a microwave and the fact that it requires rather expensive equipment. One can debate which of these two *in situ* methods is best for which type of molecular class. As *in situ* funnel-freezing does not have the ethical disadvantage, and because it can be effectively used in standard laboratory settings, this might be the preferred method.

Lipid composition in core and penumbra of WT and FHM1 mice

Oxidative stress induced by ischemic injury triggers changes in neuronal membrane phospholipid composition that are essential for cellular function and are involved in numerous signalling pathways. In **Chapter 3**, MALDI MSI-based changes were identified in the core and penumbra region for the Na⁺/K⁺-lipid ratio and phospholipids such as phosphatidylcholine, Lyso-phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. In MSI, compounds are identified using their m/z value, where “m” is the mass and “z” stands for charge number of ions. The most interesting finding was a clear difference between the infarct core and penumbra for compound m/z = 965.5. The compound showed a specific increased signal intensity in the penumbra and a decreased intensity in the infarct core, already 8 hours after stroke induction. Follow-up MS/MS analysis revealed that the identity of this compound is PIP₁(38:4) (phosphatidylinositol 4-phosphate). PIP₂(38:4), phosphatidylinositol 4, 5-bisphosphate, (m/z = 1045.5) and PI (38:4) (phosphatidylinositol) showed the same pattern in the border zone, however less profoundly. The respective signal transduction pathway involving hydrolysis of polyphosphoinositides (poly-PI) in the central nervous system is highly energy consuming, requiring several moles of ATP via PI and PIP kinases.⁶ With the depletion of ATP during ischemia, PIP₂ high-energy-dependent turnover from PIP might be abolished, which could be the reason for the increased PIP concentration compared to PIP₂ in the penumbra. Also, stimulation of the poly-PI pathway in the border zone might be part of an early event resulting in ischemia, which is also suggested by the rapid and transient increase of Ins(1,4,5)P after global cerebral ischemia shown before.⁶ These results suggest that there is a biphasic change in poly-PI levels towards the fate of border zone tissue becoming necrotic. The fact that this change in signal intensity already occurs 8 hours after ischemic infarct onset may make it an interesting biomarker for subsequent neuronal apoptosis in the ischemic penumbra, which begins several hours to days after infarct induction.⁷

Comparison of MALDI MSI data from the various areas after stroke (*i.e.* healthy, penumbra, core) did reveal a difference between WT and FHM1 mice that is mainly present in the borderzone. At every time point, increased sodiated species were observed in the penumbra in FHM1 mutant mice. In contrast, in WT mice, the overall spectrum was mainly driven by potassiated species. This may reflect the massive failure of sodium/potassium pumps during early ischemic events that results in a large influx of sodium, displacing potassium.⁸ These high intracellular levels of sodium ions lead to the production of [M+Na]⁺ pseudomolecular ions during the ionization process. Together with the competition between K⁺- and Na⁺-ions for the same molecular species, it leads to an apparent increase in the detected levels of sodiated species and a concomitant reduction in the detected levels of potassiated species. Hence there seems to be a direct correlation in the tissue between sodium/potassium pump failure and a higher proportion of sodiated species. This interesting difference between WT and FHM1 mutant mice might be a result of the increased peri-infarct depolarizations (PIDs) and accompanying increased infarct volume shown in FHM1 mice.⁹

Automated segmentation of infarct volumes in experimental stroke research

Measuring infarct volume from MRI data is one of the most widely used readout parameters in experimental stroke research. However, until now, volume measurements heavily rely on time-consuming manual tracing or, at best, semi-automated segmentation. An inter- and intra-observer bias is easily introduced, which make intra-laboratory comparisons hardly feasible, especially when different scanners and analysing protocols are used. Therefore, in **Chapter**

4, an automated infarct segmentation method was developed for MRI mouse stroke data. This new method reliably segments ischemic infarcts in a mouse brain from T2 MR images and reduces the analysis time incredibly. Although we regard our method more reliable than manual tracing of infarcts, our method still may introduce some bias, especially with respect to tracing the shape of ventricles, so a visible check of the segmentation should be performed to make sure that large errors in measuring infarct sizes are avoided. An important asset of our method is that it is compatible with different MRI hardware and software configurations, and therefore can be used in most laboratories. When widely used, it will likely improve reproducibility in pre-clinical stroke research and will make testing of potential treatment options for stroke in multiple laboratories feasible. Therefore, as part of the dissemination of the research, the algorithm and the raw MRI data were made freely available.

The interplay between neuronal and vascular pathology in stroke and migraine

Evidence is accumulating that pathophysiological mechanisms in stroke (vascular dysfunction) and migraine (cortical spreading depolarization (SD)) are related. In fact, there seems to be a bi-directional pathway, where ischemia can cause SD and SD can cause ischemia, at least under specific conditions.

1. The migraine-ischemia connection

Cortical SD, *i.e.* waves of depolarization of neurons and glial cells, is considered the underlying mechanism of the migraine aura.^{10,11} Studies in transgenic migraine mice have shown that the enhanced susceptibility to experimentally induced cortical SD is caused by a hyperexcitability of the brain,¹¹ likely as the result of an imbalance between excitatory and inhibitory synaptic transmission. In experimental and clinical stroke, SDs (called PIDs) circle around the infarct core.^{9,12,13} PIDs are triggered by loss of membrane integrity due to hypoxia and energy depletion. Due to the energy mismatch created by the PIDs, part of the penumbra tissue will turn into a permanently depolarized and necrotic state,^{12,14,15} and therefore increase infarct volume.^{9,12}

In brains which are more susceptible for SD (as seen in migraine), infarct evolution might be increased and the threshold for the occurrence of an infarct might be decreased. The relation between *having* migraine and an (increased risk of) ischemic stroke may, therefore,

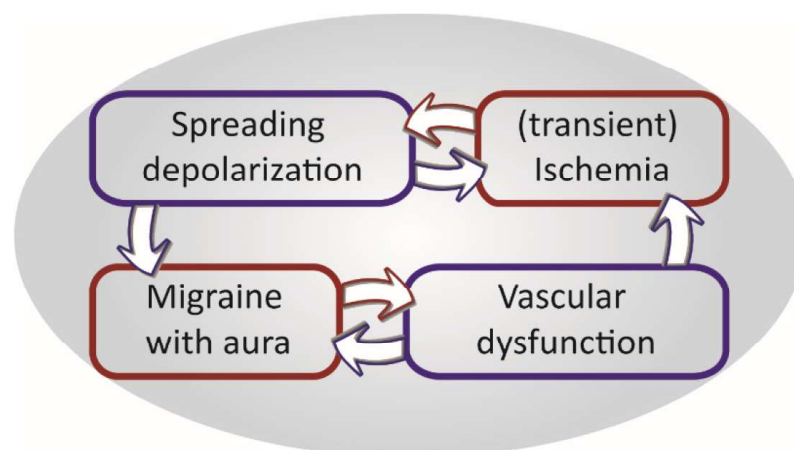


Figure 1. The detrimental circle supporting the stroke-migraine-SD-vascular dysfunction hypothesis.

be explained by the occurrence of cortical SD events and accompanying changes in blood flow in cerebral small vessels.¹⁶ Both are considered to follow the increased and subsequently reduced metabolic demand of neurons and glial cells during an SD (Figure 1). It was shown that this accompanying reduction in blood supply does not reach the ischemic threshold in healthy brain,¹⁷⁻¹⁹ but it might be the last push towards ischemia in pathological tissue.

2. The ischemia-migraine connection

In contrast, it is argued that there might be a scenario of *having* a vascular event and *getting* a migraine-like-aura, which could be explained by changes in blood flow in cerebral small vessels and accompanying occurrence of cortical SD events. There is experimental data for this “reversed” SD-ischemia mechanism, which suggests that, even in normal brains, a vascular event can trigger a SD²⁰⁻²² and eventually cause a migraine aura (Figure 1).²¹ Most relevant here, vasomotor changes in the cerebral cortex have been shown to travel (I) at an increased speed, (II) in an altered pattern, and (III) in an extended territory compared with neuronal changes.²⁰ Thus, vascular alterations could precede neuronal activity, which is in contrast to the general belief that vascular changes follow alterations in neuronal dysfunction (e.g. SD, altered ion transport and brainstem dysfunction).²⁰ There is much support for a relation between vasculopathies and migraine given that e.g. endothelial dysfunction,²³ hypercoagulability²⁴ and pathological vascular reactivity²⁵ are more common in stroke and migraine patients. This evidence points towards a clear role of (cerebral) blood vessels in migraine pathophysiology. Results in **Chapters 5 and 6** are also in support of this theory, where it was shown that RVCL-S mice do have increased infarct volume (likely due to the vasculopathy), but do not have increased cortical SD susceptibility, (a process more linked to neuronal and glial activity). Therefore one might argue that the migraine seen in RVCL-S patients might have its origin in the vasculopathy.

Experimental and clinical data seems to point towards a bi-directional effect of neuronal and vascular events and pathology, but the exact link is still debated and therefore should be investigated further.

Vascular and neuronal mechanisms in the stroke-migraine association: evidence from monogenic mouse models

Whereas it is well-established that vascular mechanisms play a key role in stroke, the importance of the vascular role in migraine is still debated.²⁶ The last decades, at times, either the vascular²⁷ or the neuronal¹⁰ theory got the popularity vote. However, mainly because of basic science research, genetic and neurobiological evidence is accumulating that instead of a pure vascular or neuronal origin, there seems to be an intimate interplay of neuronal and vascular components in migraine pathogenesis. Therefore, the opportunity to experimentally investigate stroke and migraine characteristics in transgenic models for monogenic disorders as CADASIL, RVCL-S and FHM1, in which patients have clinical features of both disorders, might be informative with respect to the influence of and interplay between vascular (smooth muscle cell or endothelial) dysfunction and increased susceptibility to cortical SD. Whereas vascular involvement was shown earlier in FHM1⁹ and CADASIL,²⁸ in **Chapter 5**, it was demonstrated that vascular involvement is also present in RVCL-S mutant mice, as evidenced by a reduced response after hyperemia in mutant mice of all age groups and attenuated relaxant responses to acetylcholine in 2-year-old mutant mice. The observation in **Chapter 6** that RVCL-S mice show unaffected susceptibility to cortical SD may suggest that neuronal involvement is not key in RVCL-S. This is different for FHM1 mice in which susceptibility to cortical SD was clearly

affected. The number of CADASIL mutant mice studied, however, was too small to assess whether susceptibility to cortical SD was affected or not.

Infarct phenotype in RVCL-S

Instead of a neuronal dysfunction, the increased infarct volume seen in RVCL-S mutant mice might be caused by vascular dysfunction and exacerbated neuroinflammation, given that an increased immune response has been suggested in RVCL-S patients.²⁹⁻³³ Both vascular deficiency and neuroinflammation could explain increased edema after infarction, which is surrounding lesions in RVCL-S patients.³⁴ The vascular deficiency and increased infarct volume shown in **Chapter 5** is in line with clinical features of RVCL-S, including periventricular white matter T2 hyperintensities and infarct calcifications,³⁵ areas of ischaemia and necrosis secondary to an occlusive endotheliopathy of small-sized and medium-sized arteries³⁵ and inflammatory lymphocytic infiltration in lesions, which are maybe due to BBB disruption (which could explain the increased infarct volume).³⁵ However, it remains somewhat unclear to what extent migraine is present in RVCL-S patients as such association has been reported for some families with a C-terminal truncating TREX1 mutation but not in others.^{35,36}

The earlier-mentioned monogenetic mouse models give us the opportunity to study the complex comorbidity of stroke and migraine, hopefully providing better insight on the underlying pathophysiological mechanisms in relation to neuronal activity and vasculature.

In this light, for future studies, it may be interesting to also include other monogenic conditions in which the stroke-migraine association is represented in the phenotypic characteristics, like MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes),^{37,38} HIHRATL (Hereditary Infantile Hemiparesis, Retinal Arteriolar Tortuosity and Leukoencephalopathy)^{39,40} and HHT (Hereditary Hemorrhagic Telangiectasia).⁴¹

Discrepancy of stroke and SD characteristics in monogenic mouse models between studies

In **Chapters 5 and 6**, stroke experiments in FHM1, CADASIL and RVCL-S KI mouse models are described. In earlier studies an increased infarct volume was shown in FHM1 mice, which was said to be due to increased cortical SD susceptibility and decreased AD latency.^{9,42,43} In experiments conducted for this thesis findings concerning increased cortical SD susceptibility and decreased AD latency in FHM1 mice with the R192Q missense mutation could be confirmed, but no increased infarct volume after transient MCAO was observed. Possible explanations for this discrepancy are that different MCAO protocols were used and infarct volumes were analysed in different ways. Regarding the latter, for the study in this thesis (**Chapter 3**), ischemic changes were measured from T2 MRI data and an automated infarct segmentation method was used to calculate lesion volume, whereas in the published studies^{9,42} DWI MRI was used to assess early ischemic change and *ex vivo* tissue staining was used for infarct calculation. For the calculations in those studies,^{9,42} manual delineation of the ischemic territory was performed, making the results vulnerable for bias. Even more problematic, the finding of an increased infarct volume could not be reproduced by the same researchers in a follow-up study in which they tested the effect of drugs on infarct parameters in the same FHM1 strain.⁴² A very plausible explanation is that in the first study⁹ the infarct size in the WT mice was particularly low, which was not the case in the follow-up study,⁴² so the comparison of infarct sizes between genotypes was inflated in the first study. Hence, not finding a larger infarct size in the FHM1 R192Q mutant after all, may be an appropriate reflection of reality. In this thesis the more severe FHM1 S218L mutant (that showed even larger infarct sizes than the R192Q mutant and had normal values in the respective controls⁹) were not tested, so it is

still likely that the link between stroke and FHM1 remains.

In the CADASIL mutant mice described in **Chapter 6** we could not confirm the increased cortical SD susceptibility reported for CADASIL mutant and knock-out mice,⁴⁴ although no definite conclusion can be drawn from our study as the group sizes were low. In a future study, group sizes should be increased to allow for proper group comparison and statistical analysis. One possibility for the discrepancy is that different CADASIL mutant *NOTCH3* mouse lines were used in both projects. For both CADASIL strains, an archetypical cysteine-changing *NOTCH3* mutation was used, but in **Chapter 6** the Arg90Cys mutation was present on a human genomic background,²⁸ whereas in the previous study the Arg90Cys mutation was present in a cDNA under the control of the SM22 α smooth muscle cell promoter.^{44,45} Possibly, the different genetic background of both mouse strains influences disease phenotype and/or severity. The conflicting results are likely not due to the methodology used to investigate CSD characteristics as in this thesis it was possible to confirm the abnormal CSD characteristics in FHM1 mutant mice.⁹

The therapeutic time window after infarct onset in patients with and without migraine

If patients that suffer from migraine with aura experience an SD in the acute infarct phase, and SD results in an increased infarct volume, infarct evolution could be faster. If so, we may need to consider a different or a faster therapeutic strategy in this stroke-subpopulation. It is possible, but at present unclear, whether the same applies to migraine without aura patients that may experience so-called silent SDs.^{46,47} However, the existence of this phenomenon is debated.⁴⁸⁻⁵⁰ In a retrospective clinical study,⁵¹ it was suggested that penumbra turnover is indeed faster in migraine patients (with and without aura). However, in **Chapter 7**, in a large prospective cohort of ischemic stroke patients, participants who also suffer from migraine (with or without aura) did not have an increased infarct volume, nor more secondary damage after stroke, or a poorer outcome after treatment. Not finding such effects in migraine patients could be due to the method used: MRI (DWI and PWI)⁵¹ vs. CT (non-contrast and CTP) (in the present study), difference in stroke-to-imaging time inclusion criterion (<72 hours⁵¹ vs <9 hours (in the present study)), the large spread of data in both studies, and the small number of patients included in the retrospective study.⁵¹ Besides that, recall bias might have occurred in our study. Our population was in general about 60 years old at time of their stroke, but migraine is most active at younger age. The higher susceptibility for SDs in migraineurs might be associated with status of migraine activity, but no data on this was present in our study. As the number of young stroke in our cohort was low, the possibility of an effect of migraine on brain injury after stroke in this category of patients cannot be excluded.

Whether the therapeutic time window for revascularization therapy (iv thrombolysis or thrombectomy) is shorter in migraineurs with an acute ischemic infarct cannot be confirmed by our study and remains unclear. Besides that, no diminished treatment effect of intravenous thrombolysis or mechanical thrombectomy was found in participants with migraine, which could also plead for identical therapeutic time windows.

The gap between experimental and clinical stroke research: and how to go from here

Although major progress has been made in the last decades with respect to the understanding of the pathophysiology of stroke in an experimental setting, translation of findings to the clinic is essentially lacking as evidenced by the numerous failed clinical trials based on results of basic science (with the exception of intravenous thrombolysis with recombinant

tissue plasminogen activator and mechanical thrombectomy).^{7,52,53} Hence, bench-to-bedside translation remains, to a large extent, a black box due to (unchangeable) factors like species differences, heterogeneity of clinical acute ischemic stroke characteristics, and complex relationships between structural brain damage and clinical outcomes.⁵⁴ Despite the failure of experimental therapeutic treatments in clinical trials, pre-clinical stroke research did produce relevant information on basic pathophysiological mechanisms, which help predict human pathophysiology, clinical phenotypes and therapeutic strategies. For example, the differentiation between core and penumbra was first described in monkeys subjected to experimental stroke.^{55,56} Based on extensive animal research and using new imaging techniques such as MRI and CTP, the penumbra-phenomenon has evolved into an important factor for treatment decision-making and outcome prediction in today's clinical stroke care.⁵⁷ SD, first described as spreading depolarization by Leão in 1945,⁵⁸ was also examined in great detail in animal models.⁵⁹ SD events were also found in patients with various disorders,⁵⁹ including patients with an ischemic stroke or subarachnoid hemorrhage.^{13,60-63} Experimental stroke research is an important step in unravelling the pathophysiological mechanisms involved in stroke, developing treatment targets and therapeutics itself. However, we need to pay closer attention to study designs to reduce bias and bridge the translational gap. Many factors should be considered to reduce confounding due to study quality, for example by using the ARRIVA-criteria.^{64,65}

When translating experimental work towards clinical practice, we should keep the different techniques used to investigate stroke characteristics in mind, for example imaging, which plays a major role in diagnostic, therapeutic, and prognostic clinical decision making.⁶⁶⁻⁶⁸ Both MRI and CT can be used to investigate the critical four “P’s” of acute stroke imaging: parenchyma, pipes, perfusion and penumbra.⁶⁹

Non-contrast CT (NCCT),⁷⁰⁻⁷² CT angiography (CTA)⁷³⁻⁷⁵ and CT perfusion (CTP)⁷⁶⁻⁸¹ are often used in modern stroke diagnostics, decision-making concerning treatment and outcome prediction. MRI data acquisition can include T1- and T2-weighted imaging, diffusion-weighted imaging (DWI)⁸² and perfusion-weighted imaging (PWI)^{83,84} and MR angiography.⁸³ For both modalities, debate is ongoing concerning the detection of the penumbra (using mismatch between NCCT and CTP or between DWI and PWI, therefore multiple different methods are in play.⁸⁵ Due to technical reasons, MRI is the preferred technique in the experimental stroke setting (**Chapters 2-6**), but due to some practical limitations concerning MRI, CT is the most often used imaging technique in the acute stroke setting in the clinic (**Chapters 7-8**).

To be successful, uniform robust experimental models are crucial and these models should represent the clinical settings as close as possible, which is, by definition, a challenge as no perfect model exists. One important difference between pre-clinical and clinical research is that in rodent studies the aim is to obtain a similarly-sized infarct at the same location, which is achieved by performing the MCA occlusion in animals in a highly standardized manner, whereas in clinical studies, participants are included with infarcts of variable sizes at heterogeneous locations, including lacunar infarcts. Although the homogeneity of the produced infarcts is an advantage of experimental research, one needs to take this additional challenge to bridge the translational gap into account when combining and extrapolating results from animal (including **Chapters 2-6**) and clinical (including **Chapters 7-8**) research.

Bias should be minimized by proper study design,^{86,87} some of which can be rather easily included in the experimental design, such as randomization, blinding (of the researchers that perform the experimental work and the analysis of data), type of animal (sex and age), timing of treatment, etc. Unfortunately, such simple measures to prevent unnecessary bias are absent

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in (the description of) many experimental stroke studies.^{64,65} A possible solution to improve the translational quality of findings from pre-clinical research is to, also in experimental research, perform randomized controlled multi-center studies,⁸⁸⁻⁹⁰ as is common practice in clinical studies. The failure of drug testing in clinical trials, however, is also due to potential issues with the way trials are currently performed, namely amongst others, small trials and the timing of treatment.^{86,91}

Overall, better care should be taken to ensure a more rigid experimental setup and experimental results should be interpreted with more care to engage increasing translational black-box transparency.

Directions for future research

The research described in this thesis serves as a building block for future experimental as well as clinical/epidemiological research concerning the association between stroke and migraine.

Future research should:

- A) in more detail dissect stroke and migraine pathophysiology;
- B) explore new therapeutic possibilities to reduce the risk and burden of stroke;
- C) try to better bridge the existing gap between experimental and clinical research.

Possible interesting topics with respect to (A) to investigate in the future are: (I) in-depth molecular analysis of brain tissue, blood and CSF in experimental animal models to better pinpoint important disease pathways that may yield possible therapeutic targets to correct neuronal and/or vascular dysfunction; (II) investigation of additional monogenetic diseases

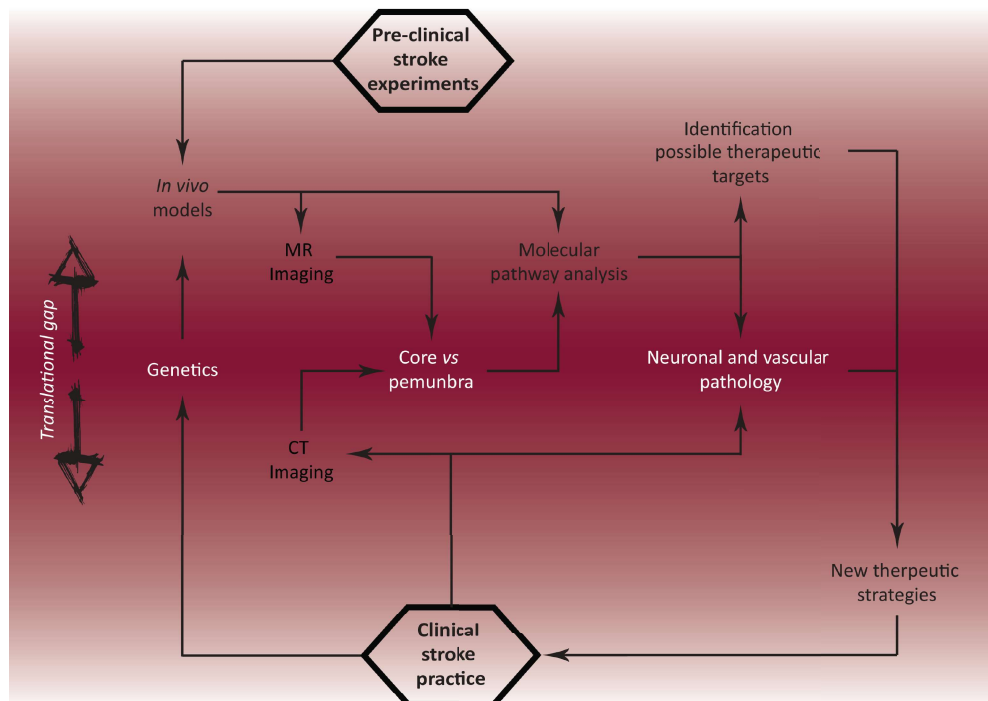


Figure 2. The detrimental circle supporting the stroke-migraine-SD-vascular dysfunction hypothesis.

with stroke and migraine in their clinical spectrum, foremost MELAS, HIRATL or HHT, to further substantiate the role of SD, vasculature, and stroke vulnerability in disease pathology; (III) investigation whether or not the induction of multiple SDs in the monogenic mouse models of FHM1 and CADASIL can induce ischemic damage; and (IV) investigation of SD mechanisms and ischemia in *in vivo* and *in vitro* models, such as a “brain-on-a-chip”, which has become a realistic possibility,⁹² already used in multiple fashions.⁹²⁻⁹⁴ Next generation sequencing and microarray technology are powerful methodologies to unravel changes in gene expression in such models, in a particular cell type and/or in response to a particular condition, for example SD or ischemia. Such experiments can yield knowledge on molecular pathways that the brain uses to overcome these events (neuroprotection), which may provide possible avenues for developing novel therapies.⁹⁵

Possible interesting topics with respect to (B) to investigate in the future are: (I) test new (and existing) pharmacological compounds that can reduce risk and burden of stroke in experimental mouse models, more precisely to investigate their effect on infarct growth and outcome and SD, and thereby pinpoint the primary site of action (neuronal and vascular) and their interplay in migraine and stroke; and (II) explore non-pharmacological therapeutic strategies, such as nervus vagus stimulation, which has shown first promising results in animal models⁹⁶ and patients⁹⁷⁻⁹⁹ and specifically investigate effects of the procedure on for instance infarct outcome.

Lastly, possible interesting topics with respect to (C) to investigate in the future are: (I) increase of diversity in animal models for example by comparing male and female mice, but also older mice in experimental designs, as was done in this thesis, to be better able to extrapolate data from experimental research to the heterogeneous patient population; (II) make the important step towards pre-clinical phase III trials and prospective clinical cohort studies of acute stroke. In such pre-clinical trials, possible stroke and SD prophylactic drugs could be evaluated. These trials could also identify other unknown variables which effect the risk for stroke onset and increased infarct evolution (for example altered coagulation factors and endothelial dysfunction).

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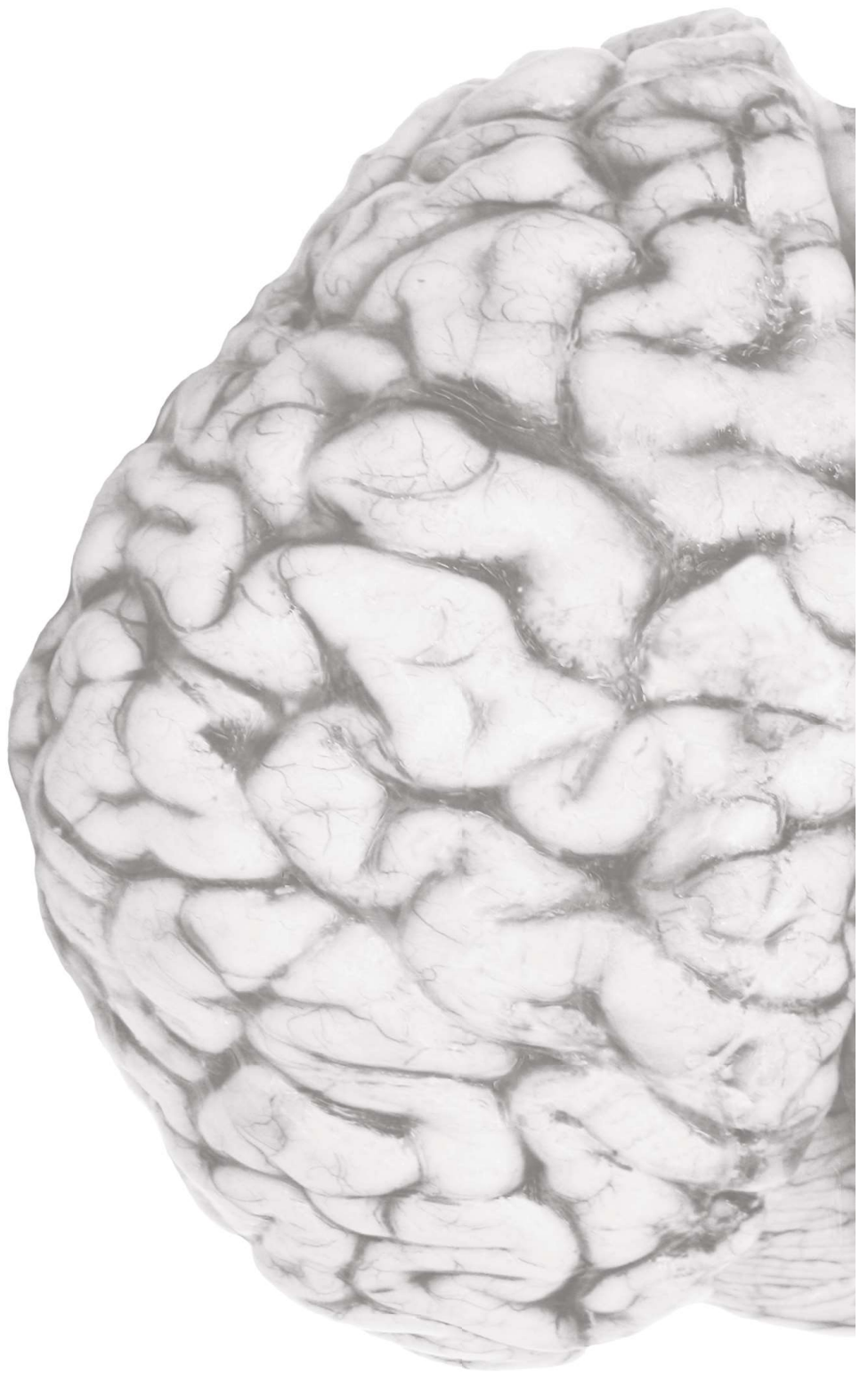
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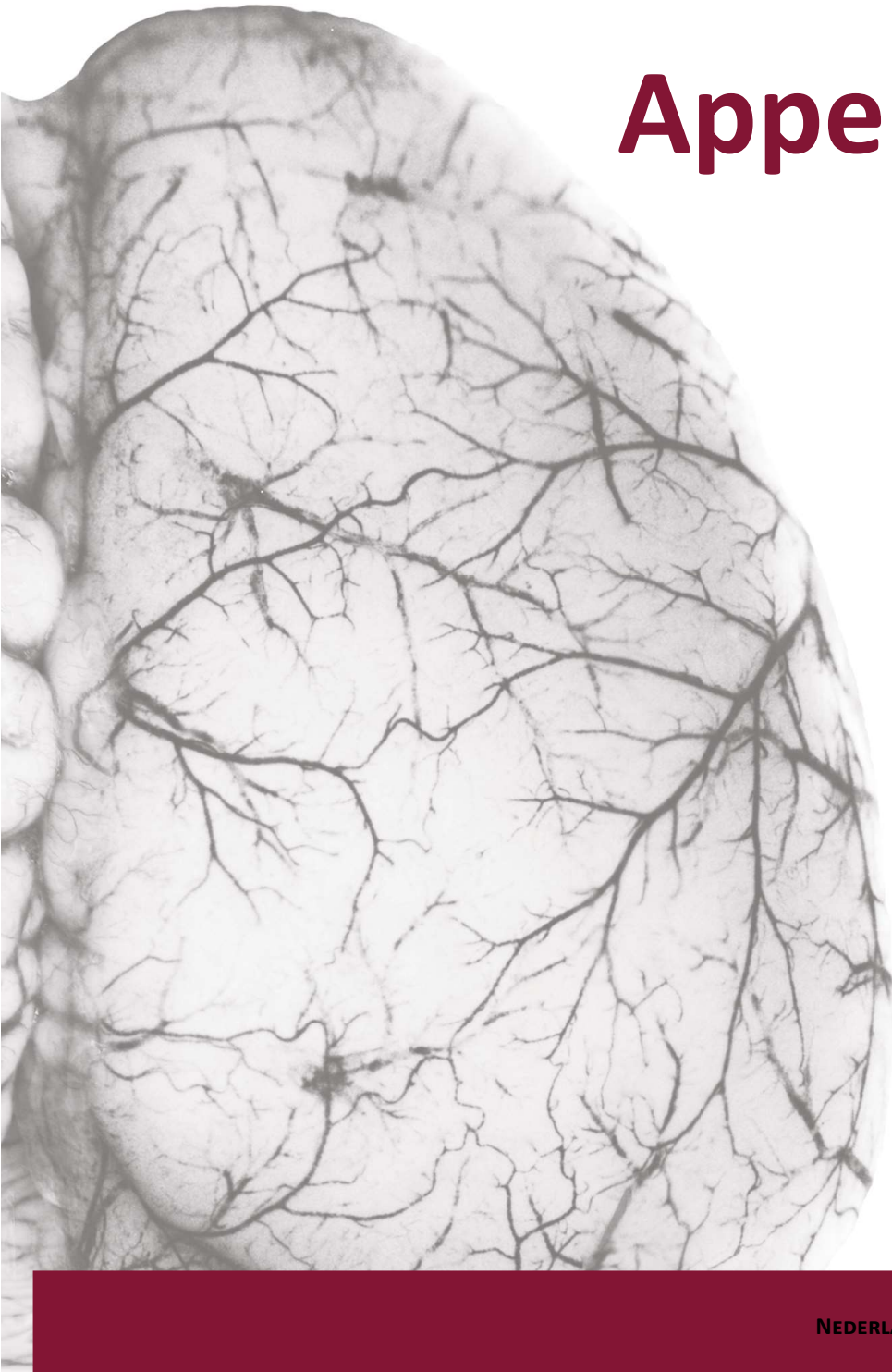
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Appendix

SUMMARY
NEDERLANDSE SAMENTATTING
LIST OF PUBLICATIONS
CURRICULUM VITAE
DANKWOORD

SUMMARY

The research described in this thesis was aimed at identifying and understanding biological mechanisms and molecular pathways involved in the pathophysiology of stroke and migraine, including the detrimental connection between them. The thesis consists of two parts. Part 1 describes multiple experimental stroke research projects in mice in which we set out to: (I) improve the methodology of stroke research, and (II) unravel the stroke-migraine connection using different research strategies, methods, and transgenic mouse models. The mouse models express human pathogenic mutations found in CADASIL, RVCL-S and FHM1 and represent the clinical spectrum of monogenic disorders linking ischemic stroke and migraine. Part 2 includes multiple clinical projects in which we set out to study a large cohort of ischemic stroke patients with and without migraine in search for means to investigate stroke characteristics and vascular pathology.

Part I describes the pre-clinical stroke research. In experimental stroke research, tissue preparation is key for obtaining reliable results, as rapid post-mortem changes can negatively influence the analysis of molecular compounds in affected tissue from MSI approaches that detect changes in peptide and metabolite composition with detailed spatial resolution.

Chapter 1 provides a general introduction to the topics described in this thesis.

In Chapter 2 the methodology to sacrifice and process mouse brains for MSI analysis was optimized in a way less sensitive to *post mortem* degradation. Two methods were compared namely *ex vivo* heat stabilization and *in situ* funnel-freezing, of which *ex vivo* heat stabilization is most widely used in experimental stroke research (but prone to *post mortem* degradation). *In situ* funnel-freezing, in which liquid nitrogen is poured over the intact skull (from which the skin had been removed) of the animal has the benefit that blood circulation is maintained until the cold reaches a vessel so compounds are frozen almost instantly, so time for degradation is kept at a minimum. Direct comparison of both sacrificing and two processing methods (that involve thawing of samples so they can be analysed with MSI apparatus) revealed that *post mortem* metabolic stability in brain sections was best maintained using *in situ* funnel-freezing in combination with fast thaw-mounting. This methodology was used for experiments described in Chapter 3.

In Chapter 3 the MCAO model was used to investigate the specific molecular lipid signature after experimental transient infarction in various areas of affected (core, penumbra) and healthy brain tissue in WT and FHM1 mice that harbour the human pathogenic R192Q missense mutation. At 4, 8 and 24 hours after infarct onset, mice were sacrificed using the *in situ* funnel-freezing technique of Chapter 2 and brain sections were analysed using TOF-SIMS MSI, MALDI MSI and MS/MS. Infarct evolution was clearly shown by 2D renderings of multiple (lyso-) phosphatidylcholines isoforms. The penumbra was especially visible starting at 8 hours post-MCAO by $m/z = 965.5$ and $m/z = 1045.5$ corresponding to Phosphatidylinositolphosphate (PIP_1) and Phosphatidylinositol 4,5-bisphosphate (PIP_2) which could be an interesting observation as they may serve as early ischemic markers and provide information on tissue health in the penumbra. Together with the finding of increased sodiated species in FHM1 penumbra compared to increased potassiated species in the WT penumbra might reflect an increased ischemic vulnerability of penumbral tissue especially in migraine mutant mice. The observed changes may help our understanding of the mechanisms involved in the first hours after stroke onset.

In Chapter 4 a novel method was developed to calculate experimental infarct volume in



mice from T2-weighted MRI images in a manner that is less labour intensive than manual demarcation of stroke regions, which is common practice in experimental stroke research. Quantitative analysis of data from manual thresholding and tracing not only takes a lot of time but is also subjected to potential researcher bias. Hence, a fully automated approach was developed for the analysis of longitudinal MRI data to quantify ischemic lesion volume progression in the mouse brain, which reduces the analysis time by more than 10-fold and avoids researcher bias. The method is based on a level-set lesion segmentation algorithm that is built using a minimal set of assumptions and requires only one MRI sequence (T2) as input. The method shows good agreement with manual segmentation and is accurate on heterogeneous data of various time points (4, 24, 48 hours and 8 days), different MRI hardware (7T Pharmascan Bruker Biospin and 11.7T Biospec Bruker BioSpin) and acquired by centers in Leiden and Cologne. This method was thereafter used to calculate the infarct volumes described in Chapters 5 and 6.

In [Chapter 5](#) mortality, stroke susceptibility and vascular characteristics were investigated in a recently generated transgenic knock-in mouse model with a human C-terminal frameshift mutations in the *TREX1* gene. In patients, such mutations result in RVCL-S that is characterized by vasculopathy, especially in densely vascularized organs, white matter lesions, ischemic stroke and migraine. In line with the severe phenotype in patients, homozygous mutant mice show an increased mortality starting at midlife. The mutant mice also showed a vascular phenotype as evidenced by attenuated PORH responses (across all age groups) and lower acetylcholine-induced relaxations in aortae (in 20- to 24-month-old mice). A vascular phenotype is also suggested by the increased infarct volume seen in 12- to 14-month-old mutant mice at 24 hours after infarct onset. Therefore, these RVCL-S KI mice (showing increased mortality, signs of abnormal vascular function, and increased sensitivity to experimental stroke) can be instrumental to investigate the pathology seen in RVCL-S patients.

In [Chapter 6](#) ischemic stroke and migraine characteristics were investigated in transgenic mouse models with pathogenic gene mutations for FHM1 (neuronal mechanisms), CADASIL and RVCL-S (vascular mechanisms). Cortical spreading depolarization (CSD) and MCAO, as experimental surrogates of migraine and ischemic stroke, respectively, were induced in young (3- to 6-month-old; both CSD and MCAO) and older (12- to 14-month-old; only MCAO) FHM1, CADASIL and RVCL-S mutant mice. Only FHM1 mutant mice showed abnormal, increased CSD frequency and propagation rate. Anoxic depolarization latency was decreased in both age groups of FHM1 and CADASIL mutant mice. Infarct volume was increased only in RVCL-S mutant mice (an effect that was mainly driven by the older group of mice) and that correlated with increased neurologic deficit after infarct. Both CSD as well as vascular dysfunction and the interplay between the two seem to play an important role in monogenic diseases as FHM1, CADASIL and RVCL-S since increased CSD susceptibility does not always result in increased infarct volume and *vice versa*, increased infarct volume cannot always be attributed to increased CSD susceptibility.

Part II describes the clinical research aiming to investigate stroke- and migraine-related infarct characteristics, vascular dysfunction and spreading depression in several patient cohorts.

Patients with a history of migraine might be susceptible for spreading depolarizations which are known to affect vascular and neuronal function and penumbra recovery after stroke. In an observational study in [Chapter 7](#) we investigated whether these patients have more brain damage in the first days after stroke and less favourable outcome after recanalization therapy compared with patients without migraine. To do so, we included patients from a prospective

multicenter ischemic stroke cohort. Lifetime migraine history was based on International Classification of Headache Disorders-II criteria. Patients underwent non-contrast CT, CT-angiography and CT-perfusion ≤ 9 hours of stroke onset and follow-up CT after 3-5 days. In total, we included 600 patients of whom 43 had migraine (24 with aura). Patients with migraine had as often as patients without migraine a perfusion deficit on admission, similar infarct core area, a penumbra area, mean blood-brain barrier permeability, malignant edema, hemorrhagic transformation, final infarct volume and poor clinical outcome after 3 months. Our results suggest that patients with migraine do not have significantly more secondary brain damage, poor outcome after ischemic stroke and have similar effect after recanalizing treatment, compared with patients without migraine.

A possible mechanism for the stroke-migraine comorbidity is vascular dysfunction. Although this is an extremely broad concept of multifactorial origin, vascular dysfunction almost always involves (primary, secondary or even tertiary) arteriosclerosis. In fact, several studies have suggested that patients with migraine more often have signs of atherosclerosis in the systemic circulation. Therefore, we aimed in [Chapter 8](#) to investigate the association between migraine and cerebrovascular atherosclerosis in a cohort of 656 patients with acute ischemic stroke. Using state-of-the-art CT imaging technique, we assessed intra- and extra-cranial atherosclerotic changes and found that migraine patients and controls did not have more atherosclerotic changes in intracranial or extracranial (semi-) large vessels. Our results suggest that the biological mechanisms by which migraine results in ischemic stroke are not related to cerebral atherosclerosis, on a macroscopic level.

[Chapter 9](#) provides a general discussion about the main findings described in this thesis.

NEDERLANDSE SAMENVATTING

Het onderzoek beschreven in dit proefschrift is gericht op het identificeren en ontrafelen van de (neuro) biologische mechanismen en moleculaire netwerken die betrokken zijn bij de pathofysiologie van een beroerte/herseninfarct en van migraine, inclusief het verband tussen beide aandoeningen. Het proefschrift bestaat uit twee delen. Deel 1 beschrijft meerdere experimentele onderzoeksprojecten in muizen waarin we ons gefocust hebben op: (I) het verbeteren van de methodologie die gebruikt wordt in beroerte-onderzoek, en (II) het uitdiepen van het verband tussen een herseninfarct en migraine, met behulp van verschillende onderzoeksstrategieën, methodes en transgene muismodellen. De muismodellen die in dit proefschrift zijn gebruikt, geven expressie aan menselijke pathogene mutaties die aanwezig zijn in FHM1, CADASIL en RVCL-S patiënten. Deze drie mutaties vertegenwoordigen een (groot) deel van het klinische spectrum van monogene aandoeningen waarbij een herseninfarct en migraine samen voorkomen. Deel 2 bevat klinische projecten gericht om een groot cohort van patiënten met een herseninfarct met en zonder migraine te onderzoeken op zoek naar een verschil in infarctkenmerken, secundaire schade en herstel na behandeling.

Deel I van dit proefschrift beschrijft het pre-klinische herseninfarct-migraine onderzoek. Bij experimenteel onderzoek naar de mechanismen van een herseninfarct is de methode waarop weefsel bij de muis wordt afgenomen en wordt verwerkt van essentieel belang voor het verkrijgen van betrouwbare resultaten, aangezien snelle post-mortem veranderingen de analyse van moleculaire verbindingen in hersenweefsel sterk kunnen beïnvloeden. Dit is vooral van belang bij technieken als “mass spectrometry imaging” (MSI), waarbij de specifieke samenstelling van peptiden en metabolieten in biologisch weefsel gedetecteerd worden met een zeer hoge ruimtelijke resolutie.

Hoofdstuk 1 bevat een algemene introductie over de onderwerpen welke in dit proefschrift worden beschreven

In Hoofdstuk 2 werd de methode van opofferen en vervolgens de extractie en verwerking van een muizenbrein voor MSI-analyse geoptimaliseerd zodat de methode minder gevoelig is voor post-mortem degradatie. Twee methoden werden vergeleken, namelijk *ex vivo* stabilisatie met behulp van warmte en *in situ* bevriezing met behulp van vloeibare stikstof, waarvan *ex vivo* warmte-stabilisatie het meest gebruikt wordt bij experimenteel herseninfarct onderzoek met behulp van MSI (maar die ook gevoelig is voor *post mortem* degradatie). *In situ* bevriezing, waarbij vloeibare stikstof over de intacte schedel (waarvan de huid is verwijderd) van de muis wordt gegoten, heeft het voordeel dat de bloedsomloop wordt behouden totdat de kou een bloedvat bereikt. Hierdoor wordt het hersenweefsel vrijwel onmiddellijk bevroren om zo de tijd van moleculaire degradatie zo kort mogelijk te kunnen houden. Bij directe vergelijking van beide opofferings technieken en twee verschillende verwerkingsmethoden van het hersenweefsel bleek dat *post mortem* metabolische stabiliteit in de weefselsecties het best behouden blijft door gebruik te maken van *in situ* bevriezing in combinatie met een snelle verwerkingsmethode om de weefselsecties gereed te maken voor MSI analyses. Deze methode is vervolgens gebruikt voor de experimenten beschreven in Hoofdstuk 3.

In Hoofdstuk 3 werd het transient “Middel Cerebral Artery occlusion” (MCAO) model gebruikt om de moleculaire profiel van lipiden te onderzoeken na een herseninfarct in verschillende gebieden in en om het geïnfarceerde hersenweefsel (kern, grenszone en gezond weefsel). Hiervoor zijn wild-type (WT) en FHM1 migraine muizen, die de menselijk pathogene R192Q missense mutatie dragen, gebruikt. Op 4, 8 en 24 uur na het begin van

het infarct werden muizen opgeofferd met behulp van de in-situ bevriezingstechniek zoals in Hoofdstuk 2 besproken. Vervolgens zijn de hersencoupees geanalyseerd met behulp van massa spectrometrie beeldvormingstechnieken. Evolutie van het infarct werd duidelijk aangetoond door 2D-weergaven van meerdere isoformen van (lyso-) phosphatidylcholines. Het grensgebied tussen de kern van het infarct en het gezonde weefsel was vooral zichtbaar vanaf 8 uur na MCAO met $m/z = 965,5$ en $m/z = 1045,5$ die overeenkomen met phosphatidylinositolphosphate (PIP1) en Phosphatidylinositol 4,5-bisphosphate (PIP2). Deze stoffen kunnen wellicht interessante vroege ischemische markers zijn voor de uiteindelijke staat (levend of dood) van het weefsel in de grenszone. Samen met de vondst van toegenomen natrium-gebonden moleculaire structuren in de FHM1 grenszone in vergelijking met toegenomen kalium-gebonden moleculaire structuren in de WT grenszone, kunnen de resultaten van dit onderzoek duiden op een verhoogde ischemische kwetsbaarheid van het weefsel in het grensgebied bij de mutante muizen. De waargenomen veranderingen kunnen ons helpen om de mechanismen welke actief zijn in de eerste paar uur van een beroerte beter te begrijpen.

In Hoofdstuk 4 wordt een nieuwe methode beschreven om experimentele infarct volumes in een muizenbrein te berekenen vanuit T2 gewogen MRI beelden. Op deze manier is de analyse minder arbeidsintensief dan de meest gebruikte analyse techniek in experimenteel onderzoek waarbij een infarct gebied handmatig wordt omlind. Deze handmatige kwantitatieve analyse van infarct volumes kost niet alleen veel tijd, maar is ook zeer vatbaar voor potentiële bias tussen verschillende onderzoekers. Om deze nadelen te voorkomen hebben we een volledig geautomatiseerde methode ontwikkeld voor de analyse van longitudinale MRI-gegevens, om zo de progressie van het infarctvolume in het muizenbrein te kwantificeren. Hiermee wordt de analysetijd drastisch verminderd en wordt bias zoveel mogelijk vermeden. De methode is gebaseerd op een 'level-set' segmentatie algoritme dat gebruik maakt van een minimale set van aannames en die slechts één MRI-sequentie (namelijk T2) vereist als input. Deze nieuwe methode toont een goede overeenkomst met handmatige segmentatie en is nauwkeurig op heterogene data van zowel verschillende tijdstippen (4, 24, 48 uur en 8 dagen na het ontstaan van het infarct), als van verschillende MRI apparaten (7T Pharmascan Bruker Biospin en 11.7T Biospec Bruker BioSpin) en als ook wanneer de data komen van verschillende onderzoekscentra. Deze methode is vervolgens gebruikt om de in Hoofdstukken 5 en 6 beschreven infarct volumes te berekenen.

In Hoofdstuk 5 zijn de mortaliteit, gevoeligheid voor het ontstaan van een herseninfarct en vasculaire eigenschappen onderzocht in een recent gegenereerd transgeen 'knock-in' muismodel met een menselijke C-terminale frameshift mutatie in het *TREX1* gen. Bij patiënten resulteert deze mutatie in RVCL-S, een aandoening die wordt gekenmerkt door vasculopathie in sterk gevasculariseerde organen, die leidt tot witte stof afwijkingen, cerebrale infarcten en migraine. In lijn met het ernstige fenotype bij patiënten, tonen onze mutante muizen ook een verhoogd sterftecijfer, beginnend op middelbare leeftijd. In de mutante muizen hebben we ook tekenen gevonden van vasculair pathologie, die zowel zichtbaar is in de afgezwakte PORH reactie gezien over alle leeftijdsgroepen als in de lagere acetylcholine geïnduceerde relaxaties van geïsoleerde aortae in de 20- tot 24-maanden oude mutante muizen. Een vasculair fenotype was wellicht ook de oorzaak van het verhoogde infarct volume dat werd gezien in 12- tot 14-maanden oude mutante muizen op 24 uur na het ontstaan van het infarct, waarbij in parallel ook verergerde gedragsscores zichtbaar waren. Door deze verhoogde sterfte, tekenen van abnormale vaatfunctie en verhoogde gevoeligheid voor experimentele herseninfarct zijn deze RVCL-S KI muizen een nuttig model om de pathologie te onderzoeken

die bij RVCL-S patiënten wordt gezien.

In Hoofdstuk 6 is de gevoeligheid voor een herseninfarct en migraine onderzocht in transgene muis modellen voor FHM1, CADASIL en RVCL-S. Muizen van 3-6 en 12-14 maanden werden onderworpen aan de MCAO operatie of KCl geïnduceerde CSD om infarct evolutie en CSD gevoeligheid te bestuderen. Alleen FHM1 mutante muizen vertoonden abnormale, verhoogde CSD frequentie en snelheid. De tijd tot het ontstaan van anoxische depolarisatie was verminderd in beide leeftijdsgroepen van FHM1 mutante muizen en CADASIL. Infarct volume was alleen verhoogd in de RVCL-S mutante muizen (een effect dat voornamelijk werd gedreven door de oudere groep muizen) wat ook correleerde met een verslechterde gedragsscore na het infarct. Zowel CSD als vasculaire dysfunctie en de interactie tussen beide lijken een belangrijke rol te spelen in deze monogene aandoeningen. Deze hypothese komt voort uit het feit dat een verhoogde CSD-gevoeligheid niet altijd resulteert in een vergroot infarct volume en *vice versa*, een vergroot infarct volume kan niet altijd toegeschreven aan een verhoogde CSD gevoeligheid.

Deel II beschrijft het klinisch onderzoek gericht op beroerte- en migraine-gerelateerde karakteristieken van een herseninfarct, vasculaire dysfunctie en CSD in verschillende patiënten cohorten.

Patiënten met een voorgeschiedenis van migraine kunnen vatbaar zijn voor CSD, een fenomeen waarvan bekend is dat ze de vasculaire en neuronale functie en het herstel van de penumbra na een beroerte beïnvloeden. In een observationeel onderzoek beschreven in Hoofdstuk 7 hebben we onderzocht of deze migraine patiënten in de eerste dagen na een beroerte meer hersenschade hebben en minder gunstige uitkomst hebben na intraveneuze trombolysen of intra-arteriële trombectomie vergeleken met patiënten zonder migraine. Om dit te onderzoeken, hebben we data van patiënten met een herseninfarct uit een prospectief multicenter cohort gebruikt. De geschiedenis van levenslange migraine was gebaseerd op de 'International Classification of Headache Disorders-II' criteria. Patiënten ondergingen non-contrast CT, CT-angiografie en CT-perfusie scans binnen 9 uur na het begin van de beroerte. Ook kregen ze een follow-up CT na 3-5 dagen. In totaal hebben we 600 patiënten geïnccludeerd, van wie er 43 migraine hadden (24 met aura). Patiënten met migraine hadden net zo vaak een perfusiedefect bij opname als patiënten zonder migraine. Daarnaast hadden ze een vergelijkbare omvang van: het infarct gebied, het penumbra gebied, het gemiddelde bloed-hersen barrière permeabiliteit, mate van ernstig oedeem, hemorrhagische transformatie, infarctvolume en vergelijkbaar herstel na 3 maanden. Onze resultaten suggereren dat patiënten met migraine niet significant meer secundaire hersenschade hebben, geen slechter eindresultaat hebben na een herseninfarct en een vergelijkbaar effect hebben na een rekanaliserende behandeling, vergeleken met patiënten zonder migraine.

Een mogelijk mechanisme voor de relatie tussen een herseninfarct en migraine is vasculaire dysfunctie. Hoewel vasculaire dysfunctie een extreem breed concept is van multi-factoriële oorsprong, omvat het wel vaak (primaire, secundaire of zelfs tertiaire) atherosclerose. Verschillende studies hebben aangetoond dat patiënten met migraine vaker tekenen van atherosclerose in de systemische circulatie laten zien. Daarom hebben we in Hoofdstuk 8 geprobeerd om de associatie tussen migraine en cerebrovasculaire atherosclerose in een cohort van 656 patiënten met een acuut herseninfarct te onderzoeken. Met behulp van moderne CT beeldvorming hebben we intra- en extra-craniale atherosclerotische veranderingen beoordeeld. Hieruit bleek dat patiënten met migraine geen verhoogde atherosclerotische veranderingen hadden in de intracraniale of extracraniale (semi-) grote vaten vergeleken

met de controle groep. Dit resultaat suggereert dat de biologische mechanismen waardoor migraine resulteert in ischemische beroerte waarschijnlijk niet gerelateerd zijn aan cerebrale atherosclerose op macroscopisch niveau.

Hoofdstuk 9 bevat een algemene discussie over de belangrijkste bevindingen die in dit proefschrift zijn beschreven.

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**/# Authors contributed equally*

CURRICULUM VITAE

Inge A. Mulder was born March 3th 1986 in Oosterhout, Noord-Brabant. She attended secondary school at the Sondervick College De Locht in Veldhoven (1998-2004), where after she moved to Enschede to start in 2004 with her bachelor in Technical Medicine at the University of Twente. In the beginning of 2012 she received her Master in Technical Medicine (track Reconstructive Medicine). In May 2012, she started her PhD project at the department of Neurology and Human Genetics of Leiden University Medical Center (LUMC). The results of her PhD research are described in this thesis. Inge received a Van Leersum Grant of the Royal Netherlands Academy of Arts and Sciences and a Fellowship grant from the International Headache Society in order to continue her scientific career with a post-doctoral research fellowship in 2017/2018 in the neurovascular research Lab of C. Ayata, MD PhD, at the department of Radiology, Massachusetts General Hospital (MGH), Harvard Medical School, Boston, USA. In 2020 Inge received the yearly Saxena award of the Dutch Headache Society for her work performed at MGH. Currently Inge is working as a senior post-doctoral researcher at the Amsterdam University Medical Center (Amsterdam UMC) in Amsterdam, location AMC and VUmc.

DANKWOORD

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