Stroke and migraine: Translational studies into a complex relationship

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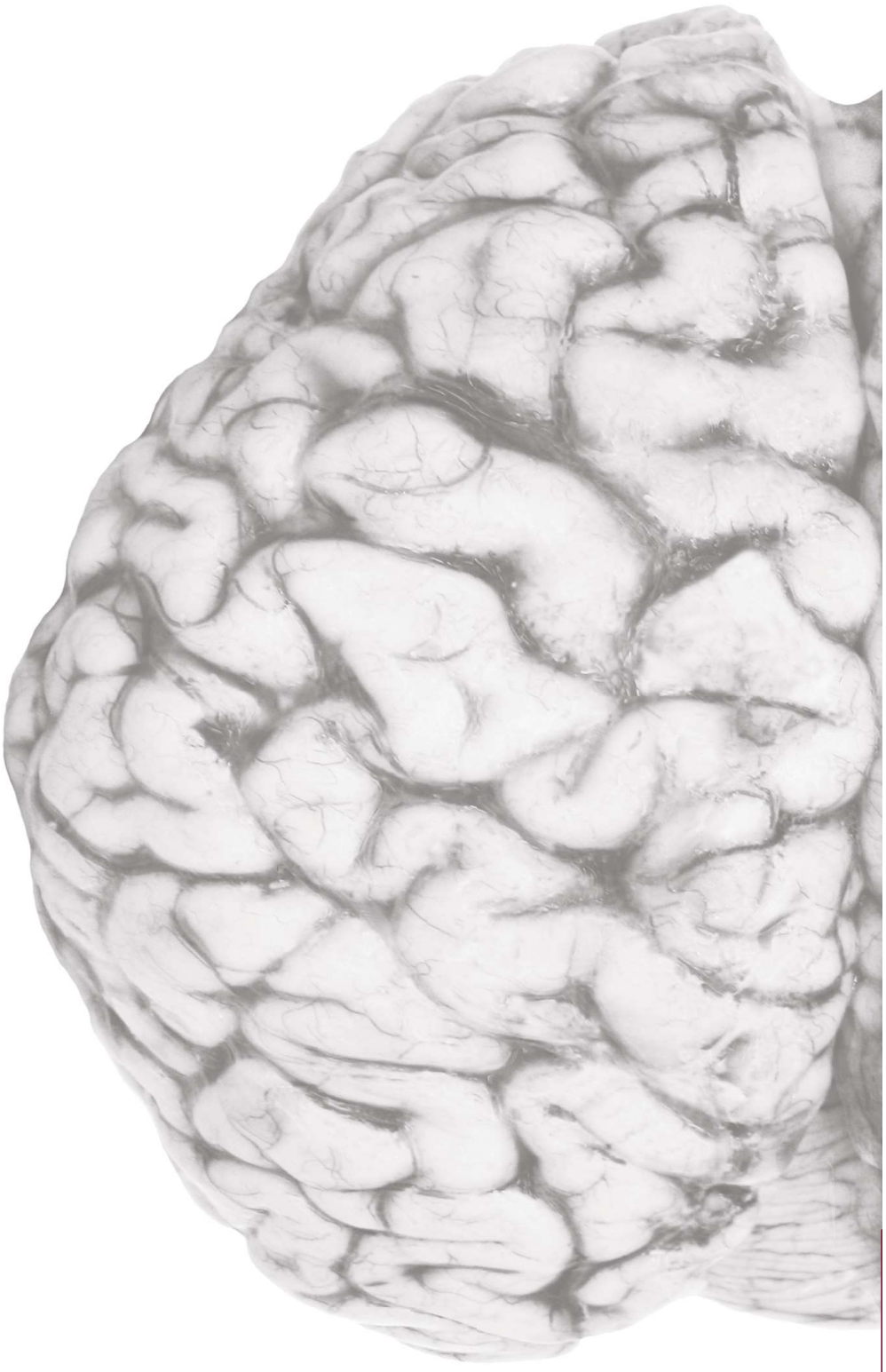

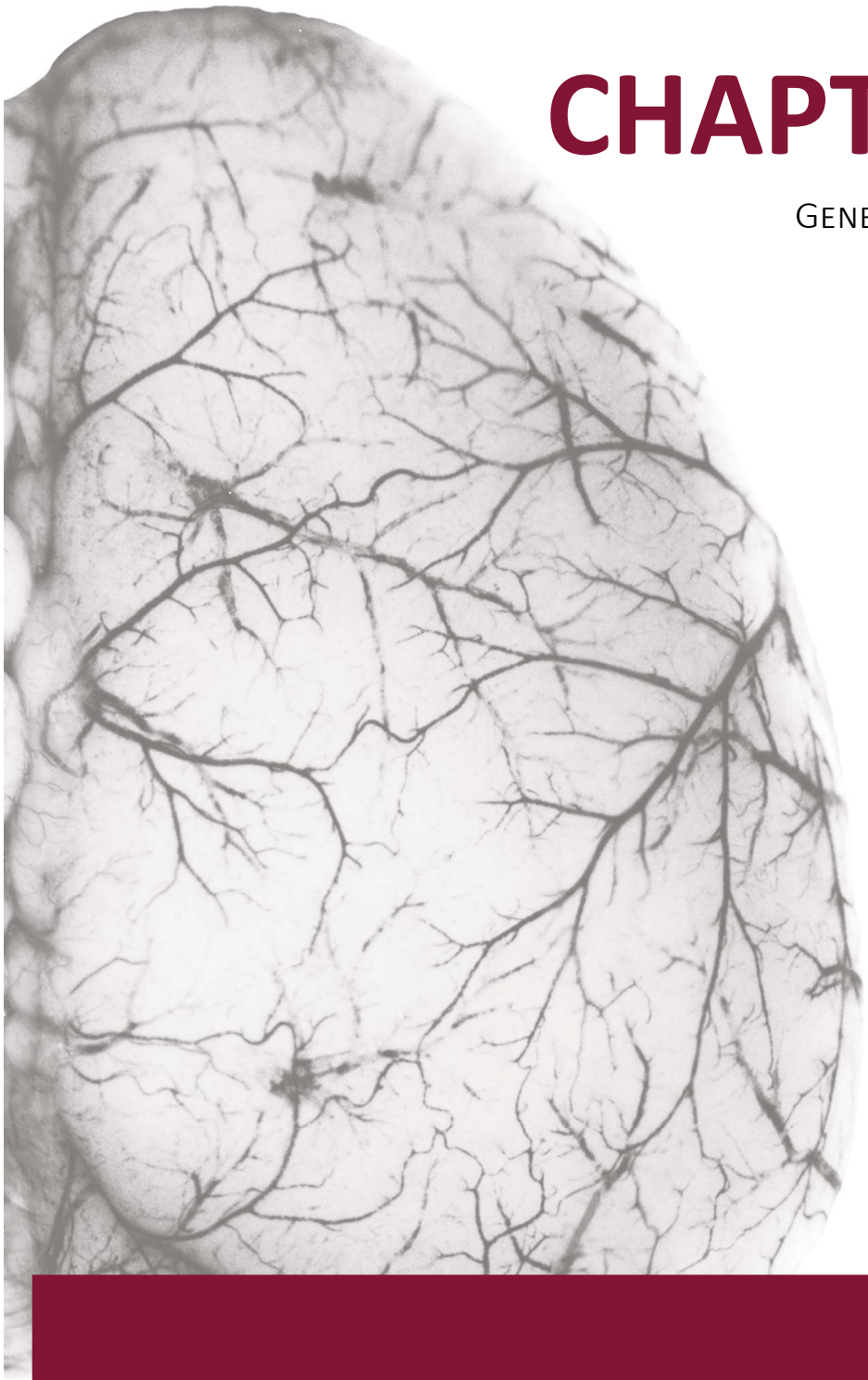

CHAPTER 9

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 asphosphatidylcholine, 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). PIP_2(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, PIP_2 high-energy-dependent turnover from PIP might be abolished, which could be the reason for the increased PIP concentration compared to PIP_2 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 faith 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,

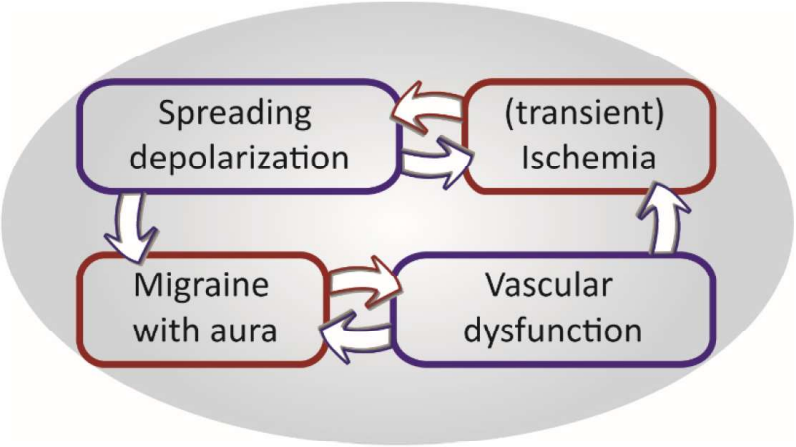

Figure 1. The detrimental circle supporting the stroke-migraine-SD-vascular dysfunction hypothesis.

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 SD[20-22] 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 believe 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] hypercoagulability[24] and pathological vascular reactivity[25] 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 vascular[27] or the neuronal[10] 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 FHM1[9] 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 arteries[35] 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 study[9] 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 controls[9]) 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 SM22α 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 hours[51] *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 subarachnoidal 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

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

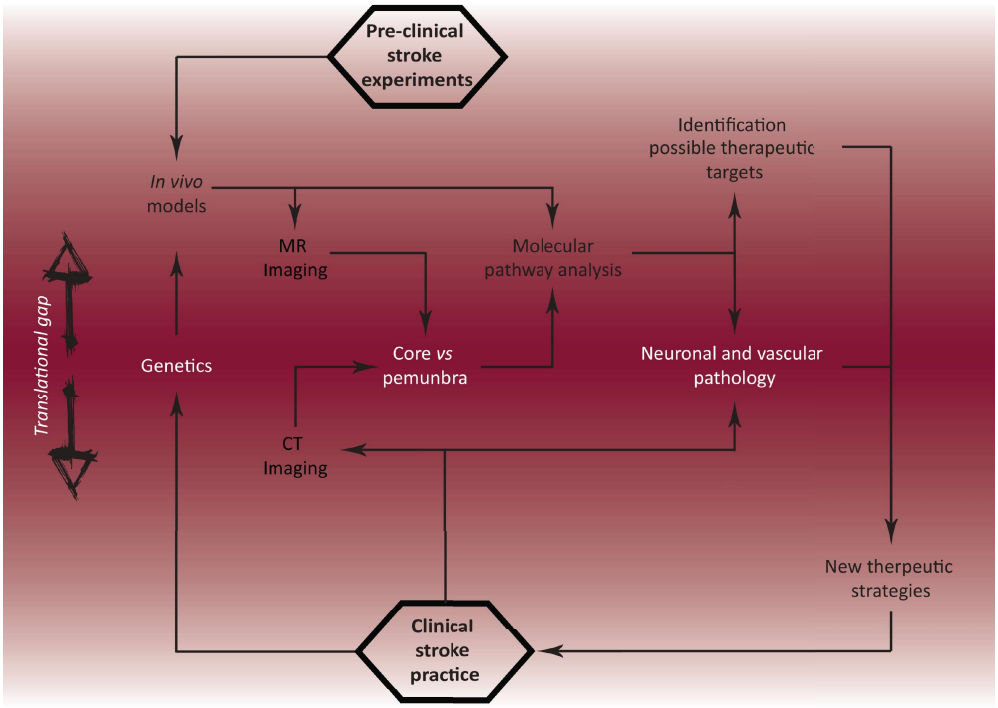

Figure 2. The detrimental circle supporting the stroke-migraine-SD-vascular dysfunction hypothesis.

with stroke and migraine in their clinical spectrum, foremost MELAS, HIHRATL 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 fasions.[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 models[96] and patients[97-99] 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 heterogenous 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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