Dissertation

Understanding the pathophysiology of cerebral small vessel disease: The Graz Lacunar Stroke Study II (GLAS-II)

submitted by

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Statutory Declaration

*I hereby declare that this doctoral thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "**Guidelines of the Medical University of Graz on Good Scientific Practice**". I have read and understood the Medical University's regulations and procedures concerning plagiarism.*

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Graz, July 2025

Disclosures

Publications

The current thesis was the basis of the scientific papers listed below, which are already published. The respective manuscripts were drafted by the doctoral candidate, Melanie Haidegger, which explains similarities between the published work and parts of this doctoral thesis:

1. Haidegger, M[1]; Lindenbeck, S[1]; Hofer, E[1,2]; Rodler, C[1]; Zweiker, R[3]; Perl, S[3]; Pirpamer, L[1]; Kneihsl, M[1]; Fandler-Höfler, S[1]; Gattringer, T[1,4]; Enzinger, C[1]; Schmidt, R[1]. Arterial stiffness and its influence on cerebral morphology and cognitive function. Therapeutic Advances in Neurological Disorders. 2023; 16: doi: 17562864231180715.

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2. Haidegger, M[1]; Klock, N[1]; Kneihsl, M[1,2]; Fandler-Höfler, S[1]; Eppinger, S[1,2]; Eller, K[3]; Sailer, S[1]; Enzinger, C[1], Gattringer, T[1,2]. Recurrent cerebrovascular events after recent small subcortical infarcts. Journal of Neurology. 2024; 271: 5055-5063.

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Public presentations

Parts of the current doctoral thesis were presented at international conferences listed below and the respective abstracts were published. Certain similarities with this doctoral thesis are therefore probable:

1. Congress of the European Academy of Neurology 2022, Vienna, Austria

Haidegger, M; Lindenbeck, S; Hofer, E; Rodler, C; Zweiker, R; Perl, S; Pirpamer, L; Kneihsl, M; Fandler-Hofler, S; Gattringer, T; Enzinger, C; Schmidt, R. Arterial stiffness and its influence on cerebral morphology and cognitive function. European Journal of Neurology, EAN 2022 Abstract Book. 2022; 9th European Academy of Neurology Congress. July 2022; Vienna, Austria. [Poster]

2. Conference of the European Stroke Organisation 2024, Basel, Switzerland

Haidegger, M; Klock, N; Kneihsl, M; Fandler-Höfler, S; Eppinger, S; Seiler; S, Enzinger, C; Gattringer, T. Recurrent cerebrovascular events after recent small subcortical infarction. European Stroke Journal, ESOC 2024 Abstract Book. 2024; 10th European Stroke Organisation Conference; May 2024; Basel, Switzerland. [Poster]

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Abbreviations and Definitions

ADC	Apparent diffusion coefficient
ASCOD	Atherosclerosis, small vessel disease, cardioembolism, other and dissection
ASPS	Austrian stroke prevention study
BBB	Blood brain barrier
BOLD	Blood oxygen level dependent sequence
BHI	Breath holding index
CAA	Cerebral amyloid angiopathy
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CBF	Cerebral blood flow
CT	Computed tomography
CKD	Chronic kidney disease
CMB	Cerebral microbleeds
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CSVD	Cerebral small vessel disease
CVR	Cerebrovascular reactivity
DTI	Diffusion tensor imaging
DWI	Diffusion weighted imaging
e.g.	exempli gratia
EPVS	Enlarged perivascular spaces
ESO	European Stroke Organisation
FDR	False discovery rate
FGF-23	Fibroblast growth factor 23
FLAIR	Fluid attenuated inversion recovery
GFR	Glomerular filtration rate
HbA1c	Glycated haemoglobin A1c
HDL	High density lipoprotein
ICH	Intracerebral haemorrhage
ICrH	Intracranial haemorrhage

IgG	Immunoglobulin G
IQR	Interquartile range
LDL	Low density lipoprotein
MCA	Middle cerebral artery
MEDOCS	medical and nursing documentation and communication network of Styria
MPRAGE	Magnetization prepared rapid acquisition with gradient echoes
MRI	Magnetic resonance imaging
mRS	Modified Ranking scale
NIHSS	National Institute of Health Stroke Scale
nt-pro BNP	n-terminal pro B-type natriuretic peptide
PI	Pulsatility index
PSMD	Peak width of skeletonized mean diffusivity
PWV	Pulse wave velocity
RSSI	Recent small subcortical infarct
SD	Standard deviation
SIFAP	Stroke in Young Fabry Patients
STRIVE	Standards for reporting vascular changes on neuroimaging
SPSS	Statistical package of social sciences
SWI	Susceptibility weighted imaging
TCD	Transcranial Doppler ultrasound
TIA	Transient ischaemic attack
TOAST criteria	Trial of Org 10172 in Acute Stroke Treatment
TOF	Time of flight angiography
WMH	White matter hyperintensities

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Zusammenfassung

Einleitung:

Die zerebrale Kleingefäßerkrankung (engl. CSVD) ist eine der führenden Ursachen für ischämische Schlaganfälle sowie kognitive Beeinträchtigung und Demenz. Die Pathophysiologie ist bisher noch nicht vollständig verstanden, so dass bisher keine spezifischen Therapieansätze vorhanden sind. Störungen der zerebrovaskulären Reaktivität (engl. CVR), eine erhöhte arterielle Pulsatilität, sowie die arterielle Steifigkeit sind mögliche Pathomechanismen der CSVD. Die CSVD verursacht lakunäre Schlaganfälle, welche sich bildgebend meist als rezente, kleine subkortikale Infarkte (engl. RSSI) zeigen. Über die Inzidenz und Ätiologie von rezidivierenden zerebrovaskulären Ereignissen in RSSI-Kohorten ist bisher wenig bekannt. Die Identifikation von klinischen und bildgebenden Risikofaktoren ist dafür von entscheidender klinischer Bedeutung.

Methoden:

In einer retrospektiven Studie wurde das Langzeit-Outcome inklusive wiederkehrender zerebrovaskulärer Ereignisse und dessen Ätiologien erhoben, sowie Assoziationen zu klinischen und bildgebenden Faktoren ermittelt. Außerdem wurde die Assoziation von arterieller Steifigkeit mit CSVD und Hirnatrophie in einer gesunden Studienkohorte untersucht. In einer prospektiven Studienkohorte wurde die klinische Anwendung eines transkraniellen Ultraschallprotokolls (engl. TCD) mit Messung der CVR und Atemmodulation einer RSSI-Kohorte als Pilotstudie getestet.

Resultate:

In einer retrospektiven Studie von 332 RSSI-PatientInnen (mittleres Alter 68 Jahre, 36% weiblich) mit einer Follow-Up Zeit von 12 Jahren traten 70 ischämische (21%) und 26 hämorrhagische zerebrovaskuläre Ereignisse (8%) auf. Chronische CSVD in der MRT zu

Baseline sowie auch der Summenscore dieser Veränderungen war mit Rezidiven assoziiert. In 70% der Fälle wurden wiederkehrende Ereignisse erneut durch CSVD verursacht. Die arterielle Steifigkeit, gemessen als Pulswellengeschwindigkeit (PWV), konnte in einer Kohorte mit 89 Probanden (mittleres Alter 72 Jahre, 51% weiblich) mit einem reduziertem globalen Hirnvolumen assoziiert werden ($p \leq 0.001$). Eine Assoziation zu CSVD-Parameter konnte nicht nachgewiesen werden. Die Messung der CVR mit TCD und Atemmodulation wurde in einer kleinen prospektiven RSSI-Kohorte (n=13) pilotiert.

Diskussion:

Wir konnten in einer Langzeitanalyse einer RSSI-Kohorte zeigen, dass das Risiko von wiederkehrenden zerebrovaskulären Erkrankungen besonders bei vorhandenen chronischen CSVD Markern in der MRT hoch ist. Mehrheitlich war CSVD die Ätiologie neuer zerebrovaskulärer Ereignisse. Die arterielle Steifigkeit war mit reduziertem Hirnvolumen, nicht aber mit CSVD-Markern assoziiert. In einer Pilotstudie zeigten wir, dass die CVR-Messung mittels TCD und Atemmodulation in einer RSSI-Kohorte anwendbar ist.

Abstract

Introduction:

Cerebral small vessel disease (CSVD) is one of the leading causes of ischaemic stroke, cognitive impairment and dementia. The pathophysiology is incompletely understood, therefore specific therapeutic approaches are not available yet. Cerebrovascular reactivity (CVR), increased arterial pulsatility and arterial stiffness are possible mechanisms of CSVD. CSVD causes lacunar ischaemic stroke, depicted as recent small subcortical infarcts (RSSI) on neuroimaging. Little is known about the incidence and aetiology of recurrent cerebrovascular events in RSSI patients. The identification of clinical and imaging risk factors is therefore of substantial clinical importance.

Methods:

In a retrospective study the long-term outcome including recurrent cerebrovascular events and their aetiology was assessed and associations with clinical and imaging features were investigated. In a further project, the association of arterial stiffness with CSVD imaging parameters, brain atrophy and cognitive function was investigated in a healthy, elderly cohort. In a prospective study the feasibility of a transcranial Doppler ultrasound (TCD) protocol including CVR assessment with breathing modulation was tested in an RSSI cohort as a pilot study.

Results:

In a retrospective study of 332 RSSI patients (mean age 68 years, 36% female), 70 recurrent ischaemic cerebrovascular events (21%) and 21 intracranial haemorrhages (8%) were detected in a follow-up period of 12 years. Chronic CSVD features on baseline MRI as well as the CSVD summary score were associated with recurrent events. In 70% of recurrent events the aetiology was CSVD again.

Arterial stiffness, measured as pulse wave velocity (PWV), was associated with reduced total brain volume ($p \leq 0.001$) in a cohort of 89 participants (mean age 72 years, 51% female). An association of PWV and CSVD-related imaging parameters could not be shown.

CVR assessment with TCD and breathing modulation was tested in a small RSSI cohort (n=13) as pilot study.

Conclusion:

We were able to show the risk of recurrent cerebrovascular events in an RSSI cohort is high in long-term observations, particularly in case of accumulating chronic CSVD changes on MRI. In most cases recurrent cerebrovascular events were caused by CSVD again. Arterial stiffness was associated with reduced total brain volume, but not classical CSVD changes on MRI. In a pilot study it was shown, that the CVR protocol using TCD and breathing modulation is feasible in an RSSI cohort and applicable in clinical practice.

Introduction

Ischaemic stroke

Stroke is the second most common cause of death and the leading cause of disability and dependency in adulthood worldwide. Ischaemic stroke accounts for about 80-85% of all strokes, whereas about 10-15% of stroke is due to an intracranial haemorrhage. The risk of recurrent events is substantial, with one in five strokes being a recurrent event (1). Furthermore, stroke is associated with neurological sequelae such as cognitive decline, dementia, epilepsy and post-stroke depression, representing a global health and economic burden (2).

The clinical definition of a stroke is characterized by the sudden onset of a neurological deficit. Brain imaging is paramount in differentiating between an ischaemic and hemorrhagic cause. In most cases the National Institutes of Health Stroke Scale (NIHSS) is used to describe stroke severity.

Ischaemic stroke is due to the occlusion of cerebral arteries and consecutive hypoxia in the respective brain tissue. There are different pathomechanisms that lead to cerebral artery occlusion including cardioembolism, large artery stenosis or occlusion and cerebral small vessel disease (CSVD) as well as other and cryptogenic causes. There are different classification systems regarding aetiologic subtypes of ischaemic stroke such as the TOAST criteria or the ASCOD classification (3, 4). The distinction of different aetiologies of ischaemic stroke is crucial as they warrant different treatment strategies in the acute stroke setting and in secondary prevention.

Cerebral small vessel disease

CSVD is one of the most common causes of ischaemic stroke aside from cardioembolism and large-artery atherosclerosis and represents the main cause of intracerebral haemorrhage (ICH) in patients above 65 years of age (5-7). CSVD is a chronic, progressive pathology of the small penetrating cerebral vessels below 1mm in diameter, that is further associated with several different clinical and radiological manifestations (**Table 1**). Besides acute stroke events CSVD is further related to cognitive decline, depression, extrapyramidal symptoms, urinary incontinence and gait disturbances with increased risk of falls and fractures in the long-term (8, 9).

CSVD is a heterogenous disease that is used as an umbrella term for several pathophysiological processes with different aetiologies and clinical presentations (10). The underlying pathophysiological processes of CSVD are still incompletely understood and are the focus of many ongoing studies (6, 9, 11, 12). An association of CSVD with increasing age and accumulation of vascular risk factors has been shown (10, 13). Other known, rarer causes of CSVD are cerebral amyloid angiopathy (CAA), which results from beta amyloid deposition in the leptomeningeal and cortical small vessels and is the leading cause of lobar haemorrhage in elderly patients, and monogenetic CSVD such as the CADASIL syndrome (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) due to a mutation in the NOTCH3 gene (14). CAA-related CSVD and monogenetic CSVD shall not be addressed in this thesis.

In comparison to other stroke subtypes specific treatment strategies for CSVD-related ischaemic stroke (lacunar stroke/microangiopathic stroke) and chronic CSVD and its progression have not yet been implemented in clinical practice.

Table 1: Overview of clinical and radiological manifestations of cerebral small vessel disease

Clinical manifestations	
Acute	**Chronic**
Lacunar ischaemic stroke	Cognitive decline (mild cognitive impairment, vascular dementia)
Intracerebral haemorrhage	Mood disorders (Depression, anxiety)
Transient ischaemic attack	Extrapyramidal symptoms
Transient focal neurological episode	Gait disturbances
	Urinary incontinence
Radiological manifestations	
Acute	**Chronic**
Recent small subcortical infarct	White matter hyperintensities
Covert small subcortical infarct	Lacunes of presumed vascular origin
Deep intracerebral haemorrhage	Enlarged perivascular spaces (deep/lobar)
Lobar intracerebral haemorrhage	Cerebral microbleeds (deep/lobar)
Convexal subarachnoid haemorrhage	Brain atrophy
	Cortical microinfarct
	Superficial cortical siderosis

Considering the increasingly aging population worldwide, CSVD represents a critical economic and global health burden as it is highly associated with reduced quality of life, long-term functional and cognitive disability and consequently with the increasing demand

of a supportive environment in the elderly population (15). The prevalence of CSVD is high as it occurs in about 5% of patients above 50 years and in almost 100% of patients above 90 years of age (9).

Lacunar ischaemic stroke - Clinical and radiological presentation

CSVD is accountable for approximately 25-30% of ischaemic strokes (16). Lacunar ischaemic stroke is a clinical definition that is historically based on the neurological presentation in the acute stroke setting that is assumed to be caused by intrinsic CSVD in the supply area of a single small penetrating arteriole. In this context about 20 different clinical lacunar stroke syndromes have been described. The five most common syndromes are shown in **Table 2**. A common finding is the absence of an involvement of higher cortical functions due to the subcortical location of the infarct. Therefore, the presence of symptoms such as aphasia, homonymous hemianopsia, neglect, apraxia or other cortical symptoms almost always excludes the differential diagnosis of a lacunar stroke syndrome (17).

The stroke severity of lacunar ischaemic stroke is often lower compared to other stroke subtypes according to the National Institute of Health stroke scale (NIHSS) (18). However, about 20-30% of patients show a worsening of clinical symptoms in the first hours or days after the acute stroke onset, which is referred to as progressive lacunar stroke (19, 20) Furthermore, it has been shown that about 20% of lacunar ischaemic stroke patients suffer from transient neurological symptoms prior to the acute stroke event (transient ischaemic attack (TIA), capsular warning syndrome) (21, 22).

Table 2: The five most common lacunar stroke syndromes

Lacunar stroke syndrome	Clinical presentation
Pure sensory stroke	Acute sensory deficit that involves more than one body region
Pure motor stroke	Acute motor deficit that involves more than one body region
Sensorimotor stroke	Acute sensory and motor deficit that involves more than one body region
Ataxic hemiparesis	Acute motor deficit with ataxia
Dysarthria clumsy hand syndrome	Dysarthria and ataxic paresis of the upper extremity

With the rapid advances in neuroimaging techniques in the last decades, magnetic resonance imaging (MRI) has become the preferred method to diagnose lacunar ischaemic stroke. Small subcortical lesions might often not be reliably detected in computed tomography (CT) in the early phase of the disease, especially in a brainstem location. The term recent small subcortical infarct (RSSI) is the imaging correlate of an acute lacunar stroke (e.g., the consequence of the occlusion of a single small brain artery / arteriole) that has been defined according to the Standards for reporting vascular changes on neuroimaging (STRIVE). RSSI is also associated with the presence of other chronic CSVD-related MRI parameters (23).

The STRIVE criteria are MRI-based and include the following points to define an RSSI:

- A lesion that has a hyperintense signal in diffusion-weighted imaging (DWI) and correlates in location to a hypointense signal in the apparent diffusion coefficient (ADC) sequence.
- An axial diameter of or below 2 cm.
- A lesion that is located in the basal ganglia, the thalamus, the brainstem or the centrum semiovale.

According to the recently published STRIVE-2 criteria, the term RSSI should only be used in patients in whom the lesion on MRI corresponds to the acute neurological deficit in time and location. Thus, clinically silent subcortical DWI-positive lesions that are often incidentally diagnosed on brain imaging are not included in this terminology (24, 25).

The correlation of clinical presentation and imaging criteria is however not always present. Studies have shown that about 20-25% of patients presenting with a clinical lacunar ischaemic stroke syndrome show a different infarct morphology on neuroimaging (e.g., a cortical lesions) thus representing a clinical-radiological mismatch in these cases (26). On the other hand, it is known that not every RSSI pattern on imaging is caused by intrinsic CSVD. In 10-15% of cases it might be due to other stroke aetiologies such as branch atheromatous disease, atherosclerotic large vessel stenosis/occlusion or cardioembolism. Furthermore, different stroke aetiologies might also coexist (24, 27). It is therefore important that the diagnostic work-up of lacunar stroke/RSSI patients includes – besides brain imaging – at least neurovascular ultrasound of extra- and intracranial vessels and screening for atrial

fibrillation in order to detect other or coexisting stroke aetiologies as respective findings warrant different therapeutic approaches in secondary stroke prevention (7).

Lacunar ischaemic stroke - secondary prevention and recurrent events

As the underlying pathophysiology of CSVD is incompletely understood so far, there is currently a lack of specific treatment options for the stroke subtype lacunar ischaemic stroke. In line with different possible pathophysiological mechanisms of CSVD, there are several new treatment approaches which are currently investigated in large clinical trials (28, 29). However, there is not enough evidence yet to include any of them in current guideline recommendations (7).

In this context the standard treatment of lacunar stroke patients in secondary prevention consists of antiplatelet therapy and control of vascular risk factors such as hyperlipidaemia, diabetes mellitus and arterial hypertension. Furthermore, life-style modifications such as smoking cessation, regular physical activity, maintaining a healthy body weight and limiting alcohol consumption are also recommended (7).

The main objective of secondary prevention is the reduction of recurrent cerebrovascular and other vascular events. It has been shown in several studies that the short-time risk of recurrent cerebrovascular events in lacunar ischaemic stroke patients is lower compared to other subtypes of ischaemic stroke (30). The lower rate of recurrent events in short-term observations, the small lesion size and often low stroke severity of lacunar stroke patients might also be reasons why this stroke subtype has often been described as a more benign subtype of ischaemic stroke. However, data about recurrent cerebrovascular and other

vascular events in long-term observations suggest another scenario. Single studies could show that the risk of recurrent cerebrovascular events did not differ in long-term follow-up studies stratified by stroke aetiology, even though current literature on this topic is still limited (31-33). In this context, chronic CSVD-related imaging parameters have been associated with the occurrence of recurrent cerebrovascular events (34, 35). Little is known about aetiologies of recurrent ischaemic cerebrovascular events and their association with progressive CSVD. Furthermore, there are almost no data on the incidence of intracranial haemorrhage in RSSI cohorts, even though a shared underlying pathophysiology, intrinsic CSVD of deep perforating arterioles and its progression, seems likely.

Neuroimaging of cerebral small vessel disease

The diagnosis of CSVD-related brain changes is predominantly based on MRI. As the small penetrating vessels themselves are not yet visualizable with clinically available MRI-techniques, the main focus of imaging relies on CSVD-related parenchymal brain changes (12). In this context it is important to distinguish between acute and chronic MRI abnormalities. Acute radiological findings comprise an RSSI or deep intracerebral haemorrhage (**Figure 1, Panel A** and **E**).

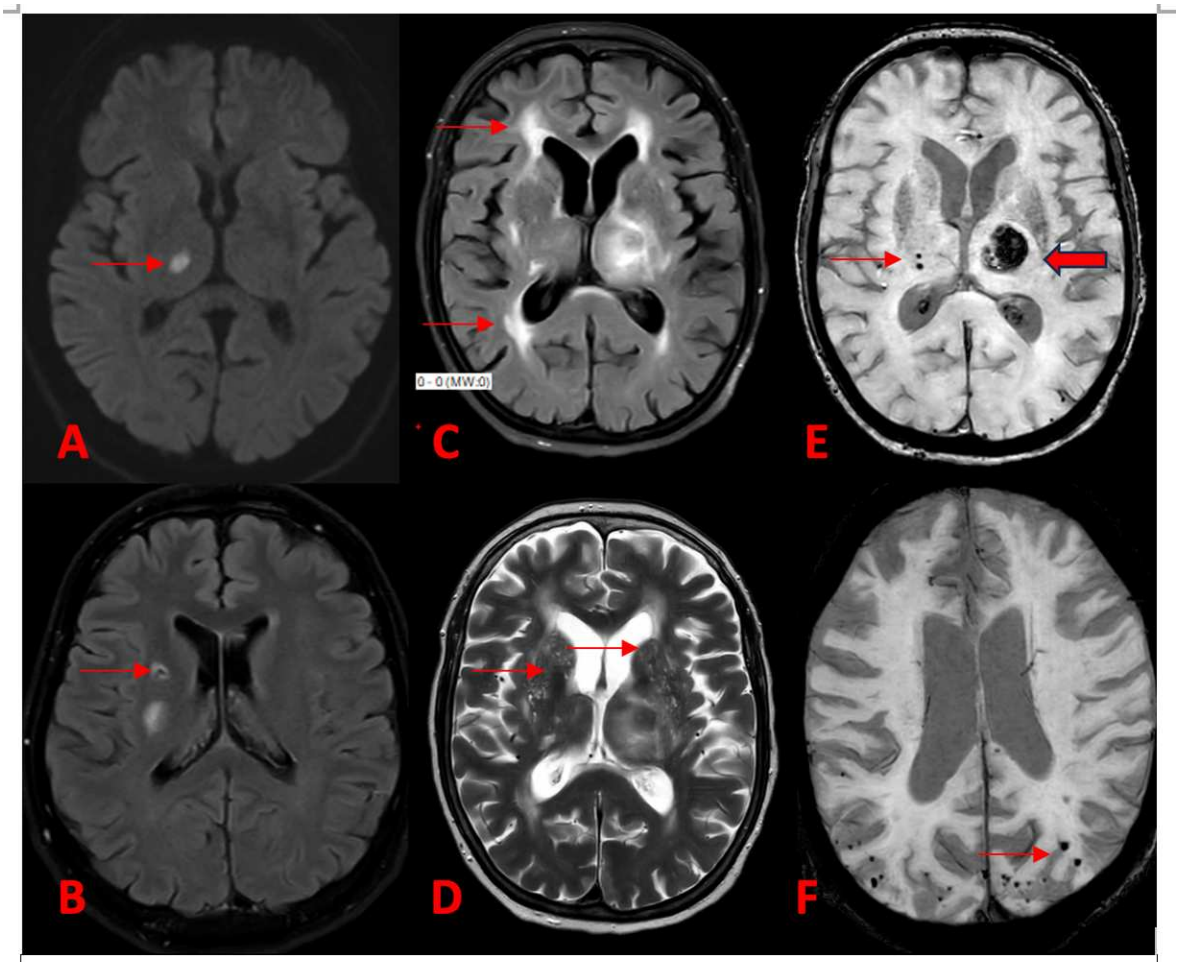
There are several chronic brain lesions that are associated with intrinsic CSVD and are also often found in clinically asymptomatic individuals. These include white matter hyperintensities (WMH), enlarged perivascular spaces (EPVS), lacunes of presumed vascular origin and cerebral microbleeds (CMB) (**Figure 1**). The terminology and definition of these MRI features have been summarized in the STRIVE criteria (24). CSVD lesions on

neuroimaging are a common finding in ischaemic stroke, especially lacunar stroke, and are associated with stroke outcome (34-36).

WMH of presumed vascular origin are defined as hyperintense lesions on T2-weighted and fluid attenuated inversion recovery sequences (FLAIR) located in the subcortical white matter (**Figure 1, Panel C**). While their appearance is usually symmetric in both hemispheres, their form and size may vary. WMH are a common finding on MRI in middle-aged and older adults and their progression has been related to vascular risk factors and aging (23). Extensive WMH burden is associated with stroke risk, reduced cognitive function and mortality (37, 38). The most common rating method for WMH is the Fazekas score that differentiates 4 categories of WMH severity (Fazekas 0-3) in two locations (periventricular and deep) (39). With the use of tissue segmentation programs, the calculation of the WMH volume itself is also possible.

EPVS are fluid-filled cavities that are located near small penetrating vessels in the subcortical white or deep grey matter with a maximal diameter of 3 mm (**Figure 1, Panel D**). Their signal on MRI is similar to that of cerebrospinal fluid (CSF). They appear round or linear in shape depending on the imaging plane. According to their location, deep EPVS can be distinguished from lobar EPVS (24). Deep EPVS are associated to CSVD and lacunar stroke. Lobar EPVS are associated to CAA and are therefore also part of the modified Boston Criteria (40). Although the number of EPVS increases with age and the presence of vascular risk factors such as arterial hypertension and diabetes, an association of a high number of EPVS with lacunar ischaemic stroke, WMH occurrence and cognitive decline has been shown (41, 42). EPVS are usually graded and counted with visual rating on T2 MRI sequences (43).

Figure 1: Radiological manifestations of CSVD (with approval of Medical University of Graz, Department of Radiology, Division of Neuroradiology, Vascular and Interventional Radiology)



A: Recent small subcortical infarct in the thalamus; **B**: Lacune of presumed vascular origin in the basal ganglia; **C**: White matter hyperintensities; **D**: Enlarged perivascular spaces; **E**: Cerebral microbleeds in deep location (thin arrow), deep intracerebral haemorrage (bold arrow); **F**: Cerebral microbleeds in lobar location

Lacunes of presumed vascular origin appear as round or ovoid shaped lesions with fluid-filled cavitations, isointense to the signal of CSF, located in the subcortical white or deep grey matter or the brainstem (**Figure 1, Panel B**). They are hyperintense on T2 and hypointense on FLAIR and T1 sequences. Their size can vary between 3 to 15 mm (43). Lacunes might develop after an RSSI (12, 23), but a lacune can also be the result of a haemorrhage or appear as a de-novo lesion in healthy brain tissue or WMH and should therefore not be classified as a subacute/chronic stroke event unless it can be linked to a

corresponding transient or persistent neurological deficit in time and location (44). Furthermore, it is known that not all RSSI evolve into a lacune, some also evolve into non-cavitated white matter lesions or even disappear entirely. It could be shown in single studies that different pathophysiological processes such as leakage of the blood brain barrier (BBB) might play a role in the morphologic evolution of an RSSI (45).

CMB are defined as round-shaped foci of small chronic intracerebral haemorrhages, usually below 5 mm in diameter that are best diagnosed with T2* or susceptibility weighted imaging sequences (SWI) as hypointense lesions (**Figure 1, Panel E** and **F**) (24). Their presence and number increases the risk for overall ischaemic stroke and intracerebral haemorrhage (46). CMB are an MRI features of intrinsic CSVD especially when located in the deep subcortical grey or white matter or the brainstem. A lobar location of CMB has been associated with CAA (40, 47).

Considering these four CSVD-related imaging features, Staals et al. proposed the use of a CSVD summary score to reflect the overall CSVD burden (43). This score has already been widely used in international studies and is also part of the recently published STRIVE-II criteria (24). The four categories (score 0-4) are defined accordingly:

- Deep WMH Fazekas 2 or higher and/or periventricular WMH Fazekas 3
- Ten or more EPVS in one hemisphere in the basal ganglia region
- Presence of one or more lacune of presumed vascular origin
- Presence of one or more CMB in the deep white or grey matter

Brain atrophy is not a specific imaging feature for CSVD, as it overlaps with other causes for neurodegeneration in many cases. However, deep (widening of the ventricles) and cortical atrophy (deepening of cerebral sulci) have been associated with CSVD (23, 48). Subcortical lesions such as an RSSI or extensive WMH might induce loss of connected cerebral cortex volume due to degeneration of large white matter tracts. Thus, leading to secondary neurodegeneration and further an effect on the total brain volume (49).

Apart from these conventional CSVD imaging abnormalities, microstructural changes in the subcortical tissue detected with diffusion tensor imaging (DTI) such as peak width of skeletonized mean diffusivity (PSMD) are related to CSVD and its clinical and radiological manifestations, especially cognitive dysfunction (50, 51).

Future directions of imaging-techniques in CSVD might eventually arise with the introduction of high-resolution 7 Tesla MRI and thus the possibility to visualize the small perforating arteries directly in non-invasive in-vivo imaging (52, 53).

Pathophysiology of cerebral small vessel disease

Autopsy and histopathological findings of lacunar ischaemic stroke patients showed lipohyalinosis, arteriolosclerosis, microatheroma and microaneurysms in the affected perforating small arteries and arterioles (17, 54). Also, an increased thickness of the tunica media of the small arterioles was found, that was associated with a reduced vessel lumen and consecutive chronic hypoperfusion and hypoxia of the related brain tissue (55). The main hypothesis was that these factors were predominantly related to natural ageing processes and the exposure to arterial hypertension. Thus, leading to a progression of

pathological processes and occlusion of the arteriolar lumen resulting in subcortical infarction. Leakage and rupture of microaneurysms in the cerebral microvasculature might be an explanation of CMB and haemorrhagic stroke (12, 17).

However, even though arterial hypertension is still the most common vascular risk factor in patients with lacunar ischaemic stroke, CSVD and CSVD-related stroke also occur in individuals without arterial hypertension (54). Thus, suggesting that the underlying pathogenesis of CSVD might not be so simple after all and that age and vascular risk factors might rather be accelerating factors than the prior cause or initiator of CSVD and its progression. This assumption is also supported by several studies that showed no difference in the prevalence of common vascular risk factors between different stroke aetiologies (30, 56).

Newer theories suggest that the underlying pathogenesis of CSVD affects the whole or several parts of the neurovascular unit, which includes endothelial cells, smooth muscle cells, pericytes and glial cells besides the neuron itself. Parts of the neurovascular unit also form the BBB. In this context, possible pathomechanisms of CSVD have been investigated that might also be important targets of new therapeutic approaches in the future. These include endothelial dysfunction and BBB leakage, inflammation, arterial stiffness and haemodynamic changes in the microvasculature such as impaired cerebrovascular reactivity (CVR) or consequences of increased pulsatility (6, 9, 57-60). This is supported by findings that suggest that markers for arterial stiffness and arterial pulsatility indices are higher in CSVD-related strokes than in other stroke aetiologies (56).

Damage in the microvasculature is associated with a higher permeability of the BBB resulting in leakage of fluids, blood products and proteins. Possible consequences are wall thickening due to edema, initiation of inflammatory processes and consecutive damage to glial cells (6, 55). Unsurprisingly, dysfunction of BBB is associated to lacunar ischaemic stroke, vascular dementia and CSVD-related imaging findings such as WMH (11).

Table 3: Overview of potential mechanisms that play a role in the pathogenesis of cerebral small vessel disease development and progression

Mechanisms in CSVD pathogenesis
Arteriolosclerosis
Lipohyalinosis
Blood brain barrier leakage
Endothelial dysfunction
Inflammation
Glial cell damage
Reduced cerebral blood flow
Arterial stiffness
Increased pulsatility of small vessels
Impaired cerebrovascular reactivity

There are hypotheses which argue that thickening of the arteriolar or capillary vessel wall induced by either edema or arteriolosclerotic processes leads to an increased stiffness and therefore reduced elasticity of the affected vessel. As a consequence, vasodilatory capacities of the small vessels (cerebrovascular reactivity) are impaired and shear stress on the vessel wall is increased which leads to extensive small vessel damage on a cellular and tissue basis

(61, 62). CVR and arterial stiffness will be discussed further in the next chapters of this thesis.

Different inflammatory markers such as interleukins or c-reactive protein (CRP) have been associated with CSVD-related lesions on MRI. Inflammation might induce endothelial cell dysfunction and consecutive lipohyalinosis and BBB leakage (58, 59). This process might be intensified by exposure to vascular risk factors such as arterial hypertension (11). Inflammation might also lead to the dysfunction of oligodendrocytes which results in loss of myelin and consecutive gliosis which can ultimately be visualized on MRI-sequences as WMH or lacunes (55).

However, it is unknown how different pathophysiological mechanisms interact with each other, in which order they occur and which factor might be the initiator of the pathophysiological cascade of CSVD. Moreover, the role of genetic and heritable factors in the disease process of CSVD is also still very much under debate (14). An overview of relevant mechanisms that play a role in CSVD pathogenesis are displayed in **Table 3**.

Furthermore, associations of CSVD and small vessel disease in other organ systems have been investigated, especially in organs that are dependent on a high oxygen supply, like the brain itself. Examples are the kidney or the retina. In this context several recent studies could show that CSVD-related stroke and impaired renal function share similar prognostic biomarkers (9, 63, 64). Furthermore, microvascular abnormalities in the retina have been associated with lacunar stroke and CSVD (65-67).

These findings might support the hypothesis that small vessel disease might be a systemic rather than an organ-specific disease. Nevertheless, sufficient data on this matter are still limited.

Impairment of cerebrovascular reactivity

CVR is defined as the ability of the cerebral vessels to adapt to metabolic changes with modification in vessel diameter (vasodilatation or vasoconstriction) in order to guarantee a stable cerebral blood flow (CBF) and therefore oxygen and nutrient supply to the brain tissue. In case of a hypercapnic stimulus CBF increases, in case of a hypocapnic stimulus CBF decreases. If this capacity is impaired the brain's demand for constant oxygen and nutrient supply in highly active regions or in phases of low perfusion cannot be met and brain tissue damage can occur (ischaemia) (68-70). Endothelial dysfunction and progressive arterial wall stiffening might play a role in CVR impairment (71). In this context, CVR impairment itself has been associated with CSVD-related stroke, WMH and EPVS (61, 72).

In order to assess CVR impairment, CBF changes have to be visualized. In this context, visualization with transcranial doppler ultrasound (TCD) is a non-invasive method to evaluate CBF changes via measurement of changes in blood flow velocities in the large intracranial vessels (73, 74). Another option is using MRI sequences that show changes in cerebral perfusion in different brain regions such as blood-oxygen level dependent functional MRI sequence (BOLD), arterial spin-labeling or quantitative flow phase contrast MRI (75). With currently available imaging techniques it is not possible yet to visualize haemodynamic changes including CVR in the small penetrating arteries directly. However, a transmission

of haemodynamic changes from large or medium size vessels to the small penetrating arterioles seems likely.

A vasoactive stimulus can be provided with non-invasive breathing modulation such as breath holding (hypercapnia) and hyperventilation (hypocapnia) or otherwise with the use of CO2 inflations or vasoactive drugs such as acetazolamide (69, 76).

Most current studies use MRI-based methods, in most cases BOLD sequences, and CO2 inflations in order to assess CVR (72, 77, 78). TCD in combination of breathing modulation with breath holding and hyperventilation might be an uncomplicated method to assess CVR in a bed-side setting. Prior studies have investigated CVR impairment in patients with carotid artery stenosis and related stroke and could prove the feasibility of this method in this patient cohort (79). However, there are no studies yet that have tested this protocol in RSSI/lacunar stroke cohorts or investigated a possible relationship of CVR impairment measured via TCD and breathing modulation and CSVD-related brain changes and their progression.

Arterial stiffness and pulse wave velocity

Arterial stiffness of large vessels, especially the aorta has been identified as a possible mechanism in CSVD pathogenesis and has been associated with different CSVD-related brain changes such as WMH, brain atrophy and acute ischaemic stroke. There are different hypotheses how arterial stiffness could play a role in the pathophysiological cascade of CSVD development and progression.

First of all, increased stiffness of the aortic wall leads to a reduced elasticity of the vessel wall itself. Consequently, the pulse pressure and pulsatility in small peripheral vessels such as the cerebral microvasculature is increasing. With increased shear stress on the small cerebral vessel wall, consecutive endothelial dysfunction and BBB leakage are direct consequences (80, 81). In this context, it has been shown that pulsatility indices measured with TCD are especially high in patients with extensive CSVD-related brain changes and vascular dementia (82).

Furthermore, increased aortic stiffness is associated with a higher aortic impedance and a consecutive impedance-mismatch between the aorta and peripheral vessels. That means that less wave reflections take place on vessel bifurcations and more pulsatile power is directly transmitted in the small microvasculature of sensitive organs such as the brain or the kidney. Exposure to extensive pressure and abnormal pulsatile power prevent the cerebral small vessels from adapting to pressure changes and therefore impairs CVR and CBF. The consequence is the occurrence of barotrauma in the cerebral microvasculature that leads to brain tissue damage (83).

Arterial stiffness is highly depended on arterial blood pressure and therefore a circadian rhythm is assumed, however, the influence of circadian changes of arterial stiffness on cerebral morphology and cognitive function have not been investigated so far.

The gold standard of arterial stiffness measurement is carotid-femoral pulse wave velocity (PWV) measurement (84). However, newer and easier techniques to measure PWV have been developed such as pulse wave analysis that is integrated in an 24-hour blood pressure monitoring device and calculates PWV from these repetitive measurements. Thus, it is possible to differentiate between day-time and night-time measures and further assess a possible circadian dynamic of PWV and arterial stiffness (85).

Aims and scope

This thesis has three major aims which are addressed in three separate projects:

A.) Evaluation of long-term outcome events of a large RSSI cohort.

For this purpose, a large, well-defined cohort of RSSI patients was retrospectively evaluated in a follow-up period of more than ten years. The aim was to identify clinical and MRI parameters that are associated with mortality, recurrent (cerebro)vascular events and intracranial haemorrhage. Also, the aetiology of recurrent cerebrovascular events was investigated. Data on these research questions are limited in scientific literature so far.

B.) Investigation of the relationship of arterial stiffness and cerebral small vessel disease.

Arterial stiffness has been discussed as a possible mechanism in CSVD pathogenesis and can be measured as PWV with ambulatory blood pressure monitoring devices. In this context, a retrospective study was conducted that investigated the association of PWV and CSVD-related brain changes. For this purpose, we used data from the Austrian Stroke Prevention Family study.

C.) Recruitment of a prospective patient cohort of RSSI patients according to the STRIVE criteria as a longitudinal follow-up study.

The aim of this project is to better understand the underlying pathophysiology of CSVD and its acute hallmark feature acute lacunar stroke with the morphologic MRI correlate of an RSSI. This prospective study is designed as a longitudinal follow-up study. Two follow-up visits are planned (3 and 15 months after the index RSSI). Particular focus will be set on the

assessment of CVR and its measurement via TCD and breathing modulation, which has not been tested in an RSSI population so far (pilot study).

The main goal is the investigation of the following hypotheses:

- The method of CVR measurement with TCD and breathing modulation is feasible in an RSSI population
- Impaired CVR is associated with CSVD and its progression
- The CSVD burden correlates with small vessel disease-related changes in other organ systems, e.g., impaired kidney function, implying an underlying systemic disease process of (cerebral) small vessel disease

In this context, it is planned to assess and analyse following data of the patient cohort:

- Demographic and life style parameters
- Vascular risk factors and medication
- Clinical characteristics of the index stroke (including validated clinical scores)
- MRI features of CSVD-related brain changes
- Neurovascular ultrasound including CVR measurement
- Neuropsychological assessment
- Laboratory parameters including studies on kidney function and markers of axonal damage (e.g., neurofilament light)
- Clinical outcome variables (functional outcome, mortality, recurrent vascular events)

Material and Methods

Project A: Long-term outcome and recurrent cerebrovascular events

Study design and patient selection

The retrospective study included 332 patients that were diagnosed with an MRI-defined RSSI at the University Hospital / Medical University of Graz between 2008 and 2013. The baseline data of this RSSI cohort have already been the basis of several other scientific publications (86, 87). The aim of this present study was to investigate the long-term outcome, mortality and the occurrence and aetiology of ischaemic and haemorrhagic cerebrovascular reevents as well as the frequency of other vascular events.

All patients older than 18 years who showed an MRI pattern corresponding to a single RSSI in the supply area of a small perforating cerebral artery were included. The RSSI was defined according to the STRIVE criteria (23, 24). Patients who had more than one acute ischaemic lesion or a different stroke morphology not corresponding to the RSSI definition according to the STRIVE criteria were excluded. Further exclusion criteria were an insufficient quality of the available MRI data. The presence of coexisting stroke aetiologies (e.g., atrial fibrillation or upstream extracranial/intracranial large vessel stenosis) was not considered an exclusion criterion as the definition of an RSSI was based on MRI imaging and not on clinical characteristics.

The respective data of the patients at baseline included demographic data, common vascular risk factors, respective medication at the time of the index event, clinical presentation as well

as validated clinical scores (e.g. NIHSS, modified Rankin Scale (mRS)) (18, 88), information on acute stroke treatment (e.g., intravenous thrombolysis), laboratory parameters (e.g., lipid parameters, HbA1c, renal function parameters, c-related peptide) and imaging data. The patient data was obtained using the hospital information system "*medical and nursing documentation and communication network of Styria*" (MEDOCS), an electronical documentation system that contains medical information of all public hospitals in the province of Styria.

At the time of the index stroke event, all patients received brain MRI, neurovascular ultrasound of the extracranial and intracranial brain-supplying arteries, assessment of modifiable vascular risk factors and cardiac diagnostic work-up (24-hour electrocardiogram and echocardiography), if indicated.

Follow-up data

Follow-up data was obtained using MEDOCS. The patient cohort was screened for the reoccurence of ischaemic cerebrovascular events (TIA or ischaemic stroke), intracranial haemorrhage (intracerebral, subarachnoid or subdural haemorrhage) and other vascular events (myocardial infarction, peripheral limb ischaemia). Also, mortality and if available the cause of death was assessed.

Furthermore, available laboratory parameters of interest (e.g., lipid profile, HbA1c, renal function parameters) as well as the most recent medication status was documented. In case of recurrent ischaemic stroke/TIA, the aetiology of the event was assessed according to the ASCOD classification which differentiates between five different stroke aetiologies

(atherosclerosis, small vessel disease, cardioembolism, other and dissection) (4). Recurrent events were defined as clinical outcome events, therefore silent DWI-positive lesions were not taken into account in this context.

Imaging parameters

All included patients received 1.5 Tesla brain MRI within the first five days of the index RSSI. The MRI protocol comprised axial T2-weighted, axial T2-weighted FLAIR, gradient-echo T2*-weighted sequences and an axial DWI as well as intracranial time-of-flight (TOF) angiography. Imaging parameters of interest were evaluated by two experts in neuroimaging who were blinded to clinical outcome information (87).

RSSI were defined according to the STRIVE criteria (23). Information about the index RSSI including its location and side was assessed. Furthermore, imaging findings related to chronic CSVD (WMH of presumed vascular origin, EPVS, CMB, lacunes of presumed vascular origin) were evaluated. WMH were graded according to the Fazekas Score and differentiated in periventricular and deep WMH (39). In this context severe WMH were defined as periventricular WMH 3 or deep WMH 2 or 3. The presence of EPVS was evaluated in the basal ganglia and the single lesions were counted. A number of more than 10 EPVS in the basal ganglia in one hemisphere was considered relevant in this context. Furthermore, the presence and number of lacunes were assessed. CMB were assessed and counted in gradient-echo T2*-weighted sequences. In this context, CMB were further distinguished in deep and lobar CMB.

Additionally, a widely-used CSVD summary score was used to describe the overall CSVD burden which takes all four categories into account that were described above (deep WMH Fazekas 2 or higher or periventricular WMH Fazekas 3, presence of one or more CMB, presence of one or more lacune, ten or more EPVS in one hemisphere in the basal ganglia region). A score between 0 and a maximum of 4 was calculated (43).

Furthermore, brain atrophy was evaluated using the quantitative scale of the SIFAP protocol that was already used in several scientific publications to describe the width of the cerebral sulci and ventricles in eight subcategories (V1-8 and S1-8) (89, 90).

Data availability and ethical approval

The data of this project are available upon reasonable request. The study was approved by the Ethics Committee of Medical University of Graz (EK 34-507 ex 21/22). Due to its retrospective study design written informed consent was not necessary.

Project B: Arterial Stiffness and cerebral small vessel disease

Study design and patient selection

The study population consists of participants from the Austrian Stroke Prevention Family study (ASPS). This prospective, single-center study investigated the impact of common vascular risk factors on cerebral morphology and cognitive function in a community dwelling population and their first-degree relatives. Therefore, participants with a preexisting diagnosis of stroke or dementia of any kind were not enrolled. Patients aged 50-75 years were included. The study took place between 2006 and 2013 at Medical University of Graz and recruited 419 participants in total. For this analysis, all patients of the ASPS Family cohort who underwent brain MRI and 24-hour blood pressure measurement including pulse wave analysis were included (n=89). As pulse wave analysis was added to the study protocol in 2011, only participants of the years 2011-2013 were considered in this study. Additionally, a subgroup of this cohort underwent neuropsychological testing (n=82). Demographic data including age and sex as well as vascular risk factors of the patient cohort was assessed.

Pulse wave velocity measurement

Ambulatory 24-hour blood pressure measurement and pulse wave analysis was performed in all included participants according to the recommendations of the European Society of Hypertension (91). In this regard, an oscillometric blood pressure monitoring device was used (Mobil-O Graph; I.E.M. Stolberg, Germany). A measurement was performed every 15 minutes during the day (6am - 10 pm) and every 30 minutes during the night (10 pm - 6 am)

on the non-dominant upper arm of the participant. After every diastolic blood pressure measurement, the device automatically recorded the pulse wave form for ten seconds. From this record a mean value was calculated, which was used by an integrated software (ARCSolver algorithm) of the Mobil-O Graph to estimate the aortic pulse wave for pulse wave velocity (PWV) calculation (software HMS CS 5.1). The device and its integrated PWV calculation software have also been validated in respective studies (92, 93). Of these records, the 24-hour PWV and the PWV during day- (6 am - 10 pm) and night-time (10 pm - 6 am) was calculated respectively.

MRI findings

All participants received 3-Tesla brain MRI (TimTrio Siemens, Erlangen, Germany). The study protocol comprised standard T1 and T2 sequences, FLAIR, DTI, magnetization transfer imaging (MTI) and a high-resolution T1 weighted 3D sequence (MPRAGE).

WMH were identified visually. In this process, every single lesion was outlined using the IDL programme (Exelis Visual Information Solutions, USA). Afterwards every lesion was segmented with a combined algorithm (region seeding and thresholding) and WMH volume was calculated by multiplying the lesion size with slice thickness with the help of the software FSLMATHS (FSL, Oxford, www.fmrib.ox.ac.uk).

DTI data was used to generate Peak width of Skeletonized Mean Diffusivity (PSMD) according to a freely-available script initially described by Baykara et al (50). A white matter skeleton map was created with fractional anisotropy maps. In a next step, a histogram analysis of the mean diffusivity of the integrated voxels of the white matter skeleton maps was performed. Thus, PSMD was defined as the difference between the 95th and the 5th

percentile of mean diffusivity level distribution of all integrated voxels of the white matter skeleton.

The different brain volumes of interest (total brain volume, grey matter volume, white matter volume) were calculated with the software SIENAX (FMRIB Software Library 6.0). With this software extracted brain and skull images were compared to standardised samples. Afterwards, segmentation according to tissue type was performed (total brain volume, white matter volume, grey matter volume, basal ganglia volume, volume of neocortex) (94).

Neuropsychological testing

A subgroup of the study cohort (n=82) was evaluated for cognitive function by experienced neuropsychologists using a predefined neuropsychological test battery (95). Different cognitive domains were investigated: executive function (Wisconsin Card Sorting Test, the Trail Making Test (part B), and the Digit Span Backwards Test), visuopractical skills (Purdue Pegboard Test) and memory and learning (Bäumler's learning and memory test). Summary measures were subsequently converted to Z-scores. Furthermore, a global cognitive performance score was calculated summarising the scores of the different domains. This global cognitive performance score was also converted to a Z-score.

Data availability and ethical approval

The data of this project are available upon reasonable request. The study was approved by the Ethics Committee of Medical University of Graz (EK number 17-088 ex 05/06). Written informed consent was obtained of all study participants.

Project C: Cerebrovascular reactivity

Study design and patient selection

This study was designed as a prospective, longitudinal follow-up study. The main aim was to test a TCD protocol with breathing modulation in an RSSI cohort as a pilot study. Patients with an MRI-defined RSSI were included. RSSI was defined according to the STRIVE criteria (23):

- Presence of a hyperintense lesion on DWI corresponding to a hypointense lesion on the ADC map
- Maximal axial diameter of ≤ 2 cm
- Detected in one of the following regions: basal ganglia, internal capsule, centrum semiovale, thalamus or brainstem

The inclusion and exclusion criteria are presented in **Table 4**. Patients were recruited from the stroke unit or general wards of the Department of Neurology, University Hospital / Medical University of Graz between May 2021 and March 2024.

All screened and eligible patients signed the informed consent form. The collected data was documented using the electronical database Archimed (Research Documentation and analysis, RDA).

Table 4: The inclusion and exclusion criteria of the prospective project

Inclusion	Exclusion
- Single recent small subcortical infarct according to the STRIVE criteria - Above 18 years and under 80 years of age - Available MRI	- Recent cortical infarction - Multiple recent subcortical infarcts - Contraindications for MRI - mRS > 2 before the index stroke - Coexisting disease that limits the life expectancy below 6 months

All included patients received brain MRI (1.5 or 3 Tesla) within 5 days of the index stroke. Furthermore, neurovascular ultrasound including CVR measurement, neuropsychological assessment and study-specific laboratory tests were performed. Also, cardiac diagnostic work-up including transthoracic echocardiography and at least 24-hour cardiac rhythm monitoring was performed, if indicated, as part of the routine stroke work-up of our University Hospital. Clinical characteristics including validated scores (mRS, NIHSS, Barthel index) (18, 88, 96) and neurological deficits as well as vascular risk factors and lifestyle factors (smoking status, alcohol consumption, body mass index) were documented. Vascular risk factors were defined according to recent guidelines (97-99).

Follow-up visits

Follow-up visits were performed 3 and 15 months after the index stroke at our specialized stroke outpatient department. These follow-up visits comprised neurological examination, assessment of validated clinical scores (NIHSS, mRS, Barthel index) (18, 88, 96),

assessment of vascular risk factors, blood and urine tests, again neurovascular ultrasound of extra- and intracranial arteries including measurement of CVR, 3 Tesla brain MRI and neuropsychological testing. Also, lifestyle parameters such as smoking, regular alcohol consumption and the body mass index were assessed. All diagnostic tests were performed during one visit and took about 4 hours to complete.

Neurovascular ultrasound and measurement of cerebrovascular reactivity

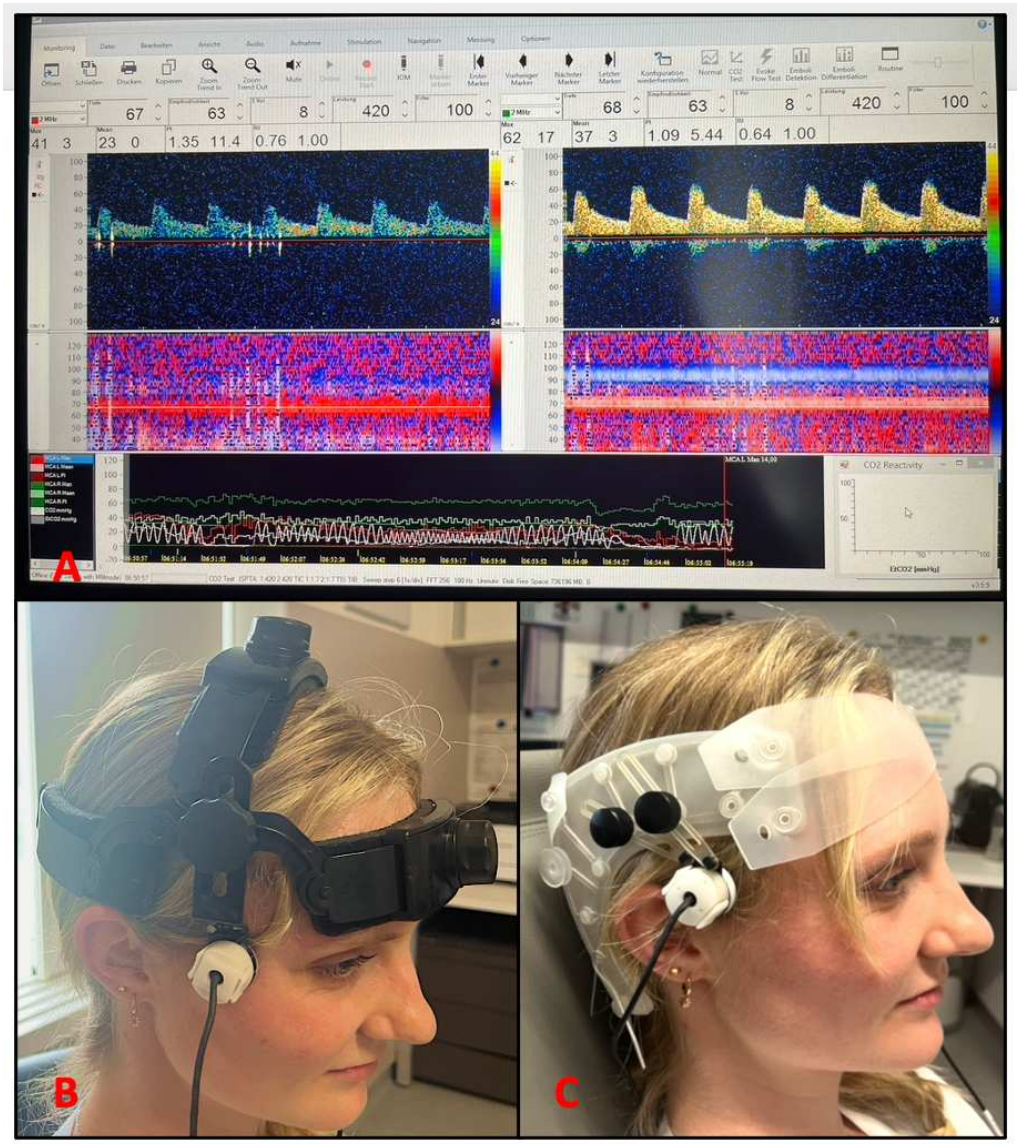
Every study participant received an ultrasound examination of the extra- and intracranial cerebral arteries. Thus, large artery stenosis or atherosclerotic plaques were identified and documented accordingly. The ultrasound protocol was the same for the baseline visit and the two follow-up visits.

The study-specific neurovascular ultrasound protocol included the mean blood flow velocities and pulsatility indices of the internal carotid artery, the middle cerebral artery (MCA), the basilar artery and the ophthalmic artery on both sides. Afterwards CVR was assessed using the Multidop X device (DWL Elektronische Systeme GmbH, Singen, Germany), which allows TCD measurement of both MCA at the same time with the use of a head device that includes two transcranial doppler probes (2 MHz, **Figure 2 Panel B and C**). Also, a nasal tube was applicated to measure oxygen and carbon dioxide levels of the patient's breath and validate the correct performance of the breathing manoeuvres.

After resting in a stable, supine position for at least five minutes the patient was asked to hold breath for 30 seconds. The patient was then asked to continue normal breathing for about 2 minutes. Then hyperventilation was performed for 45 seconds. The whole procedure

was performed twice in every patient and a resting phase of five minutes was implemented between measurements. The whole examination takes about 20 to 30 minutes and the transcranial Doppler curves as well as the oxygen and carbon dioxide curves were recorded for each patient and saved with the software Compumedics DWL (version QL 3.5.9) of the Multidop X for off-line analysis. The interface of the software and the two different head devices are presented in **Figure 2 (Pannel A-C)**.

Figure 2: The software interface of the Multidop X device is shown in Panel A. Two different head devices were used (Panel B and C).



In an off-line session, the curves were analyzed. To assess the mean blood flow velocity of the MCA during breath holding, the average mean velocity of the MCA during the last 5 seconds of the breath holding phase was calculated, as the maximal velocity change could be observed in the last seconds of the breath holding period. To assess the mean blood flow velocity of the MCA during hyperventilation, the average mean velocity of the MCA during the last 5 seconds of hyperventilation was assessed. Furthermore, the pulsatility indices of the MCA were assessed during normoventilation, breath holding and hyperventilation.

The breath holding index (BHI) was calculated using the formular below that was previously described by Markus et al (73). The BHI was calculated for both sides separately and a mean value of both sides was calculated. All blood flow velocities (v) are displayed in cm/seconds and as mean blood flow velocities. A threshold below 0.67 was suggested to identify impaired CVR (73).

$$\frac{(v_{mca}(breath\ holding) - v_{mca}\ (baseline)) \div v_{mca}(breath\ holding)}{time\ of\ breath\ holding\ (seconds)} \times 100$$

The full range of vasodilation or vasomotor reserve was calculated using the formular below previously described by Ringelstein et al (74). Full range of vasodilatation was calculated for both sides separately and a mean value was created. All blood flow velocities (v) are displayed in cm/seconds and as mean blood flow velocities.

$$\frac{v_{mca}(breath\ holding) - v_{mca}(hyperventilation)}{v_{mca}\ (baseline)} \times 100$$

Imaging studies

All patients received a 1.5 or 3 Tesla brain MRI at the time of the index RSSI. In this context a routine protocol for stroke patients was performed which comprises standard T1 and T2-weighted sequences, FLAIR, SWI, DWI and 3D time of flight angiography (TOF).
At the follow-up visits, a 3 Tesla brain MRI (TimTrio Siemens, Erlangen, Germany) was performed. The MRI study protocol comprised DWI including a 64 directions sequence, 3D FLAIR sequence, 3D T2 sequence, sagittal T1 (isotropic, 1mm slice thickness), SWI and TOF MRA sequences.

Laboratory parameters

At baseline, routine laboratory parameters were assessed as part of the routine diagnostic stroke work-up during hospitalization (blood count, lipid profile, HbA1c, nt-pro BNP, renal and liver parameters, electrolytes, c-reactive protein, coagulation parameters). Additionally, fibrinogen, lipoprotein(a), interleukin 6, cystatin C, fibroblast growth factor 23 (FGF23) and vitamin D was assessed as part of the study-specific laboratory protocol.

At the follow-up visits, the laboratory protocol comprised a blood count, electrolytes, renal parameters including cystatin C, liver parameters, c-reactive protein (CRP), lipid profile including lipoprotein (a), interleukin 6, HbA1c, nt-pro BNP, coagulation parameters including fibrinogen, FGF23 and vitamin D.

A urine sample was taken at every visit (baseline, follow-up 1 and 2). Total protein, albumin, immunoglobulin G (IgG), beta 2 microglobulin and n-acetyl-beta-D glucosaminodase (beta NAG) was tested.

At every visit (baseline, follow-up 1 and 2) one EDTA (6mL) and two serum samples (6mL) were additionally taken and put in the freezer (temperature -80°C) for potential future analysis.

Neuropsychological assessment of cognitive function

A neuropsychological assessment of cognitive function was performed at every visit (baseline, follow-up 1 and 2) and the test battery did not differ between the visit types. The test battery comprised the Montreal cognitive assessment (MoCA), the hospital anxiety and depression scale (HADS), the EQ5D-5L to assess quality of life, the symbol digit modalities test (SDMT) and the comprehensive trail making test (CTMT, Trail 2, 4 and 5).

Data availability and ethical approval

The data of this project are available upon reasonable request. The study was approved by the Ethics Committee of Medical University of Graz (EK 24-260 ex 11/12). Written informed consent was obtained of every participating patient.

Statistical analysis

The software IBM SPSS (Versions 27-29) was used to perform the statistical analyses on all three projects. Continuous variables are presented as mean and standard deviation or as median and interquartile range; nominal variables are shown in absolute numbers and percentages.

To compare nominal data Pearson's Chi-square test was used. For the comparison of continuous data Student's t test or Mann-Whitney U Test was used. In this context, gaussian distribution was assessed with Kolmogorov-Smirnov test (Project A and C) or Shapiro Wilk test (Project B).

In Project A, a multivariable Cox regression model was fitted. This model was adjusted for age, sex, vascular risk factors (arterial hypertension, hyperlipidaemia, diabetes mellitus) and imaging parameters of interest. As time variable the time span between the index event and the follow-up date was used. As collinearity between the single CSVD parameters and the CSVD summary score was assumed, the multivariable model was applied separately to single CSVD parameters and the CSVD summary score.

In Project B, the patient cohort was corrected for outliers according to the variable "total brain volume". Outliers were defined as variables outside the range of the standard deviation multiplied by 2.5. To identify variables that correlate to PWV Spearman rank correlation was used. A multivariable mixed linear model was fitted. This model was adjusted for age, sex and vascular risk factors (systolic blood pressure, diabetes mellitus, coronary artery

disease). Afterwards, false discovery rate correction was performed to adjust for family structure.

Statistical significance was defined as a probability value below 0.05 in all conducted studies (Project A, B and C).

Results

Parts of this chapter show similarities to the publications of Haidegger et al (100, 101). Permission to reproduce tables originally published in *Therapeutical Advances in Neurological Disorders* and *Journal of Neurology* was obtained from the respective copyright holders respectively (*Sage Journals*, *Springer Nature*).

Project A: Long-term outcome and recurrent cerebrovascular events

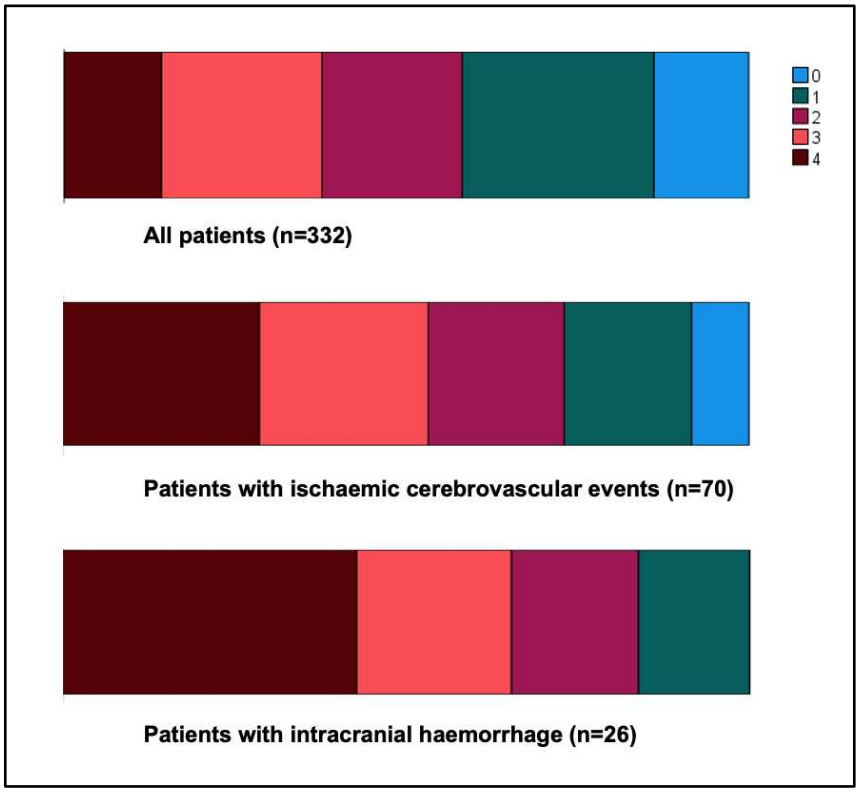
Baseline characteristics

The final study cohort comprised 332 RSSI patients. The mean age was 68 years and 36% of the cohort were women. The median follow-up time of the study cohort was 12 years (3 years IQR). The baseline characteristics (demographics, clinical presentation, vascular risk factor profile, infarct morphology, medication at baseline) are displayed in **Table 5**.

The infarct location was almost equally distributed between the anterior cerebral circulation (n=169; basal ganglia, internal capsule and centrum semiovale) and the posterior cerebral circulation (n=163; thalamus and brainstem). RSSI were predominantly located in the brainstem (n=87, 26%), followed by the thalamus (n=76, 23%), centrum semiovale (n=64, 19%), basal ganglia (n=60, 18%) and the internal capsule (n=45, 14%). The most common vascular risk factors in this study cohort were arterial hypertension (84%) and hyperlipidaemia (65%). At least one chronic CSVD imaging parameter was present in 286

patients (86.1%). The median CSVD summary score (0-4) was 2. The distribution of the CSVD summary score is shown in **Figure 3**.

Figure 3: The distribution of the CSVD summary score (0-4) in the patient cohort



30 patients (9.0%) had a concomitant ipsilateral upstream arterial large vessel stenosis of or above 50%, and 41 patients (12.3%) had atrial fibrillation. The presence of these concomitant diseases was, however, not associated with the occurrence of ischaemic cerebrovascular events during the follow-up period (OR 1.66, 95% CI 0.80–3.46; OR 1.41, 95% CI 0.59–3.31 respectively).

Table 5: Baseline characteristics including demographics, vascular risk factors, infarct morphology and medication (reproduced with permission of Springer Nature(101))

Demographics (n=332)	
Age, years (mean, SD)	67.7 (12.0)
Women (n, %)	119 (35.8)
NIHSS at admission (median, IQR)	3 (2)
mRS at admission (median, IQR)	0 (1)
NIHSS at discharge (median, IQR)	1 (1)
mRS at discharge (median, IQR)	1 (2)
Vascular risk factor profile (n, %)	
Arterial hypertension	279 (84.0)
Diabetes mellitus	89 (26.8)
Hyperlipidaemia	217 (65.4)
Chronic kidney disease <60mL/min/1.73m^2	76 (26.1)
Chronic kidney disease <45mL/min/1.73m^2	27 (8.1)
Atrial fibrillation	41 (12.3)
Ipsilateral extracranial/intracranial large artery disease (⩾50% stenosis)	30 (9.0)
Coronary heart disease	35 (10.5)
Peripheral artery disease	21 (6.3)
Previous stroke	71 (21.4)
Active smoking	107 (32.2)
Cancer	9 (2.7)
Infarct morphology (n, %)	
Anterior Circulation	169 (50.9)
Basal ganglia	60 (18.1)
Internal capsule	45 (13.6)
Centrum semiovale	64 (19.3)
Posterior Circulation	163 (49.1)
Thalamus	76 (22.9)
Brainstem	87 (26.2)
Right hemisphere	144 (43.4)
Medication after the index event (n, %)	
Antiplatelets	295 (88.9)
Oral anticoagulation	37 (11.1)
Antihypertensives	258 (77.7)
Antidiabetics	77 (23.2)
Statins	195 (58.7)

Recurrent ischaemic cerebrovascular events

During the follow-up period of 12 years, 70 patients had recurrent ischaemic cerebrovascular events (21%). Of these 70 patients, 54 patients had ischaemic stroke (16%) and 22 patients had TIA (7%). The median time to ischaemic cerebrovascular event recurrence was 3 years (6 years IQR). TIA happened earlier (median 2 years, 3 years IQR), than ischaemic stroke (median 4 years, 5 years IQR). 16 patients (5%) had multiple ischaemic cerebrovascular events during the follow-up period.

The aetiologies of recurrent stroke were CSVD (72%), cardioembolism (13%), symptomatic large artery stenosis (9%) and other/indefinite causes (6%). The aetiologies of TIA in the follow-up period were CSVD (59%), cardioembolism (32%) and symptomatic large artery stenosis (9%).

Diabetes mellitus (OR 1.86, 95% CI 1.07-3.27, **Table 6**) as well as increased HbA1c levels (p=0.014) were associated with recurrent ischaemic cerebrovascular events in univariable analysis. Also stroke/TIA recurrence was more common in patients whose index RSSI was located in the internal capsule (OR 2.11, 95% CI 1.06-4.19).

Other vascular risk factors and laboratory parameters as well as initial stroke severity, medication after the index event and further infarct locations were not associated with recurrent stroke/TIA (p>0.5 respectively).

Table 6: Clinical and imaging parameters and its association to stroke/TIA recurrence (reproduced with permission of Springer Nature (101))

	Stroke/TIA recurrence n=70	No recurrence n=262	P value	Odds ratio 95% CI	P value adjusted *	Hazard ratio 95% CI
Age, years, (mean, SD)	67.7 +/- 10.1	67.7 +/- 12.4	0.983	-	0.585	1.01 (0.98-1.03)
Women, (n, %)	22 (35.8)	97 (37.0)	0.386	0.82 (0.52-1.28)	0.210	1.39 (0.81-2.33)
Vascular risk factors (n, %)						
Arterial Hypertension	62 (88.6)	217 (82.8)	0.244	1.61 (0.72-3.59)	0.388	1.41 (0.64-3.11)
Diabetes mellitus	52 (74.3)	165 (63.0)	0.077	1.70 (0.94-3.07)	**<0.001**	**2.43 (1.44-3.88)**
Hyperlipidaemia	26 (37.1)	63 (24.0)	0.028	1.86 (1.07-3.27)	0.297	1.34 (0.78-2.30)
CKD**	19 (30.6)	57 (24.9)	0.360	1.33 (0.72-2.47)	0.894	0.96 (0.53-1.76)
Atrial fibrillation	12 (17.1)	29 (11.1)	0.170	1.66 (0.80-3.46)	0.219	1.50 (0.79-2.85)
Ipsilateral large artery stenosis >50%	8 (11.4)	22 (8.4)	0.432	1.41 (0.59-3.31)	0.157	1.73 (0.81-3.72)
Coronary artery disease	7 (10.0)	28 (10.7)	0.868	0.93 (0.38-2.22)	0.501	1.32 (0.59-2.94)
Peripheral artery disease	4 (5.7)	17 (6.5)	0.813	0.87 (0.28-2.68)	0.859	0.91 (0.33-2.55)
Previous stroke	19 (27.1)	52 (19.8)	0.186	1.50 (0.82-2.76)	0.130	1.53 (0.88-2.65)
Smoking	24 (34.3)	83 (31.7)	0.679	1.13 (0.64-1.97)	0.797	1.07 (0.63-1.84)
Active cancer	2 (2.9)	7 (2.7	0.932	1.07 (0.22-5.28)	0.692	1.34 (0.31-5.82)
Imaging parameters						
Severe WMH†, n (%)	50 (71.4)	138 (52.7)	0.005	2.29 (1.3-4.0)	**0.015**	**1.97 (1.14-3.41)**
WMH, median (IQR)	2 (2)	2 (2)	0.016	-	**0.018**	**1.41 (1.06-1.86)**
EPVS‡, n (%)	44 (62.9)	170 (64.9)	0.865	0.93 (0.54-1.64)	0.755	0.92 (0.54-1.56)
CMB(s), n (%)	32 (45.7)	76 (29.0)	0.008	2.25 (1.26-3.98)	**0.014**	**1.89 (1.32-3.14)**
Lacune(s), n (%)	36 (51.4)	105 (40.1)	0.088	1.58 (0.93-2.68)	0.320	1.28 (0.78-2.11)
CSVD summary score, median (IQR)	2 (2)	2 (2)	0.010	-	**0.044**	**1.22 (1.06-1.49)**
Atrophy ventricular, (median, IQR)	5 (2)	4 (3)	0.040	-	**0.026**	**1.20 (1.02-1.40)**
Atrophy sulcal, (median, IQR)	5 (2)	4 (3)	0.180	-	0.233	1.12 (0.93-1.34)

*adjusted for age, sex, arterial hypertension, diabetes, hyperlipidaemia and MRI parameters of CSVD

** chronic kidney disease (CKD) with GFR<60 mL/min/1.73m^2

† Severe white matter hyperintensities: deep Fazekas Score ≥2 or periventricular Fazekas Score 3

‡ Enlarged perivascular spaces: >10 in one hemisphere in the basal ganglia

Regarding the chronic CSVD-related imaging parameters, severity of WMH (OR 2.29, 95% CI 1.3-4.0), presence of more than one CMB (OR 2.25, 95% CI 1.26-3.98) and an increased CSVD summary score (p=0.010, **Table 6**) were associated with ischaemic cerebrovascular event recurrence in univariable analysis. When differentiating CMB between deep and lobar localization, there was no statistical significance for both subgroups (p>0.5 respectively). EPVS and the presence of more than one lacune were not associated with recurrent ischaemic events. Ventricular atrophy was related to recurrent ischaemic cerebrovascular events (p=0.040), whereas sulcal atrophy was not associated.

In multivariable Cox regression analysis adjusted for age, sex and other vascular risk factors, the association of recurrent ischaemic cerebrovascular events and diabetes mellitus was also evident (HR 2.43, 95% CI 1.44-3.88, **Table 6**). The location of the index RSSI was no longer predictive in this context.

Regarding the presence of chronic CSVD MRI findings, severity of WMH (HR 1.97, 95% CI 1.14-3.41) and presence of CMB (HR 1.89, 95% CI 1.32-3.14) were predictive of stroke/TIA recurrence. The median CSVD score was also higher in the subgroup of patients who suffered from recurrent ischaemic cerebrovascular events in the multivariable Cox regression model (HR 1.22, 95% CI 1.06-1.49, **Table 6**). Ventricular atrophy remained associated with recurrent ischaemic cerebrovascular events (HR 1.20, 95% CI 1.02-1.40).

Intracranial haemorrhage occurrence

Intracranial haemorrhage (ICrH) occurred in 26 patients (7.8%) during the follow-up period of a median time of 12 years and included intracerebral haemorrhage (n=14; deep n=13, lobar n=1), subdural haemorrhage (n=7) and subarachnoid haemorrhage (n=5; aneurysmal

Table 7: Clinical and imaging parameters and its association to intracranial haemorrhage occurrence after index RSSI (reproduced with permission of Springer Nature (101))

	ICrH occurrence n=70	No ICrH n=262	P value	Odds ratio 95% CI	P value adjusted*	Hazard ratio 95% CI
Age, years, (mean, SD)	69.4 +/- 11.6	67.5 +/- 12.0	0.445	-	0.106	1.04 (0.99-1.09)
Women, (n, %)	9 (34.6)	110 (35.9)	0.892	0.95 (0.44-2.06)	0.769	1.14 (0.47-2.74)
Vascular risk factors (n, %)						
Arterial Hypertension	23 (88.5)	256 (83.7)	0.521	1.50 (0.43-5.18)	0.864	1.12 (0.32-3.96)
Diabetes mellitus	5 (19.2)	84 (27.5)	0.364	0.62 (0.23-1.72)	0.909	0.94 (0.34-2.60)
Hyperlipidaemia	18 (69.2)	199 (65.0)	0.666	1.21 (0.51-2.87)	0.747	0.87 (0.37-2.04)
CKD**	5 (21.7)	71 (26.5)	0.618	0.77 (0.28-2.15)	0.215	0.48 (0.15-1.52)
Atrial fibrillation	4 (15.4)	37 (12.1)	0.624	1.32 (0.43-4.05)	0.710	1.23 (0.41-3.70)
Ipsilateral large artery stenosis >50%	1 (3.8)	29 (9.5)	0.336	0.38 (0.05-2.94)	0.494	0.49 (0.07-3.72)
Coronary artery disease	2 (7.7)	33 (10.8)	0.672	0.69 (0.16-3.05)	0.829	1.18 (0.27-5.18)
Peripheral artery disease	0 (0.0)	21 (6.9)	0.168	-	0.982	-
Previous stroke	9 (34.6)	62 (20.3)	0.087	2.08 (0.89-4.90)	0.126	1.96 (0.83-4.62)
Smoking	5 (19.2)	102 (33.3)	0.140	0.47 (0.18-1.30)	0.236	0.56 (0.20-1.56)
Active cancer	0 (0.0)	9 (2.9)	0.375	-	0.979	-
Imaging parameters						
Severe WMH†, n (%)	20 (76.9)	168 (54.9)	0.030	2.74 (1.07-7.01)	0.049	2.61 (1.00-6.82)
WMH, median (IQR)	2 (1)	2 (2)	0.268	-	0.169	1.38 (0.88-2.16)
EPVS‡, n (%)	19 (73.1)	195 (63.7)	0.442	1.70 (0.66-4.40)	0.332	1.64 (0.60-4.45)
CMB(s), n (%)	16 (61.5)	92 (30.1)	0.004	3.87 (1.64-9.07)	**0.006**	**3.25 (1.39-7.58)**
Lacune(s), n (%)	14 (53.8)	127 (41.5)	0.222	1.64 (0.74-3.67)	0.264	1.59 (0.70-3.61)
CSVD summary score, median (IQR)	3 (2)	2 (2)	0.005	-	0.011	1.57 (1.11-2.22)
Atrophy ventricular, (median, IQR)	4 (1)	4 (3)	0.482	-	0.849	0.98 (0.75-1.26)
Atrophy sulcal, (median, IQR)	5 (2)	4 (3)	0.984	-	0.682	0.94 (0.70-1.26)

*adjusted for age, sex, arterial hypertension, diabetes, hyperlipidaemia and MRI parameters of CSVD

** chronic kidney disease (CKD) with GFR<60mL/min/1.73m^2

† Severe white matter hyperintensities: deep Fazekas Score ≥2 or periventricular Fazekas Score 3

‡ Enlarged perivascular spaces: >10 in one hemisphere in the basal ganglia

n=2, convexal n=2, traumatic n=1). The median time from the index RSSI to the intracranial bleeding event occurrence was 5 years (IQR 5 years).

Vascular risk factors, stroke severity, RSSI location, medication and analysed laboratory parameters were not associated with ICrH occurrence in the long-term follow-up of this RSSI cohort. In univariable analysis, severity of WMH (OR 2.74, 95% CI 1.07-7.01) and presence of CMB (OR 3.87, 95% CI 1.64-9.07) were related to ICrH occurrence. Both the presence of deep (OR 2.56, 95% CI 1.11-5.93) and lobar CMB (OR 4.96, 95% CI 2.18-11.38) were associated with ICrH occurrence. Neither lacunes, EPVS nor ventricular or sulcal atrophy were predictive in this context.

In multivariable analysis, only the presence of CMB remained a predictor for ICrH occurrence (HR 3.25, 95% CI 1.39-7.59, **Table 7**).

Table 8: The distribution of the applied CSVD summary score in the subgroups of patients with recurrent ischaemic and haemorrhagic cerebrovascular events

CSVD summary score	Event (n, %)	No Event (n, %)	P value
Recurrent ischaemic cerebrovascular event (n=70)			
0	4 (5.7)	42 (16.0)	0.026
1	18 (25.7)	75 (28.6)	0.630
2	14 (20.0)	54 (20.6)	0.910
3	20 (28.6)	58 (22.1)	0.259
4	14 (20.0)	33 (12.6)	0.114
Occurrence of intracranial haemorrhage (n=26)			
0	0	46 (15.0)	0.033
1	6 (23.1)	87 (28.4)	0.559
2	5 (19.2)	63 (20.6)	0.869
3	7 (26.9)	71 (23.2)	0.667
4	8 (30.8)	39 (12.7)	0.011

The applied median CSVD summary score was evidently higher in the subgroup of patients suffering from an ICrH during follow-up (3 versus 2, p=0.027, **Table 7**), which was also predictive in multivariable analysis (HR 1.57, 95% CI 1.11-2.22). It is of note, that the subgroup of patients with a maximum CSVD summary score of 4 was especially high in the group of ICrH patients (31%, OR 3.04, 95% CI 1.24-7.47, **Table 8**).

Other vascular events

Other vascular events occurred in 21 patients (6.3%) and included myocardial infarction (n=17, 5.1%) and peripheral limb ischaemia (n=4, 1.2%). The median time to myocardial infarction or peripheral limb ischaemia occurrence was 2 years (IQR 6 years). Diabetes mellitus (OR 3.29, 95% CI 1.34-8.03), chronic kidney disease (OR 2.69, 95% CI 1.00-7.26), coronary artery disease (OR 5.06, 95% CI 1.88-13.56) and atrial fibrillation (OR 3.15, 95% CI 1.15-8.66) were associated with the occurrence of myocardial infarction or peripheral limb ischaemia. Sex and other vascular risk factors were not associated with myocardial infarction or peripheral limb ischaemia occurrence.

Mortality

The mortality rate was 19.3% (n=64) during a median follow-up period of 12 years. The median time between the index stroke event and death was 5 years (IQR 6 years). The most common causes of death were cardiogenic shock (n=7, 11%), intracranial haemorrhage (n=6, 9%) and pneumonia (n=5, 8%). Only 3 patients (0.9%) died within the hospitalization period of the index RSSI.

Mortality was higher in the subgroup of patients suffering from an ICrH during follow-up (OR 2.41, 95% CI 1.02-5.70) displayed in an univariable Cox regression model. It is of note, that of 13 deep intracerebral bleeding events, 5 were fatal (38%). Mortality was also significantly higher in the subgroup of patients with myocardial infarction or peripheral limb ischaemia during the follow-up period (OR 2.80, 95% CI 1.11-7.08). On the other hand, there was no association of mortality and ischaemic cerebrovascular event recurrence (OR 0.84, 95% CI 0.42-1.67).

Project B: Arterial Stiffness and cerebral small vessel disease

Baseline characteristics of the study cohort

This analysis included 84 patients (mean age 72 years, 43% women). The most common vascular risk factors were arterial hypertension (73%), hyperlipidaemia (64%) and active smoking (50%). In a subgroup of 77 patients (mean age 68 years, 51% women) neuropsychological testing was performed.

Baseline data including demographics, vascular risk factors, imaging parameters and neuropsychological test scores are presented in **Table 9**. The median PWV during 24-hour measurement was 10m/s (3m/s IQR). Median PWV was higher during day-time measures than night-time measures (10m/s versus 9 m/s; $p<0.001$).

PWV was related to higher age ($r_s=0.942$, $p<0.001$), presence of coronary artery disease ($r_s=0.282$, $p=0.009$), arterial hypertension ($r_s=0.316$, $p<0.001$) and higher systolic blood pressure ($r_s=0.293$, $p=0.007$) in univariable analysis. Other vascular risk factors as well as sex were not predictive. Also, the use of antihypertensive medication (n=18, 21%) was not associated with PWV in univariable analysis ($r_s=0.022$, $p=0.835$) and in multivariable analysis corrected for age and sex ($p=0.804$) and did not differ between day- and night-time measures.

CSVD-related brain changes

The median WMH volume in this patient cohort was 5.2 cm^3. PWV correlated to WMH volume in univariable analysis ($r_s=0.472$, $p<0.001$; **Table 10**). Both day-time PWV ($r_s=0.436$,

p<0.001) and night-time PWV (r_s=0.439, p<0.001) were related to WMH volume in this context.

Table 9: Baseline characteristics including demographic data, risk factors, imaging parameters and cognitive testing (reproduced with permission of Sage Journals (100))

Demographics	**n=84**
Age, years (mean, SD)	72 (14.3)
Women (n, %)	43 (51.2)
Education, years (median, IQR)	10 (3.0)
Vascular risk factor profile	
Hypertension (n, %)	61 (72.6)
Diabetes mellitus (n, %)	11 (13.1)
Hyperlipidaemia (n, %)	54 (64.3)
Smoking (n, %)	42 (50.0)
Coronary artery disease (n, %)	25 (29.8)
Systolic blood pressure, mmHg (mean, SD)	122.7 (9.8)
Pulse wave velocity, m/s (median, IQR)	
PWV, 24-hours	10 (3)
PWV, day-time	10 (3)
PWV, night-time	9 (2)
MRI imaging parameters, cm^3	
White matter hyperintensity volume (median, IQR)	5.2 (8.0)
PSMD (mean, SD)	3.1 x 10^{-4} (0.4x 10^{-4})
Total brain volume (median, IQR)	1091.2 (156.8)
Basal ganglia volume (median, IQR)	530.0 (69.6)
Neocortical volume (median, IQR)	428.9 (55.0)
White matter volume (median, IQR)	557.8 (74.0)
Neuropsychological domains (Z scores)	**n=77**
Executive function (median, IQR)	0.219 (0.85)
Visuopractical skills (median, IQR)	0.133 (1.19)
Memory and learning (mean, SD)	0.027 (0.92)
Global cognitive performance (mean, SD)	0.025 (0.92)

Table 10: Presentation of MRI imaging parameters and their relationship with pulse wave velocity in univariable and multivariable linear regression analysis (reproduced with permission of Sage Journals (100))

	Univariable Analysis		Multivariable Analysis*		
	Spearman Correlation, r_s	P value	ß-Coefficient	Standard error	P value
Pulse wave velocity - 24-hours					
White matter hyperintensity volume	0.472	<0.001	-0.142	0.260	0.587
Peak width of Skeletonized Mean Diffusivity	0.452	<0.001	7.06×10^{-6}	1.07×10^{-5}	0.444
Total brain volume	-0.617	<0.001	-36.871	14.787	0.015†
Basal ganglia volume	-0.283	0.009	-17.188	8.129	0.038
Neocortical volume	-0.553	<0.001	-9.802	7.404	0.189
White matter volume	-0.503	<0.001	-19.682	8.743	0.027
Pulse wave velocity - Day-time					
White matter hyperintensity volume	0.436	<0.001	-0.261	0.199	0.194
Peak width of Skeletonized Mean Diffusivity	0.437	<0.001	-5.76×10^{-8}	8.11×10^{-6}	0.995
Total brain volume	-0.606	<0.001	-17.460	11.918	0.147
Basal ganglia volume	-0.328	0.002	-6.073	6.536	0.356
Neocortical volume	-0.528	<0.001	1.064	5.884	0.857
White matter volume	-0.498	<0.001	-11.386	6.975	0.107
Pulse wave velocity - Night-time					
White matter hyperintensity volume	0.439	<0.001	0.048	0.211	0.820
Peak width of Skeletonized Mean Diffusivity	0.472	<0.001	1.01×10^{-5}	7.01×10^{-6}	0.228
Total brain volume	-0.620	<0.001	-32.300	11.493	0.006†
Basal ganglia volume	-0.278	0.010	-15.143	6.344	0.019†
Neocortical volume	-0.560	<0.001	-10.500	5.752	0.069
White matter volume	-0.508	<0.001	-17.156	6.811	0.014†

*Adjusted for age, sex, systolic blood pressure, coronary disease, diabetes mellitus and family structure

†Statistically significant after FDR correction for multiple comparisons

However, this association could not be shown in a multivariable mixed linear regression model adjusted for age, sex, systolic blood pressure, diabetes mellitus, coronary artery disease and family structure. PDSM was related to PWV in univariable analysis (r_s=0.452, p<0.001, **Table 10**), irrespective of day and night-time measures. Again, this association was no longer evident in the multivariable mixed linear regression model (p=0.444)

Brain atrophy

Total brain volume, as well as grey matter volume (separated into basal ganglia and neocortical volume) and white matter volume were calculated. PWV correlated with all investigated brain volumes (p<0.05, **Table 10**) in univariable analysis and there was no difference between day and night-time measures. In the multivariable linear regression model adjusted for age, sex, systolic blood pressure, diabetes mellitus and coronary artery disease increased 24-hour PWV was significantly associated with reduced total brain volume, white matter and basal ganglia volume. These findings remained significant after FDR correction for multiple comparisons. Similar results could be shown for night-time, but not for day-time measures (**Table 10**).

Cognitive function

A subgroup of 77 patients received neuropsychological testing to evaluate the cognitive function in different cognitive domains (executive function, visuopractical skills, memory and learning). Also, a global cognitive performance score was calculated. PWV was associated with a reduced performance in all tested cognitive domains in univariable analysis,

however none of these associations could be shown in multivariable mixed linear regression model adjusted for age, sex and vascular risk factors (**Table 11**).

Table 11: Presentation of cognitive function (z scores) and the relationship to pulse wave velocity in univariable and multivariable linear regression analysis (reproduced with permission of Sage Journals (100))

	Univariable Analysis		Multivariable Analysis*		
	Spearman Correlation, r_s	P value	ß-Coefficient	Standard error	P value
Pulse wave velocity - 24-hours					
Executive function	-0.303	0.007	0.204	0.199	0.309
Visuopractical skills	-0.564	<0.001	-0.340	0.220	0.127
Memory and learning	-0.397	<0.001	0.022	0.257	0.931
Global cognitive performance	-0.491	<0.001	0.063	0.241	0.796
Pulse wave velocity - Day-time					
Executive function	-0.252	0.027	0.276	0.151	0.071
Visuopractical skills	-0.541	<0.001	-0.108	0.171	0.529
Memory and learning	-0.342	0.002	-0.128	0.197	0.517
Global cognitive performance	-0.448	<0.001	0.152	0.184	0.413
Pulse wave velocity - Night-time					
Executive function	-0.325	0.004	0.052	0.153	0.734
Visuopractical skills	-0.577	<0.001	-0.316	0.167	0.062
Memory and learning	-0.406	<0.001	-0.046	0.196	0.816
Global cognitive performance	-0.507	<0.001	-0.056	0.184	0.761
*adjusted for age, sex, systolic blood pressure, coronary disease and diabetes mellitus					

Project C: Cerebrovascular reactivity

Baseline characteristics

This prospective, longitudinal study was performed as a pilot study and included 13 participants. The mean age was 65 years and 3/13 participants of the study cohort were females. The most common vascular risk factors were hyperlipidaemia (100%), arterial hypertension (85%) and diabetes mellitus (39%). The baseline characteristics are presented in **Table 12**.

The most common infarct location was the thalamus (n=7) followed by the basal ganglia (n=3), the centrum semiovale (n=2) and the brainstem (n=1). The index RSSI transformed into a chronic lacune in all 13 participants (3-month follow-up visit).

At least one chronic CSVD imaging parameter was present in 10 patients. The most common chronic CSVD imaging parameter was the presence of 10 or more EPVS (**Table 13**). The median CSVD summary score was 1 (IQR: 2, **Table 13**) at baseline and 2 (IQR:1, **Table 13**) at the 3 months follow-up.

During the 15-months follow-up period new ischaemic lesions in form of WMH or lacunes were identified in 2 patients (15.4%).

Table 12: Baseline characteristics including demographics, vascular risk factors, infarct morphology and outcome parameters

Demographics and clinical characteristics (n=13)	
Age, years (mean, SD)	65 (+/- 10.2)
Women (n, %)	3 (23.1)
mRS before stroke event (median, IQR)	0 (0)
mRS at admission (median, IQR)	2 (3)
NIHSS at admission (median, IQR)	2 (3)
Intravenous thrombolysis (n, %)	2 (15.4)
Vascular risk factor profile (n, %)	
Arterial hypertension	11 (84.6)
Diabetes mellitus	5 (38.5)
Hyperlipidaemia	13 (100)
Chronic kidney disease <60mL/min/1.73m^2	2 (15.4)
Atrial fibrillation	1 (7.7)
Ipsilateral extracranial/intracranial large artery disease (>50% stenosis)	1 (7.7)
Coronary heart disease	2 (15.4)
Peripheral artery disease	2 (15.4)
Active smoking	2 (15.4)
Cancer	2 (15.4)
Infarct morphology (n, %)	
Anterior Circulation	5 (38.5)
Basal ganglia	3 (23.1)
Internal capsule	0 (0)
Centrum semiovale	2 (15.4)
Posterior Circulation	8 (61.5)
Thalamus	7 (53.8)
Brainstem	1 (7.7)
Right hemisphere	7 (53.8)
Outcome parameters (n, %)	
mRS after 3 months (median, IQR)	1 (2)
Mortality (n, %)	1 (7.7)
Recurrent ischaemic cerebrovascular event (n, %)	1 (7.7)
Intracerebral haemorrhage (n, %)	0
mRS… modified Rankin scale NIHSS… national institute of health stroke scale	

The total follow-up period was 15 months. The patients recovered well from the index RSSI with a median mRS of 1 at the 3 months follow-up visit (**Table 12**). During follow-up one patient had a recurrent ischaemic cerebrovascular event in form of a TIA. No intracerebral haemorrhages or other major ischaemic or haemorrhagic events occurred in the follow-up period. One patient died during the follow-up period due to the progression of a malignant cancer.

Table 13 Imaging parameters of cerebral small vessel disease

MRI at baseline	
Chronic cortical infarcts (n, %)	2 (15.4)
WMH* deep (median, IQR)	1 (2)
WMH* periventricular (median, IQR)	1 (1)
WMH Pons (n, %)	3 (23.1)
Cerebral microbleeds (n, %)	1 (7.7)
Lacunes (n, %)	4 (30.8)
EPVS† (n, %)	10 (76.9)
CSVD summary score (median, IQR)	1 (2)
MRI changes at 3 months	
Any new lesions (n, %)	2 (15.4)
CSVD summary score (median, IQR)	2 (1)
CSVD… cerebral small vessel disease WMH … white matter hyperintensities * Fazekas Score † Enlarged perivascular spaces: >10 in one hemisphere in the basal ganglia	

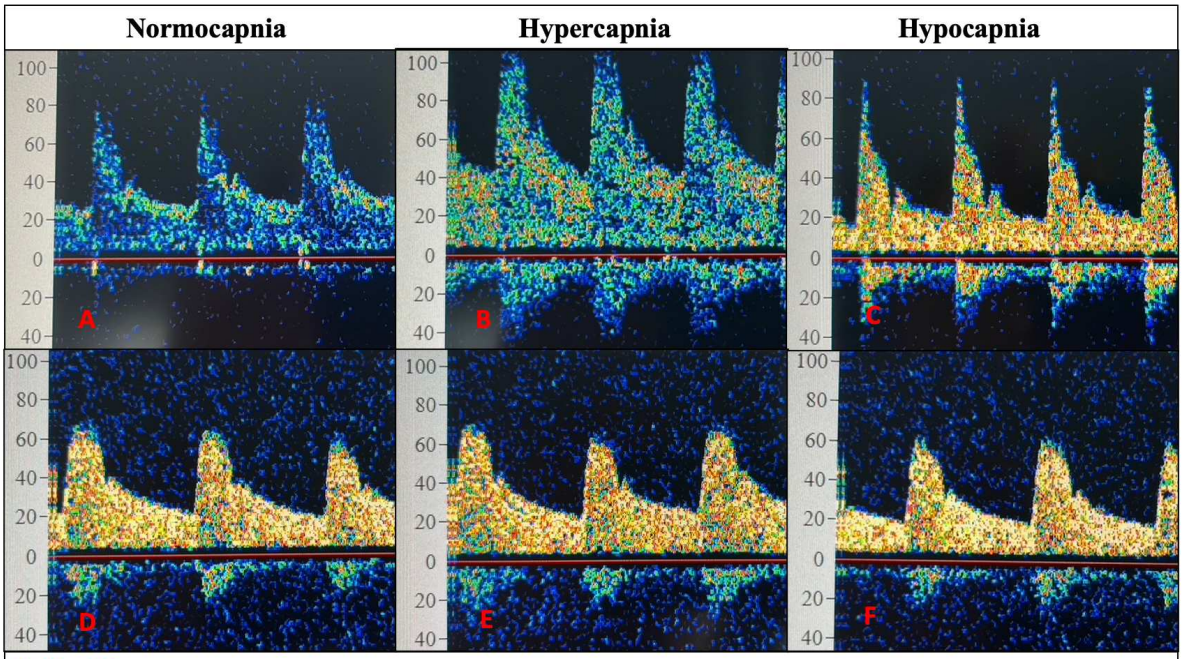
Neurovascular ultrasound and cerebrovascular reactivity

All patients received neurovascular ultrasound at the baseline and at the follow-up visits. In one patient transcranial ultrasound could not be performed due to the lack of a sufficient transtemporal window. CVR measurement was performed in all remaining 12 patients. The

examination took about 30 minutes to complete and the breathing manoeuvres were performed sufficiently by all participants.

Two examples of CVR measurements of included patients are shown in **Figure 4.** Patient 1 showed a normal CVR in the baseline examination (**Figure 4**, **Panel A-C**) with an increase of the mean blood flow velocity in the MCA during the hypercapnic phase (breath holding) and a decrease during the hypocapnic phase (hyperventilation). Also, the pulsatility index (PI) decreased during breath-holding and increased during hyperventilation. These changes could not be observed in patient 2, implying impaired CVR (**Figure 4**, **Panel D-F**).

Figure 4: Two examples of the cerebrovascular reactivity measurement via transcranial ultrasound. The mean blood flow velocities as well as the respective pulsatility index (PI) of the middle cerebral artery are presented during normocapnia (normoventilation), hypercapnia (breath holding) and hypocapnia (hyperventilation). The breath-holding index (BHI) and full range of vasodilation were calculated.



Patient 1: **A**: Normocapnia 45cm/sec, PI 1.06; **B**: Hypercapnia 71cm/sec, PI 0.80; **C**: Hypocapnia 37cm/sec, PI 1.63; **BHI**: 1.22, Full range of vasodilatation: 76%.
Patient 2: D: Normocapnia 38cm/sec, PI 1.08; **E**: Hypercapnia 41cm/sec, PI 0.98, **F**: Hypocapnia 35cm/sec, PI 1.17; **BHI**: 0.26, Full range of vasodilatation: 16%.

Of 12 sufficient baseline measurements, 4 patients showed a BHI below the threshold of 0.67 implying impaired CVR. The mean blood flow velocity in the MCA increased during the hypercapnic phase (breath holding) in all participants (mean difference 11 cm/sec, minimum 3 cm/sec, maximum 20 cm/sec, **Table 14**). The mean blood flow velocity in the MCA decreased during the hypocapnic phase (hyperventilation) in all participants (mean difference 8 cm/sec, minimum 1cm/sec, maximum 19 cm/sec, **Table 14**). The mean full range of vasodilatation was 50% (minimum 16%, maximum 76%, **Table 14**). The mean full range of vasodilatation was especially low in patients with impaired CVR compared to patients with intact CVR (26% versus 62%).

Table 14: Results of the cerebrovascular reactivity measurements of the patient cohort.

	Mean	Minimum	Maximum
Breath holding index	0.72	0.26	1.22
Difference in mean blood flow velocity during hypercapnia (cm/sec)	11.28	3.30	20.15
Difference in mean blood flow velocity during hypocapnia (cm/sec)	7.55	1.2	18.65
Full range of vasodilatation (%)	49.74	15.79	75.76
Pulsatility index normocapnia	1.15	0.80	1.60
Pulsatility index hypercapnia	1.05	0.70	1.40
Pulsatility index hypocapnia	1.40	1.00	1.80

Development of the neurovascular ultrasound protocol

Due to difficulties regarding the CVR measurement, the neurovascular ultrasound protocol was adapted after the recruitment of the first patients and recruitment was paused in 2022.

The main difficulty was that the head device in which the two transcranial ultrasound probes are embedded was uncomfortable for the patients to wear, especially since the whole ultrasound protocol took about 20 to 30 minutes to complete. It was also difficult to ensure that the position of the probes remained stable during the breathing manoeuvres. As a result, the position of the probes had to be readjusted several times, which further increased the duration of the ultrasound examination. As a solution a new head device was purchased which was made of silicon. This device was tolerated without further problems. Also, the positioning of the probes was easier and faster. The rate of readjustments of the probes during the examination was lower.

Another problem that was observed after the first measurements, was that the effect of hyperventilation, which could be observed as a decrease of mean blood flow velocity in all patients, could be seen for about 2 to 3 minutes after the breathing manoeuvre had been finished. Therefore, the time in between the individual measurements had to be extended in order to prevent the persistent effect of hyperventilation on mean blood flow velocity from influencing further measurements. As a solution the time period between two individual measurements was extended to five minutes. Furthermore, the time between breath holding and hyperventilation was extended to two minutes.

After the first examinations the changes of mean blood flow velocities were observed and the off-line analysis was adapted accordingly. The breath holding phase was performed for 30 seconds which was tolerated by all patients. The maximum change of mean blood flow velocity was observed in the last 5 seconds of the breath holding phase, therefore the mean value of these 5 seconds was measured. Hyperventilation was performed for 45 seconds. The effect of hyperventilation on mean blood flow velocity was observed earlier than the

effect of breath holding. However, the maximum effect was also observed in the last five seconds. The protocol for offline analysis was therefore adjusted accordingly:

- The baseline mean blood flow velocity (normocapnia) was defined as the mean value of the 30 seconds prior to the first breath holding phase.
- The mean blood flow velocity during breath holding (hypercapnia) was defined as the mean value of the last 5 seconds of the breath holding phase.
- The mean blood flow velocity during hyperventilation (hypocapnia) was defined as the mean value of the last 5 seconds of the hyperventilation phase.

The final version of the neurovascular ultrasound protocol is attached in the appendix of this thesis.

Discussion

CSVD is one of the leading causes for ischaemic stroke worldwide and has also been associated with further clinical manifestations such as intracranial haemorrhage and vascular dementia (5, 6). The clinical hallmark feature of CSVD is lacunar stroke, which corresponds to an RSSI on neuroimaging, preferentially MRI.

As the underlying pathophysiology of CSVD is still incompletely understood, there has been a lack of specific treatment strategies so far. In this context, further research investigating possible pathomechanisms that have been associated with CSVD and its progression are of utmost importance for further treatment development and improvement of prevention strategies (9, 102). In this thesis two possible pathomechanisms, haemodynamic changes in CBF (CVR and changes in pulsatility indices) and arterial stiffness have been investigated in two separate projects. In another project, we analysed recurrent cerebrovascular events after an RSSI and particularly focused on the aetiology of these events, which is paramount in the optimal individual poststroke care / secondary prevention.

Until specific therapeutic measures are implemented in clinical practice, the identification of clinical and imaging risk factors is essential to assess the risk of recurrent events in secondary prevention. The data on recurrent vascular events in this patient cohort is scare and little is known about the underlying aetiology of recurrent ischaemic cerebrovascular events in this population. Furthermore, there is almost no data on the incidence of intracranial or intracerebral haemorrhage in RSSI patients. However, since both lacunar

stroke and ICH are considered to be caused by intrinsic CSVD, a higher incidence of ICH in an RSSI population seems likely.

Long-term outcome and recurrent cerebrovascular events

This retrospective long-term follow-up study of RSSI patients shows that the recurrence of ischaemic cerebrovascular events and intracranial haemorrhage in this patient cohort is high. Diabetes mellitus as well as the severity of WMH, presence of lacunes and CMB and ventricular brain atrophy were associated with recurrent ischaemic cerebrovascular events, whereas only CMB were related to intracranial haemorrhage occurrence. The CSVD summary score was higher in all patients with recurrent events suggesting that the severity of CSVD has a crucial influence on the long-term outcome of RSSI patients and the frequency of recurrent cerebrovascular events. Also, our analysis could show that recurrent ischaemic cerebrovascular events were caused by CSVD again in about 70% suggesting progressive CSVD in these cases.

The definition of lacunar stroke is very heterogeneous in current literature. While clinical definitions, based on historically used lacunar stroke syndromes, can be helpful in identifying lacunar stroke patients, not all of these patients show a corresponding RSSI pattern on MRI. This study, therefore, included patients based on MRI criteria of RSSI which is distinctive to other studies that included patients according to clinical characteristics in form of lacunar stroke syndromes (33-35). In this context, the use of the STRIVE criteria for RSSI diagnosis has been proven practical in several studies (86, 103). Furthermore, patients with coexisting stroke aetiologies other than CSVD such as atrial fibrillation or upstream large vessel stenosis were mostly excluded in previous studies but were included in our

analysis as the aim was to analyse a real-world stroke cohort of RSSI patients. It is important to mention that the presence of these two potential alternative stroke causes was not related to recurrent events in our analysis. Also, it is known that several potential causes for stroke can coexist and might independently influence the long-term outcome and cerebrovascular event recurrence in this patient cohort (26, 104).

Because of their small size and often low initial stroke severity, RSSI/lacunar stroke have been described as a more benign subtype of acute ischaemic stroke. Contrary to this point of view, recent studies have shown that even though the risk for recurrent vascular events after RSSI in short-time observation is lower than in other stroke subtypes, there is no significant difference in long-term observation (31-33). Similar findings could also be shown in our analysis as the rate of recurrent ischaemic cerebrovascular events of 21% and the rate of ICrH of 8% following RSSI in a median follow-up period of 12 years is substantial. In addition, the aetiologies of recurrent ischaemic cerebrovascular events were assessed and our results showed that approximately 70% of recurrent strokes and 60% of TIAs were caused by CSVD again, suggesting a recurrent ischaemic event due to progressive CSVD. The data on the aetiologies of recurrent ischaemic cerebrovascular events in RSSI cohorts in current literature is scarce with only a single available study (105).

The results of our study identified the presence of diabetes mellitus and not surprisingly also higher HbA1c levels as a risk factor for recurrent ischaemic cerebrovascular events. This finding is supported by other recent studies. A substudy of the Secondary Prevention in Small Subcortical Strokes trial (SPS3) showed a higher rate of recurrent stroke in patients suffering from diabetes and metabolic syndrome (106). Liu et al. showed an association between

diabetes mellitus and lacunar stroke occurrence without focusing on recurrent events in a mendelian randomization analysis (107).

Only few studies can be found that investigated the association of CSVD MRI abnormalities and recurrent ischaemic cerebrovascular events in RSSI cohorts. Imaizumi et al. showed that severe WMH (Fazekas 2-3) and CMB correlated with recurrent lacunar stroke events in a lacunar stroke cohort in a follow-up time of about 4 years (34). Our investigation also showed an association of WMH and CMB with recurrent ischaemic cerebrovascular events. Furthermore, we could also show an association of the presence of lacunes and ventricular brain atrophy with ischaemic cerebrovascular event recurrence in our cohort in multivariable analysis.
However, in addition to other studies our analysis also included the CSVD summary score, which was higher in the subgroup of patients suffering a recurrent ischaemic event.

These findings clearly (and not unsurprisingly) emphasize that RSSI patients benefit equally from a structured secondary prevention protocol and stringent control of vascular risk factors as other stroke subtypes. The results of our analysis further indicate that these secondary prevention measures might be even more important in patients with severe CSVD imaging findings as these patients are more prone to cerebrovascular event reoccurrence. Furthermore, our results identified CSVD as the aetiology in about 70% of recurrent ischaemic events, suggesting that progressive CSVD is the main pathophysiological trigger of recurrent cerebrovascular events in RSSI patients.

Studies investigating the frequency of intracranial haemorrhage occurrence after RSSI or lacunar infarction are scarce. The only available study showed that severe WMH also

increase the risk of deep intracerebral haemorrhage after lacunar infarction (34). We did not find such an association in our study after correction for age and vascular risk factors. CMB remained the only CSVD-related imaging feature related to ICrH occurrence after correcting for possible confounders. However, the overall CSVD burden (based on the CSVD summary score) was evidently related to ICrH occurrence. One could argue that the presence of CMB would be a major factor regarding this association as CMB was the only imaging parameter that was associated with ICrH occurrence in multivariable analysis and a correlation of the presence of CMB and ICH occurrence has been shown in studies before (46). However, as the number of patients with a maximum CSVD summary score was particularly high in this subgroup of patients (31% versus 20% in the recurrent ischaemic event subgroup and 12% in the overall study cohort), especially deep intracerebral haemorrhage seems to be a late consequence of progressive and severe CSVD which cannot be explained by certain subcategories alone but rather by the sum of the affected deep subcortical brain structures. This assumption is supported by Goeldlin et al. who also showed a higher total CSVD summary score in intracerebral haemorrhage patients than in lacunar stroke patients (35). Even though we did not exclude other forms of intracranial haemorrhage such as subdural or subarachnoid bleeding, the associations of our analysis remained significant. This is an interesting observation that has, to the best of our knowledge, not been reported before.

In summary, our analysis showed that recurrent ischaemic cerebrovascular events and ICrH occurrence in an RSSI cohort share similar predictive chronic CSVD abnormalities on brain MRI (CMB, higher CSVD summary score). These findings suggest shared underlying pathomechanisms of these two stroke entities. While the underlying pathomechanisms of CSVD and lacunar stroke are still not fully understood, it is assumed that apart from hypertensive arteriosclerosis also endothelial dysfunction, BBB disturbance, chronic

hypoperfusion of small arterioles and haemodynamic changes play an important role in this matter (16). However, specific treatment regimes in this regard are not yet available in clinical practice and should be investigated in large randomised trials. In this context, the identification of clinical and imaging parameters that are predictive of cerebrovascular event recurrence and ICH occurrence are of importance for the clinical practice. As a consequence of the long follow-up period of about 12 years we were also able to gain important practically relevant insights regarding medication compliance and treatment adherence of our patient cohort. Here, it was evident that vascular risk factors were not always adequately controlled in long-term follow-up with a considerable part of the cohort not achieving target levels of modifiable risk factors such as arterial hypertension, hyperlipidaemia or diabetes mellitus. However, due to the retrospective design of our study not all of these parameters are documented consistently during follow-up. These results should therefore be interpreted with caution.

While some studies reported that the effect of antihypertensives, lipid-lowering drugs and antiplatelet therapy did not differ between stroke subtypes (108), several other studies described an inconsistent effect of these treatment measures in RSSI patients particularly considering extensive blood pressure lowering (109-111). The study of Ikeme et al. could show that among lacunar stroke survivors, patients with a severe WMH burden benefited the most from blood pressure control (110).

The strengths of this study lay in the long follow-up period of the patient cohort and the thorough MRI protocol that made it possible to correct for CSVD MRI findings in our analysis. This study also has some limitations which are mostly due to its retrospective study design as parameters of interest are not always consistently documented in follow-up. Also,

the lack of follow-up MRI studies limits the knowledge on (especially clinically silent or not that obvious) CSVD progression over time and its influence on recurrent ischaemic and haemorrhagic events in this patient cohort.

Arterial Stiffness and cerebral small vessel disease

Our retrospectively analysed cohort study of elderly, healthy persons without a history of dementia or cerebrovascular disease showed a significant association of increased PWV and reduced total brain volume. Whereas night-time PWV was strongly associated with reduced total brain volume, as well as with basal ganglia and white matter volume, day-time PWV was not predictive suggesting a possible impact of circadian fluctuations of PWV on brain morphology. An association between PWV and CSVD-related brain changes or cognitive function could, however, not be shown in this patient cohort.

Several studies showed an association of arterial stiffness with an increased burden of CSVD-related imaging markers such as WMH (83, 112). In contrast, we were not able to detect similar results in our study cohort. Van Sloten et al. showed an association of increased WMH burden and PWV in a meta-analysis including almost 2000 participants which is noticeably higher than the patient cohort included in our study (n=84). Considering the fact that the effect size of this analysis was rather low (OR 1.3; 95 %, Confidence interval 1.16-1.46), a weak association of WMHs and PWV could have been missed in our study cohort considering the significantly smaller sample size (112). Furthermore, Van Sloten et al. included not only community-based studies with healthy participants but also studies in selected clinical populations (e.g., individuals with stroke, TIA or dementia) in their meta-analysis, while these conditions were considered exclusion criteria in our study based on a

healthy elderly population and their first-degree relatives. This might also explain the missing link between WMH burden and PWV in our study as WMH are strongly associated to cerebrovascular disease and vascular dementia (113).

In contrast to other studies, we also included the novel imaging marker PSMD in our analysis. PSMD is a new MRI parameter initially described by Baykara et al in 2016 (50). This marker is based on DTI and white matter tract skeletonization. Several studies showed an association of increased PSMD and reduced cognitive function, especially processing speed, visuopractical skills and memory. These results suggests that PSMD might be an early parameter that indicates changes in the cerebral microvasculature and brain aging in general (114, 115). In this context, a relationship of PSMD and arterial stiffness and therefore PWV changes seems likely. However, our study did not show an association of these two parameters in a multivariable logistic regression model corrected for age, sex and vascular risk factors. A possible explanation could be the small sample size which might also be the reason why we could not prove a significant association of PWV and neuropsychological parameters unlike previous studies (116).

The effect of arterial stiffness on total brain volume is inconsistent in current literature. Whereas the community-based “Reykjavik” study could not find a significant association of PWV and global brain atrophy, (83) our findings showed an inverse relation of PWV and total brain volume as well as basal ganglia and white matter volume. These results are supported by the findings of another study, that showed an association of increased PWV and reduced total brain volume in 1255 healthy, elderly individuals (117). Tsao et al. presented similar results analysing the data of the Framingham study (118). Overall, the association of arterial stiffness and brain atrophy regarding the underlying

pathophysiological processes remains incompletely understood. Some studies supported the hypothesis that increased PWV leads to substantial change and remodelling in the cerebral microvasculature of the whole brain resulting in reduced blood flow and total brain atrophy. Our study does not corroborate this hypothesis as an association of vascular changes and arterial stiffness could not be shown in our patient cohort.

Recent studies that focused on arterial stiffness and its influence on brain morphology and cognitive function used single PWV measurements. Our study protocol also included 24-hour PWV measurement differentiating between day-time and night-time values. In this context, Aissopou et al. showed an association of 24-hour PWV and a reduced diameter of retinal vessels. These results were even more significant during night-time measurement (119). As retinal and cerebral vessels share an anatomical and developmental background; it is likely that the reaction of their terminal arterioles to an increased aortic pulse wave exposure might be similar. Our findings corroborate this hypothesis as night-time PWV measures seemed to have a stronger association than day-time measures. As PWV is highly depending on arterial blood pressure, circadian changes of systolic blood pressure could be a possible reason for this observation. However, our findings showed that night-time PWV values corrected for systolic blood pressure and its variations were still predictive for reduced total brain volume. This indicates an influence of circadian changes of PWV on the cerebral microvasculature that is independent from arterial blood pressure.

The limitations of this study are its cross-sectional design and the relatively small cohort size which might have been the reason why we could not find an association of PWV and CSVD imaging parameters and impaired cognitive function in contrast to other studies. Nevertheless, this study and its complex study design contribute to improving the

understanding of arterial stiffness and brain morphology. In comparison to previous studies, we included the novel MRI marker PSMD and circadian fluctuations of PWV in 24-hour measurement in our analysis. Our analysis indicates a connection of PWV and reduced total brain volume predominantly during night-time measurement. These findings might also be relevant in clinical practice as antihypertensive drugs, especially ACE-inhibitors, are able to significantly decrease arterial stiffness (120, 121). In this context, a respective prescription preferably in the evening could reduce night-time PWV and consequently lower the risk of global atrophy and its progression. However, these assumptions warrant further research in larger study populations with prospective study designs.

Cerebrovascular reactivity

Impaired CVR might play a role in the disease development of CSVD and its progression. Previous studies have already associated impaired CVR with CSVD features on MRI such as WMH or EPVS and their progression over time (72, 78). Almost all currently available studies focusing on CVR and CSVD used MRI techniques to measure CVR (61, 77). However, as MRI protocols including BOLD or other functional MRI sequences require long examination times that not every patient can tolerate and not all hospitals can offer, easier and non-invasive methods of CVR measurements that could possibly also be conducted on older and functionally impaired patients as a bed-side test are needed. The use of TCD might be a useful alternative in this context. Furthermore, CVR measurement requires the application of a vasoactive stimulus to visualize haemodynamic changes either with MRI or TCD. CO2 inflations or vasoactive drugs are mostly used as a vasoactive stimulus; however, these are invasive measures that could possibly be associated with side-

effects. The use of breathing manoeuvres on the other hand represents a non-invasive, simple technique that could be performed repeatedly (76, 122).

To create a simple, bed-side investigation protocol to assess CVR impairment in a CSVD cohort, we used TCD and breathing manoeuvres in a small pilot study on RSSI patients. Other studies could identify impaired CVR as a risk factor for ischaemic stroke occurrence in patients with carotid artery stenosis using a TCD protocol with either breathing modulation or CO2 inflations (123). In this context, carotid endarterectomy or stenting was also associated with an improvement of CVR in patients with high-grade symptomatic carotid artery stenosis (79, 124). To the best of our knowledge this is the first study that uses TCD combined with breathing modulation to assess CVR in a CSVD/RSSI cohort.

We tested our CVR protocol in 13 individuals that were treated at our university hospital due to an RSSI defined according to STRIVE criteria (23). The neurovascular ultrasound protocol including CVR measurement was well tolerated by all patients and all patients performed sufficient breathing manoeuvres. The mean blood flow velocities of the MCA were recorded during normoventilation, breath holding and hyperventilation. Impaired CVR was defined as a BHI below 0.67 as this threshold was suggested by Markus et al. in a stroke population with carotid artery stenosis (73). Impaired CVR was identified in 4 patients. We also calculated the full range of vasodilation, a parameter that reflects the total vasodilatory capacity of the cerebral vessels, according to a protocol previously described by Ringelstein et al (74). Interestingly, the range of vasodilatation was especially low in patients with impaired CVR compared to patients with intact CVR in our study population (26% versus 62%).

Lower, impaired CVR measured with MRI techniques was associated with a higher burden of CSVD imaging parameters (WMHs, microbleeds, lacunes) and a higher CSVD summary score in a recent study by Sleight et al., and was also associated with CSVD progression during a follow-up period of one year (61, 78). In our study all participants received brain MRI at baseline and at the two follow-up visits. The study population in our study was too small to test for similar correlations, however these research question should be addressed in further larger prospective studies and are highly relevant for clinical practice and the validation of CVR assessment with TCD. Further steps should also include longitudinal measurements of CVR in RSSI cohort to investigate if a reduction of CVR over time correlates to recurrent ischaemic stroke events or progressive CSVD imaging parameters as data on this matter is missing in current literature so far.

Arterial pulsatility indices were measured in all patients as part of the neurovascular ultrasound protocol. In patients with intact CVR and normal BHI extensive changes of PI were observed depending on the presence of a vasoactive stimulus. The PI decreased during breath holding and increased during hyperventilation in all examined patients. Overall, the PI was higher in patients with impaired CVR, however the patient cohort was too small to show a statistical significance. There are single studies that could show an association of increased arterial pulsatility measured via MRI with CSVD imaging parameters, however it is still unknown whether increased arterial pulsatility is a cause or a consequence of CSVD (60). Increased arterial pulsatility has also been associated with increased arterial stiffness, which is a further important possible pathomechanism in CSVD and its progression (82). Furthermore, it has been suggested that increased pulsatility is more common in CSVD-related stroke than in other stroke aetiologies and might therefore be a promising biomarker for risk stratification in RSSI cohorts (56).

This pilot study could show that CVR assessment with TCD and breathing modulation is feasible in an RSSI cohort and differences between normal and impaired CVR can be visualized with this method. Even though the protocol took about 30 minutes to complete, the examination was well tolerated by the participants after some protocol adaptations and no discomfort or side-effects were observed.

Further and larger trials are of need to test for correlation with CSVD, recurrent vascular events and small vessel disease in other organ systems such as the kidney or the retina. Also, CVR assessment with TCD and breathing modulations should be validated in larger cohorts and compared to respective MRI techniques such as BOLD sequences before implementation in clinical routine is established.

There are however also several limitations. First of all, the sample size is low, which is due to the study design as a pilot study that aimed to test feasibility of the investigated method in an RSSI population. The TCD protocol had to be adjusted and recruitment was temporarily paused which was also the reason that only few patients were included in this pilot study.

Furthermore, it has been described that blood flow velocity changes during breathing modulation might also be dependent on blood pressure changes (125). In this context, the participants were explicably instructed about the breathing protocol and were told not to perform a Valsalva manoeuvre while holding their breath to avoid rapid blood pressure changes. However, the exclusion of such bias without consistent blood pressure control might be difficult in clinical practice.

Another limitation is the fact, that with currently available MRI- or TCD-based techniques, it is not possible to visualize the haemodynamic changes in the small penetrating deep arteries of the brain directly. With TCD CVR and pulsatility indices can be assessed in large and medium sized intracranial arteries. However, a transmission of haemodynamic changes from large or medium size vessels to the small penetrating arterioles seems likely. This assumption is also supported by one study that could show that pulsatility indices measured with TCD are especially high in CSVD and vascular dementia patients (56).

A larger prospective trial is planned to test the TCD protocol in a larger RSSI cohort. Further investigations should focus on the correlation of CVR impairment measured with TCD and breathing modulation and CSVD-related MRI parameters.

Single studies could also show an association of renal parameters such as reduced glomerular filtration rate or proteinuria with CSVD, especially in young patients with CSVD, suggesting an underlying systemic disease of small vessels (63, 126, 127). However, it is still unknown if these associations are the result of shared risk factors or due to an underlying systemic small vessel disease. In a prospective study with a larger RSSI cohort, renal parameters in serum and urine including newer promising biomarkers such as cystatin C and fibroblast growth factor 23 should be included in the laboratory protocol. Associations of these parameters with CSVD imaging parameters and CVR impairment should be investigated.

Conclusion and outlook

We could show that the incidence of recurrent cerebrovascular events in a RSSI cohort is substantial. Most recurrent cerebrovascular events were due to CSVD again, suggesting that progressive CSVD is the main trigger for recurrent stroke events in an RSSI cohort. CSVD and its progression is further related to vascular dementia and other chronic neurological conditions associated with a reduced quality of life and an increased disability especially in the elderly population. Considering the progressively ageing population worldwide, CSVD represents a huge global health and economic burden. Understanding the underlying pathophysiological processes is paramount to develop specific secondary prevention strategies.

Arterial stiffness is a possible pathomechanism in CSVD. We could show an association of arterial stiffness and increased PWV with reduced total brain atrophy. Night-time values were of higher importance, suggesting a circadian rhythm of arterial stiffness, which has not been described in other studies before. Further prospective and larger study cohorts are needed to test arterial stiffness and its influence on CSVD.

Impaired CVR and increased arterial pulsatility might play a role in CSVD development and progression. Our study shows that a specific TCD protocol using breathing manoeuvres such as breath-holding and hyperventilation as vasoactive stimulus is feasible in this patient cohort to assess CVR. This protocol might represent a non-invasive alternative to complex MRI protocols that can also be performed as a bedside test. Further investigations should focus on the correlation of impaired CVR measured with this protocol and its association with imaging parameters of CSVD and recurrent events. Also, an association of impaired

CVR and renal parameters should be investigated, testing the hypothesis of CSVD as part of a systemic small vessel disease rather than an organ specific pathology.

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Appendix

Neurovascular ultrasound protocol for CVR Measurement

CVR-Messung – Anleitung für MULTIDOP

Version 2, 13.06.2023

Durchführen der Untersuchung:

1. Patient anlegen: „Monitoring“ → „Öffnen“
   - Baseline Untersuchung: „Neuer Patient“ (Name, Geburtsdatum ausfüllen, unter Kommentar „Baseline“ eintragen)
2. Follow-Up Untersuchung: Patient im Suchfeld suchen → „neue Sitzung“
3. Nasensonde anlegen und Doppler Sonden einstellen
   - Vorsicht: Filter der Nasensonde erst anstecken, wenn Gerät eingeschaltet und das Programm geöffnet ist, sonst findet keine entsprechende Kalibrierung statt!
4. „Monitoring“(oder „Aufnahme“) → „Record start“
5. Untersuchungsablauf: Patient soll mindestens 5 Minuten ruhig liegen
   - 30 Sekunden Luftanhalten
   - **2 Minuten** normal weiter atmen
   - Hyperventilation für 45 Sekunden
   - Normal weiteratmen
   - **5 Minuten** Pause vor zweitem Durchgang
6. „Record stop“
7. Untersuchung speichern

Auswerten der Sitzung im Offline Modus:

1. Öffnen der Sitzung
2. „Audio“ → Kontinuierliches Playback (die gesamte Aufnahme wird abgespielt!)
3. **Visuelle Auswertung der Kurven**:
   Referenz (Baseline Wert): ersten 30 Sekunden (vor erster Apnoe Phase)
   Apnoe Phase: letzten 5 Sekunden
   Hyperventilation: 45 Sekunden versus letzte 5 Sekunden
   Berechnen von BHI und Full range of vasodilatation
4. „Bearbeiten“ → Kopieren von Trends und Kurven möglich, sowie Screenshots