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Comprehensive analysis of the impairment of cerebrovascular reactivity and cardiovascular autonomic nervous system functions in patients with significant internal carotid artery stenosis

PhD thesis

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List of abbreviations

ABP: Arterial Blood Pressure

AUC: Area Under the Curve

BFV: Blood Flow Velocity

BH: Breath-Holding Test

BHI: Breath-Holding Index

BL: Baseline

CANS: Cardiovascular Autonomic Nervous System

CAR: Cerebral Arterial Resistance

CBF: Cerebral Blood Flow

CCA: Common Carotid Artery

CAR-AUC: Cerebral Arterial Resistance—Area Under the Curve

CCC: Common Carotid Artery Compression Test

CEA: Carotid Endarterectomy

CGRP: Calcitonin Gene Related Peptide

CPVR: Centro-Peripheral Valsalva Ratio

CPOI: Centro-Peripheral Overshoot Index

CTA: Computed Tomography Angiography

CVAR: Cerebrovascular Valsalva Ratio

CVR: Cerebral Vasoreactivity

ECG: Electrocardiogram

ECST: European Carotid Surgery Trial

ESVS: European Society for Vascular Surgery

GPCR: G-Protein Coupled Receptors

HV: Hyperventilation

HVI: Hyperventilation Index

ICA: Internal Carotid Artery

ICA_{op}: Side to be operated

ICA_{nonop}: Side no to be operated

ICAS: Internal Carotid Artery Stenosis

LDL: Low Density Lipoprotein

MABP: Mean Arterial Blood Pressure

MBFV: Mean Blood Flow Velocity

MCA: Middle Cerebral Artery

MLC: Myosin Light Chain

NASCET: North American Symptomatic Carotid Endarterectomy Trial

NO: Nitrogen Monoxide

NVC: Neurovascular Coupling

NVU: Neurovascular Unit

PRT: Pressure Recovery Time

PSV: Peak Systolic Velocity

ROCK: Rho Kinase

RTB: Return to Baseline

RVLM: Rostral Ventrolateral Medulla

SI: Sympathetic Index

TCD: Transcranial Doppler

THR: Transient Hyperemic Response

THRR: Transient Hyperemic Response Ratio

VM: Valsalva Maneuver

VHRR: Valsalva Heart Rate Ratio

1. Introduction

The energy requirement for the brain to maintain its constant reactivity is extremely high. Its tissue energy reserves are minimal, so the oxidative metabolism substrates necessary for its functioning are provided exclusively by the arterial blood supply. Under physiological conditions, the cerebral blood supply, with its significant reserves, abundantly provides cerebral blood demand, however, even a few minutes of ischemia causes irreversible cerebral damage. The blood supply requirement of the brain is approximately 50ml/100g brain tissue/minute, which is a total of 700-800ml/minute, which is around 15% of the total cardiac output.(1) In terms of oxygen consumption, it is about 3-3.5ml O_2/100g tissue per minute, so the brain requires roughly 20% of the total oxygen consumption of the body.(1)(2) The arterial blood supply of the brain is provided by the two internal carotid arteries, originating from the common carotid arteries, and the two vertebral arteries, originating from the subclavian arteries. An intracranial arterial anastomotic network known as the circle of Willis, formed by these systems. In addition, the anastomotic connections between the extra- and intracranial vessels, as well as the leptomeningeal connections, are of great importance. The brain's significant blood demand must be met within the nearly closed intracranial space, where, according to the Monro-Kellie doctrine, a balance must be maintained among the individual volume components (i.e., brain parenchyma, interstitial and cerebrospinal fluid, and cerebral arterial and venous blood). The significant arterial blood demand of the brain is ensured by very tightly regulated and parallel mechanisms, which, in addition to the near-constancy of global cerebral blood flow, simultaneously achieve local activity-dependent and rapidly changing regional flow enhancement.(3)

The mechanism underlying cerebral blood flow regulation has been a focus of research for centuries. In the middle of the 19th century, Dutch physiologist and ophthalmologist F. C. Donders was the first to hypothesize an early concept of functional hyperemia, given to a response in the regulation of cerebral blood flow (CBF), which is today considered the precursor of neurovascular coupling.(4) Furthermore, in the late 19th century, Italian physiologist Angelo Mosso published his research work known as "human circulation balance" – he was one of the first scientists who demonstrated the link experimentally between mental activity and CBF. A. Mosso studied patients with skull defects, allowing

him to directly observe brain pulsations. He noticed that the brain's volume and pulsation changed with mental activity. Mosso's balance table experiment showed that brain activity increases the blood flow to the brain – he demonstrated that mental activity increases blood supply to the brain which resulted in a slight tilt of his invented balanced table.(5)(6) Further substantial contributions were the experiment of Roy and Sherrington in 1890, who were the first to propose that CBF might be locally regulated in accordance with neuronal activity, which supported the metabolic hypothesis of neurovascular coupling (NVC). Their conclusions were based on the direct observation of local stimulation of the cortex, which caused dilatation of the nearby blood vessels.(7) In 1895, William Maddock Bayliss and his colleagues reported controversial findings. Based on their observations, they concluded that there was no evidence of focal vasomotor response in the brain. Instead, their findings supported that the CBF changes were passive responses to the systemic arterial blood pressure changes. His statement denying cerebral circulatory regulation proved to be wrong, but Bayliss is still cited today in connection with the "Bayliss effect," his discovery of the ability of arterioles to constrict or dilate in response to changes in arterial blood pressure.(8) In 1948, a major milestone method was developed by Seymour Kety, which was based of the inhalation of nitrous oxide to measure differences between arterial and venous concentration, allowing estimation of global cerebral blood flow over a 10-15 minute period.(9) It was later on improved by in 1961 by Niels A. Lassen and David H. Ingvar by introducing radioactive tracers such as Krypton-85 and Xenon 1-22, which enabled also the regional measurements of CBF; furthermore, these developments laid the foundation of modern functional imaging methods.(10) In the 1970s, advanced imaging techniques such as computed tomography (CT) followed by positron emission tomography (PET), single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI), enabled the examination of the anatomy and physiology of the cerebral circulation system.(11) Later on, in 1982 the introduction of transcranial Doppler (TCD) by Rune Aaslid represented a major advance in the study of CBF regulation because it enabled the real-time measurement of cerebral blood flow velocity (CBFV) with a high temporal resolution.(12)

1.1. The regulation of the cerebral blood flow circulation

The cerebral vasculature has structural differences regarding large arteries and the microcirculation system. As the vessel size decreases, the regulation transitions from global to highly localized control. The large extraparenchymal arteries – including the vessels of the Circle of Willis and the major pial arteries – are rich in smooth muscle and elastic tissue, and their regulation primarily relies on pressure-dependent myogenic responses. Then the vessels branch into penetrating arterioles and further smaller arterioles, which have fewer layers of smooth muscle and reduced elastic structures.(1) These arterioles are still responsive to autoregulatory mechanisms but are more sensitive to local concentrations of metabolic end products.(13) The regulation of blood supply occurs at several different levels: (1),(2)

1.1.1. Neural mechanism of global cerebral blood flow regulation (Autonomic regulation)

This phenomenon is understood as the importance of sympathetic, parasympathetic and trigeminal innervation.

The large cerebral arteries and extraparenchymal parts of arterioles are rich in adrenergic, cholinergic and sensory innervation, the main neurotransmitters are: adrenaline, noradrenaline, dopamine, neuropeptide Y, acetylcholine, vasoactive intestinal peptide, calcitonin gene-related peptide, substance P, and the pituitary adenylate cyclase-activity peptide.(14)(15) The most important anatomical structures are the superior cervical ganglion for sympathetic innervation, the otic and sphenopalatine ganglion for parasympathetic innervation, and the trigeminal ganglion for sensory innervation. The brainstem nuclei and the basal nucleus of Meynert are also significant structures.(3) The most significant autonomic nervous system function in terms of cerebral circulatory regulation is the arterial baroreflex. The essence of this reflex is to maintain central arterial blood pressure (ABP) within a relatively narrow range by changing heart rate (HR) and peripheral vascular resistance through a reflex mechanism induced by baroreceptors located in the aortic arch and carotid sinuses. The baroreceptors detect the wall tension-induced changes in the vascular wall, the afferent signals reach the nucleus tractus solitarius (NTS) located in medulla oblongata via the glossopharyngeal (IX.) and vagal nerves, from where the signals further reach the caudal ventromedullary (CVLM)

and rostral ventromedullary (RVLM) regions, by inducing a pressor or depressor response as needed by altering sympathetic and parasympathetic nervous system activity.(16) Sympathetic innervation plays a minor role in maintaining CBF at rest, but becomes significant when ABP increases suddenly. In previous studies, it was established that, for example, in the case of superior cervical ggl. ganglionectomy, increasing CBF can be measured. The essence of sympathetic innervation is that it prevents increase in CBF during rest periods. In the case of sudden changes in arterial blood pressure, for example, rapid blood pressure increase, however, the sympathetic response causes vasoconstrictor responses, which maintain the resting CBF and protect the blood-brain barrier. (3)(15) The effect of the parasympathetic nervous system on CBFV has not yet been fully clarified. The cholinergic projection on parenchymal neurons and the vasculature is generally associated with the release of potent vasodilator agents, including the release of NO, but it cannot be said that it has a significant role in CBF regulation.(17) The great cerebral vessels and pial arterioles are innervated by the trigeminovascular system and influence CBF mainly through the release of calcitonin gene-related peptide (CGRP), substance P and pituitary adenylate cyclase-activating peptide, which influence vascular tone.(14) CGRP has a central role in the pathophysiology of migraine, cluster headache and other trigeminal autonomic cephalalgias. It mediated nociceptive signaling within the trigeminovascular pathway and represents a key therapeutic target in the management of these disorders.(18),(19)

1.1.2. Cerebral Pressure-flow Autoregulation

The concept of cerebral autoregulation can be traced back to Lassen's publication in 1959, according to which cerebral blood flow can be considered relatively constant in a wide range of mean arterial blood pressure, i.e. between 50 and 150 mm Hg.(20) This statement is most consistent with the experiences gained with the so-called static measurement method of autoregulation, i.e. the relationship determined by pairs of values formed from the averages of CBF and ABP over relatively long periods, considered as steady state.(3) Over time, however, the assessment of cerebral vascular reactivity using the transcranial Doppler ultrasound technique invented by R. Aaslid and simultaneous non-invasive blood pressure measurement made it possible to study and numerically characterize the dynamics of autoregulation.(12),(21) Based on this, the so-called dynamic autoregulation

is a data inferred from a measurement method that quantifies the vascular response to spontaneous or induced rapid changes in arterial blood pressure that occur within a few seconds.(22),(23),(24) This so-called dynamic autoregulation is most commonly assessed using the transfer function analysis method, a mathematical approach that expresses the phase difference and amplitude ratio between arterial blood pressure and cerebral blood flow velocity oscillations.(25) The autoregulatory function is based on the myogenic response, i.e. the fundamental, intrinsic, smooth muscle-derived regulatory mechanism of cerebral arteries and arterioles. Myogenic reactivity is based on the response to changes in vascular wall tone, in which smooth muscle depolarization occurs because of an increase in arterial blood (transmural) pressure. The pressure or stretch of the vessel wall is sensed by special mechanosensors such as stretch-activated cation channels and voltage-dependent Ca^{2+} channels which transduce the physical forces into intracellular sings.(26),(27) This results in the consequent Ca^{2+} influx through the voltage-dependent calcium channels and the release of Ca^{2+} stores from the sarcoplasmic reticulum due to the local Ca^{2+} signals which causes further activation of contractile proteins – such as the phosphorylation of myosin light chain (MLC) and thus leads to vasoconstriction.(1),(28) Besides the Ca^{2+} influx, several other molecular changes contributes to the initiation of contraction: among others, myogenic tone is regulated by protein kinase C and Rho kinase (ROCK), integrins, and mechanosensitive G protein-coupled receptors (GPCRs) signal transduction. Due to the increased intracellular Ca^{2+} concentration large conductance Ca^{2+} activated K^+ channels in the cell membrane will be activated, which results in local membrane hyperpolarization and limits the vasoconstriction – the activation of K^+ channels by Ca^{2+} is also known as a negative feedback mechanism.(1),(27)

1.1.3. Chemoregulation

Chemoregulatory functions mainly refer to the effects of changes in arterial blood gases, such as changes in $PaCO_2$ and PaO_2. $PaCO_2$ is known to be one of the most potent regulators of cerebrovascular myogenic tone. However, the main mediator of the effect is not CO_2 itself, but the induced acidosis. CO_2 diffuses across the blood-brain barrier, forming carbonic acid, which will reduce the extracellular pH and mediates the regulatory mechanisms of cerebral blood circulation through the consequent pH changes.(29)

Changes in $PaCO_2$ directly affect the function of cerebral arterioles, the underlying process is governed by the carbonic anhydrase equilibrium:(13)

$$[H_2O] + [CO_2] \leftrightarrow [H_2CO_3] \leftrightarrow [HCO_3^-] + [H^+]$$

The drop of pH results in the release of nitric oxide (NO) and increases the local K^+ concentration, which induces relaxation of the vascular smooth muscle, i.e., vasodilation via cGMP signaling, membrane hyperpolarization and CO_2-driven Ca^{2+} and COX-1 mediated signaling of the astrocytes which results in PGE_4 release.(13),(30) If $PaCO_2$ level decreases, vasoconstriction develops. The response to changes in CO_2 is globally similar in different regions of the brain. In addition, other molecular and cellular mechanisms have also been revealed, including the function of NO, proteinoids, and acid-sensing ion channels.

Besides $PaCO_2$, changes of the arterial O_2 also influence the cerebral vasculature, although $PaCO_2$ has a greater role in chemoregulation. The cerebral vessels are also sensitive to hypoxia but only below PaO_2<50Hgmm. Simultaneous hypercapnia increases sensitivity to hypoxia, thereby enhancing the vasodilatory response. In contrast, hypoxia triggers ventilatory response (hyperventilation), which leads to hypocapnia and subsequent vasoconstriction.(31) Several parallel processes are also important under the influence of hypoxia, for example, the direct effect of tissue hypoxia on neurons and glia, the acidosis caused by anaerobic metabolism, and the release of vasodilator substances (e.g. adenosine).(3)

It has been studied that the regulation of CBF responds differently to changes in CO_2 levels in humans: the relationship between increasing CO_2 and the resulting increased CBF is linear between 20-60 mmHg, outside this range the response progressively decreases, following a sigmoid pattern, suggesting that resistance vessels reach the limit of their reactivity. A study identified a CO_2 threshold above which systemic blood pressure is also raised; the cerebrovascular response to CO2 can indirectly affect the ABP changes as well. (3)

1.1.4. The mechanism of neurovascular coupling and the importance of endothelium and vascular smooth muscle

Neurovascular coupling (NVC) is a complex process that links the actual neuronal activity to changes in the regional CBF in order to ensure the adequate blood supply of the activated brain regions – in other words: the essence of neurovascular regulation is that cerebral blood flow is closely linked to neuronal activity, in simple terms, if a brain area becomes more active, blood flow in that area promptly increases. These mechanisms operate at the level of the neurovascular unit (NVU). The NVU is a structural and functional relationship between the endothelial cells of the blood-brain barrier, neurons, astrocytes, pericytes, and extracellular matrix components, which together coordinate rapid changes in the vascular tone in response to shifting metabolic demands. NVU interactions take place mainly at the capillary and precapillary levels. Neuronal activation initiates signal transduction processes in astrocytes and blood vessels, as a result of which rapid vasodilation responses develop, and the increase in blood flow occurs before metabolic changes.(13)

The anatomical substrates of the NVU have different functions:

The endfeet of the astrocytes envelop almost entirely the capillary surface thus enable the localized release of vasoactive agents towards the endothelial cells and pericytes – an organization enabled by their characteristic glial fibrillary acid protein. Moreover, astrocytes can communicate via gap junctions thus allowing Ca^{2+} influx to propagate across network and coordinate responses to neuronal activation. The stimulation of astrocytes increase the intracellular Ca^{2+} levels and this induces release of vasoactive agents such as nitrogen monoxide (NO), prostaglandins, potassium which results in local hyperemia.(32)

The endothelial cells can quickly respond to the signals of the neurons and astrocytes thus releasing vasoactive substances such as prostaglandins, vascular endothelial growth factors, and NO, which act on the vascular smooth muscle and other structural parts of the NVU and regulate the local cerebral blood flow. Endothelial cells regulate muscle tone in several ways: through the production of various vasoactive substances (NO, K^+, H_2O_2, ATP, endothelium-derived hyperpolarizing factors), by communicating with smooth muscle cells through gap junctions. Endothelial responses can be triggered by

shear stress, receptor activation, and various chemical stimuli. Thus, the importance of endothelial cells can be seen, and endothelial dysfunction caused by atherosclerosis and small vessel disease has clinical significance.(3),(33)

Pericytes are specialized cells contributing to microvascular regulation. Evidence suggests functional diversity among the pericyte subtypes – with some involved in the regulation of vascular tone while others have a role in the integration of the brain-blood-barrier and sensing neuronal activity to signal upstream vasodilation.(13)

Following neuronal activation, the release of neurotransmitters (mainly glutamate) triggers the signaling pathways in the components of the NVU, leading to the generation of multiple vasoactive mediators that contribute to creating functional hyperemia.(34)

In summary, the essence of this system is that the brain quickly adjusts blood supply to the current activity through a complex system.

1.2. Atherosclerosis

Atherosclerosis is a chronic, progressive arterial vascular disease involving immunological and inflammatory processes, which mostly affects the intima layer of medium and large arteries.(35) Its clinical relevance is significant, as it is the etiological basis of considerable proportion of ischemic stroke, coronary artery disease and peripheral vascular disease, among others. Cardiovascular diseases of atherosclerotic origin are registered as the leading causes of death worldwide.(36) The pathophysiological basis of atherosclerosis is that lipoproteins deposited in the subendothelial part of large arteries, and their deposition triggers inflammatory and immunological responses.(35) The development of atherosclerosis is not uniform in the vascular system, but most often develops where shear stress occurs: a typical location for this is aortic arch and the carotid bifurcation.(37) As a result of atherosclerosis, endothelial dysfunction and lipoprotein retention develops. In areas exposed to significant shear stress, the endothelium is damaged, oxidative stress and the expression of adhesion molecules increases, and lipoproteins enter the intima. Lipoproteins entering the intima undergo oxidative and enzymatic modifications, and the modified low-density lipoprotein (LDL) has a chemotactic, cytotoxic, endothelium-damaging effect, and also activates

immune responses.(35) Under the influence of chemokines produced by the endothelium, monocytes migrate to the intima, which take up the modified phagocytes and transform into foam cells. From this point on, the foam cell is an active inflammatory cell. In addition, in atherosclerosis, T cells also appear in the plaque, which maintain inflammation by producing interleukins, which causes further lysosomal damage in the plaque, activation of inflammatory processes, which increases plaque instability.(38) As the process progresses (plaque progression), a necrotic lipid core is formed, above which a fibrous cap is located, which is basically a collagen-rich protective layer. As the cap is thick, the more stable the plaque is, while if it is thin and macrophage-rich, thin-cap fibroatheroma develops. The consequences of unstable plaque are plaque rupture, plaque erosion, and subsequent arterio-arterial thrombosis.(39) The process of atherosclerosis occurs over a long period of time, but it can also suddenly cause a potentially life-threatening condition. The neurological consequences of atherosclerotic events in the extra- and intracranial carotid and vertebrobasilar systems are transient ischemic attacks and ischemic cerebral infarction. Atherosclerotic processes in the coronary arteries lead to acute or chronic coronary syndrome and can cause claudication syndrome by limb ischemia in the peripheral vessels, among many others. In addition, the effect of damage to the cardiovascular autonomic nervous system should be emphasized.(40)

1.2.1. Internal Carotid Artery stenosis

Significant Internal Carotid Artery stenosis (ICAS) is a lumen reduction of 70% or greater, which can lead to cerebral ischemia through two main mechanisms. Stenosis is caused by atherosclerosis gradually reduces the lumen of the vessel, which can lead to a progressive deterioration of cerebral perfusion. Such a degree of lumen stenosis significantly reduces the amount of blood flow through it, which results in a decrease in the pressure ensuring perfusion in the distal vascular segment. If the anastomoses of the circle of Willis compensate for this, there are no hemodynamical consequences. In case of ineffective anastomoses, hemodynamic insufficiency may occur in the supply area of the internal carotid artery, which can lead to the development of so-called border zone infarctions, most often in the deep white matter of the hemisphere, which is the border area between the pial and perforating circulatory systems. The border zone infarctions are

frequently associated with the aforementioned severe stenosis of the internal carotid artery (ICA) (41). Moreover, in a substantial proportion of so-called territorial infarctions resulting from arterio-arterial embolization, carotid atherosclerosis is also the source of cerebral ischemia.(42) In addition to cerebral ischemic events, ICA stenosis has an adverse effect on cerebrovascular and cardiovascular autonomic nervous system reactivity.(3) Severe carotid stenosis significantly impairs cardiovascular autonomic responses through damage to the baroreceptor system. (43) It is known that patients with carotid stenosis have reduced baroreceptor sensitivity, which is associated with an increased risk of cardiovascular events. In case of ICAS, heart rate variability (HRV) is also reduced, which indicates a decrease in vagal nerve system activity.(40),(43),(44)

1.2.2 Diagnostics of Internal Carotid Artery Stenosis

Several diagnostic methods are available to confirm ICAS. Even with physical examination, carotid stenosis should be suspected in the event of a murmur heard over the carotids. The primary imaging procedure is the Carotid Doppler ultrasound examination, during which the degree of stenosis can be estimated based on blood flow velocity values. However, for a more accurate assessment, computed tomography angiography (CTA) or magnetic resonance angiography (MRA) is required, which provides a detailed picture of the plaque morphology. Another option is digital subtraction angiography (DSA), which is mostly used for planning vascular interventions. The extent of stenosis can be determined based on the NASCET (North American Symptomatic Carotid Endarterectomy Trial) and ECST (European Carotid Surgery Trial) criteria.(45),(46),(47),(48) Based on the NASCET method, the narrowest lumen diameter is compared with the diameter of a distal, normal ICA segment, while according to the ECST method, the stenosis is compared to the original diameter before the plaque.(49)

1.3. Transcranial Doppler Ultrasound

Transcranial Doppler ultrasound (TCD) is a real-time, non-invasive imaging technique for measuring arterial blood flow velocity in proximal cerebral vessels such as the middle cerebral artery (MCA) through the temporal bone (acoustic window). TCD was

introduced in 1982 by Rune Aaslid and has since become one of the most important tools in cerebrovascular experimental research.(50) The examination procedure is based on the Doppler effect, according to which the frequency of ultrasound is reflected from red blood cells approaching or moving away from the transducer, based on which the blood flow velocity can be determined. A 2MHz TCD probe is used for the examination, for which the acoustic windows of the skull bone are permeable. The cerebral blood flow velocity (CBFV) can be examined through the following windows: the transorbital window allows the examination of the ophthalmic artery and carotid syphon, the transtemporal bone window allows the examination of the anterior, middle, and posterior cerebral arteries, the transforaminal window allows the examination of the vertebrobasilar system via foramen magnum, and the submandibular window allows the examination of the distal cervical part of the ICA. There are two types of TCD devices: the classic, non-duplex (non-imaging) TCD, which identifies arteries based on the depth of examination and the direction of flow. Transcranial color-coded duplex (TCCD), combined with B-mode imaging, allows for the visual appearance-based identification of large intracranial vessels. The great advantage of the TCD examination is that it is suitable for dynamic, real-time examinations. In clinical practice, it is used, among other things, to detect cerebral vasospasm after subarachnoid hemorrhage, to investigate intracranial steno-occlusive diseases, to monitor the effectiveness of intravenous thrombolysis in ischemic stroke in the event of large vessel occlusion, to detect microembolism, to measure cerebral vasoreactivity, and to confirm global cerebral ischemia in the event of suspected brain death. A technical limitation of TCD is the thickening of the temporal bone, especially in elderly female patients, which can significantly impair or make ultrasound penetration impossible. Because TCD measures the velocity and its variation in the proximal large cerebral arteries, inference regarding regional changes in microcirculation is limited due to the black box nature of the method. In addition, TCD is highly dependent on the examiner's examination routine, meaning that the reliability and accuracy of the measurements are strongly influenced by the examiner's experience and training level.(21),(51)

1.3.1. The importance of transcranial Doppler ultrasound examinations in determining cerebrovascular reactivity

Several trials have been conducted to assess CVR based on TCD studies. These studies can be divided into two large groups. First, there are tests based on CO_2 reactivity – such as CO_2 inhalation, iv. acetazolamide test, hyperventilation (HV) and breath-holding (BH) tests – and second, the tests based on pressure-flow changes, including the Common Carotid artery Compression (CCC) test and Valsalva Maneuver (VM). Another major advantage of TCD is its ability to provide indirect insight into dynamic changes in CVR. The relationship between arterial blood pressure values measured by simultaneous non-invasive methods and flow velocity data determined by TCD has revealed the high-pass filter nature of cerebral circulatory regulation. According to this, the faster and more significant the changes in arterial blood pressure, the weaker the compensatory effect of cerebral flow resistance. In any case, for any stimulus that also changes blood pressure, the change in cerebral blood flow velocity data alone is not sufficient to estimate cerebral vascular reactivity. So TCD can be used to draw conclusions about the resistance changes of the cerebral vessels. Cerebral arterial resistance (CAR) can be calculated as the ratio of MABP and MBFV.(52) During several vasoactive stimuli MBP changes, which affects cerebral BFV. If the change of the MBP is slow then given the autoregulatory control, MBFV appears to be independent of the change in MBP. In contrast, rapid fluctuations in MBP may transiently alter MBFV before autoregulatory compensation is fully established, so conclusions can be drawn about the reaction of the resistance vessels. (52),(53)

The assessment of cerebrovascular reactivity by TCD has been a subject of extensive research for many years. Among the numerous studies with substantial clinical relevance, a few will be highlighted here:

- The method of breath holding was first introduced in 1990, which is a simple bedside test evaluating CVR.(54)
- In 1992 H.S. Markus and Harrison introduced the calculation of the breath holding index (BHI) which is nowadays a widely used index in the clinical practice.(55)
- In 1991. C.A. Giller presented the Common Carotid artery Compression test.(56)

- In 1996. P. Smielewski et al introduced the Transient Hyperemic Response Ratio (THRR) calculated from the CCC test which allowed the further examination of CVR besides BHI.(57)
- In the same year, M. Czosnyka demonstrated indices derived from spontaneous fluctuations in flow velocity and cerebral perfusion pressure reflect with the cerebrovascular pressure reactivity, and correlates with the outcomes after traumatic brain injury.(58)
- Furthermore, in 1996. F. Tiecks and colleagues investigated CVR in patients with severe carotid stenosis and demonstrated that the impairment of CVR can be identified by performing Valsalva Maneuver. (59)
- R. Panerai provided a comprehensive work for assessing CVR by using transfer function analysis in 1998 which allows the analysis of spontaneous or induced arterial blood pressure on the cerebral flow velocity, thereby providing a frequency-domain assessment of dynamic cerebral autoregulation. (60)
- In 2001. Markus HS & Cullinane demonstrated CVR as an independent risk predictor of ischemic stroke.

1.3.2. Complex evaluation of the cerebrovascular reactivity and cerebrovascular autonomic nervous system dysfunction

1.3.2.1. Hyperventilation and Breath-Holding Tests

Hyperventilation and breath-holding tests are well-known vasoactive stimuli. The implementation of the two tests is straightforward, relatively safe, and capable of inducing rapid physiological changes, which makes them particularly well suited for assessing CVR. Because the stimuli produce prompt and measurable alterations in cerebral blood flow, they allow for a clear and efficient evaluation of the vasculature's ability to respond to changes in $PaCO_2$. During HV and BH both the cerebral vasoconstriction and vasodilation responses can be evaluated. During the HV test, the patients are asked to exhale rapidly and rhythmically.

Due to the consequent decrease of blood $PaCO_2$ concentration, a cerebral vasoconstrictor response occurs, which can be detected by TCD through the decrease in the MBFV values of the MCA. (4) The opposite reaction occurs during the BH tests: patients are instructed

to perform breath-holding, causing an opposite reaction to the HV test: the blood $PaCO_2$ concentration increases, followed by vasodilation in the cerebral arterioles. The vasodilation response can also be detected by TCD as the MCA MBFV values begin to increase. (5),(6) BH test has several advantages despite the other stimuli based on the CO_2 reaction. CO_2 inhalation might have severe adverse effects such as dyspnea, discomfort, anxiety, headache, dizziness, increased heart rate and blood pressure, hypercapnia, and respiratory acidosis.(3), (7) The administration of iv. Acetazolamide might also have side effects such as headache, nausea, dizziness, tinnitus, numbness and motor weakness of the extremities, motor weakness of the extremities.(8) In contrast, the BH test in the supine position and performed under controlled medical supervision has a much more favorable side effect profile: the patient is allowed to do it for as long as it is tolerable for them, there is less fear of possible side effects in the supine position, it can be interrupted at any time, and fewer side effects are reported. Figure 1. represents the schematic illustration of HV and BH holding tests.

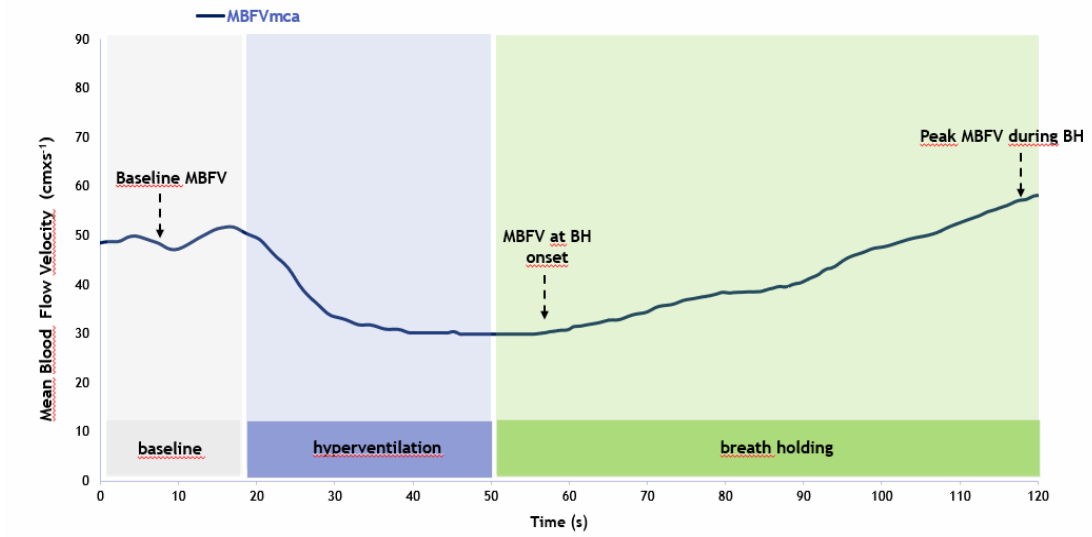


Figure 1.: *Illustration of the physiological reactions to hyperventilation and breath holding tests (original recording). $MBFV_{mca}$ refers to the mean blood flow velocity (MBFV) values of the middle cerebral artery (MCA). The baseline MBFV reflects to the baseline velocity values before HV, peak MBFV during BH reflects to the velocity values at the end of BH. The changes of the MBFV velocity values due to the stimuli are presented in the Figure. During HV vasoconstriction occurs, so a consequent reduction of MBFV values can be detected while during breath holding vasodilation occurs, thus the MBFV values increase.*

1.3.2.2.Common Carotid artery Compression test

The common carotid artery compression (CCC) test is a bedside method to assess the anastomotic capacity of the circle of Willis and the integrity of cerebrovascular reactivity (CVR). (9),(10),(11) During CCC test, a manual compression of the common carotid artery (CCA) is performed while monitoring the CBFV response of the ipsilateral MCA with TCD. Based on literature data, compression for up to 10 seconds is sufficient to elicit the maximal flow velocity response. The following changes of the CBFV during the CCC test can be detected by performing TCD measurements: due to the compression, a consequent transient decrease occurs in the MBFV of the ipsilateral MCA. The consequent decrease of the CBF results in a compensatory vasodilation of the cerebral arterioles in the supplied cerebral tissue. After the release of the compression, due to the previous cerebral vasodilation, a transient hyperemic response (THR) occurs. During CCC, F1 phase represents the MBFV values before, F2 under, and F3 after the CCC compression. The three phases are illustrated in Figure 2. below. Based on these phases of the CCC test, the Transient Hyperemic Response Ratio (THRR) can be calculated.(57) Due to safety concerns, before performing CCC test the patients must undergo Carotid Duplex Ultrasound (CDS) and Computed Tomography Angiography (CTA) examination, since manual compression of the CCA carries risk such as embolization from the atherosclerotic plaques. Moreover, due to bradycardia if the carotid sinus is stimulated, this examination requires comprehensive medical supervision with continuous blood pressure and heart rate monitoring. The CCC test has the following clinical significance: in healthy individuals with intact collateral pathways via the Circle of Willis, this reduction might rapidly be compensated. This compensatory response reflects the anastomotic capacity of circle of Willis to maintain stable perfusion despite sudden changes in the arterial supply. Furthermore, it is also used for surgical modelling of carotid clamping during CEA.

Figure 2. represents the schematic illustration of the CCC test.

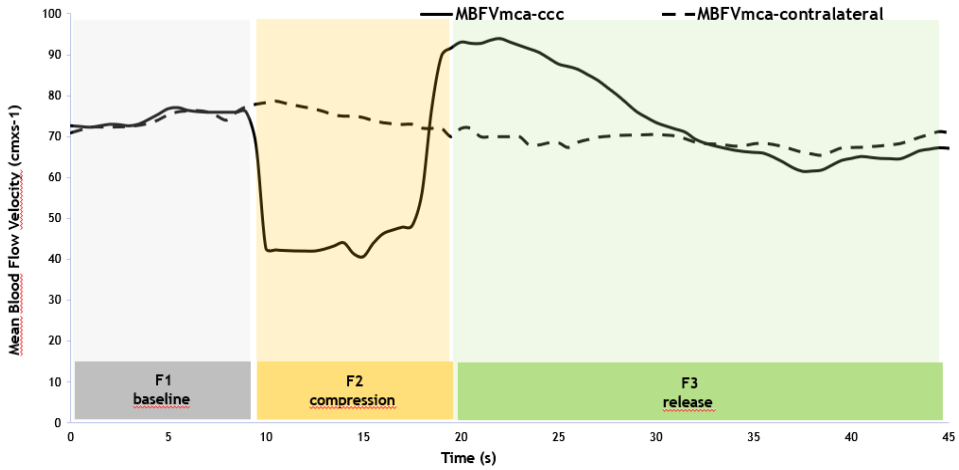


Figure 2.: *Illustration of the Common Carotid Artery Compression test (from original recording), where F1 means the MBFV values before the cessation, F2 during the cessation, and F3 after the release of the compression. $MBFV_{mca\text{-}CCC}$ refers to the side of the compression on the ipsilateral internal carotid artery (ICA), whereas $MBFV_{mca\text{-}contralateral}$ refers to the contralateral side. During the 10-second-long cessation, a consequent decrease of the MBFV values can be observed which is then followed by a transient hyperemic response (THR) after*

1.3.2.3.Valsalva Maneuver

Valsalva Maneuver (VM) is a stimulus that evokes considerable changes in central arterial blood pressure and heart rate during forced exhalation against a resistance (closed glottis or a valve). However, VM not only influences systemic arterial blood pressure but also induces characteristic changes in the cerebral circulation, thus making it a useful tool for assessing cerebrovascular reactivity. (12),(61),(62),(63),(64),(65),(66). The complex analysis of cerebral blood flow velocity response to arterial blood pressure (MABP) changes is suitable for estimating cerebrovascular reactivity, whereas changes in arterial blood pressure and heart rate indicate the regulation of cardiovascular functions by the autonomic nervous system. During VM, characteristic changes occur in cerebral BFV, ABP, and HR, which can be divided into four phases as a function of time, as illustrated in Figure 3.

The physiological phases of the VM are the following: (13)

Phase 1: at the onset of the VM, the increase in intrathoracic pressure is transmitted to the vascular system, thereby increasing central venous pressure and ABP.

Phase 2a (early): the maintained increased intrathoracic pressure reduces venous return to the heart, resulting in a decrease in the right and then in the left ventricular stroke volume, and thus ABP progressively decreases.

Phase 2b (late): A decrease in ABP activates the sympathetic nervous system, which regulates the cardiovascular system, which causes an increase in peripheral vascular resistance and heart rate, leading to an increase in ABP.

Phase 3: When forced exhalation against resistance is stopped, there is a sudden drop in the intrathoracic pressure, resulting in a rapid and temporary decrease in ABP.

Phase 4: A fast and significant rise in the ABP and HR in several seconds is observed due to the previously elevated peripheral vascular resistance and the increased venous return (overshoot; OS). Following the OS, reflex bradycardia occurs due to the activation of the baroreflex, and finally, ABP and HR return to baseline.

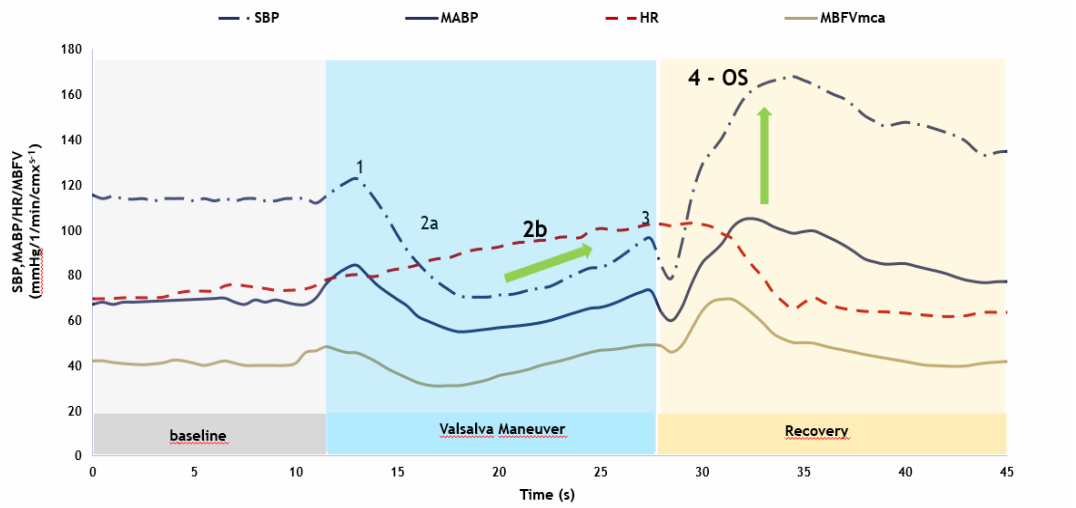


Figure 3.: *Figure 3. represents the physiological phases from an original recording of Valsalva Maneuver (VM) regarding the systolic blood pressure (SPB), mean arterial blood pressure (MABP), heart rate (HR), and MBFV (mean blood flow velocity) changes of the middle cerebral artery (MCA). The approximately 10-seconds-long baseline is followed by a 15-seconds-long VM. After the end of the VM recovery phase occurs. The numbers represent the different stages of the VM. The green colored markers reflect to the physiological reactions in 2b and OS phases.*

As discussed previously, the VM is applicable to evaluate several dysfunctions of the cardiovascular autonomic nervous system (CANS) since it directly influences the autonomic nervous system. During VM the changes of the intrathoracic pressure led to characteristic alterations in heart rate and arterial blood pressure reflecting the dynamic interaction between sympathetic and parasympathetic activity. Arterial blood pressure changes during the maneuver are mechanical in phase 1. and phase 3. while phases 2. and 4 reflect more to the cardiac function and autonomic reflexes. Sympathetic and parasympathetic systems differentially control vascular tone and heart rate, with alpha-adrenergic activity in phase 2 and beta-adrenergic effects in phase 4.

The following abnormal responses of the VM may suggest cardiovascular autonomic impairment: (13),(14)

1) The intrastrain bradycardia instead of tachycardia
2) Lack of arterial pressure decreased in early phase 2. characterized by a square-wave/flat-top pressure response
3) Diminished or absent tachycardia in phase 2.
4) Large hypotension in early phase 2 and lack of pressure recovery in phase 2
5) Lack of pressure recovery and overshoot in phase 4.
6) No bradycardia in phase 4.

These abnormal responses can be observed in patients with heart failure, valvular failure and autonomic neuropathy.

Figure 4. represents a healthy and two pathological reactions to VM.

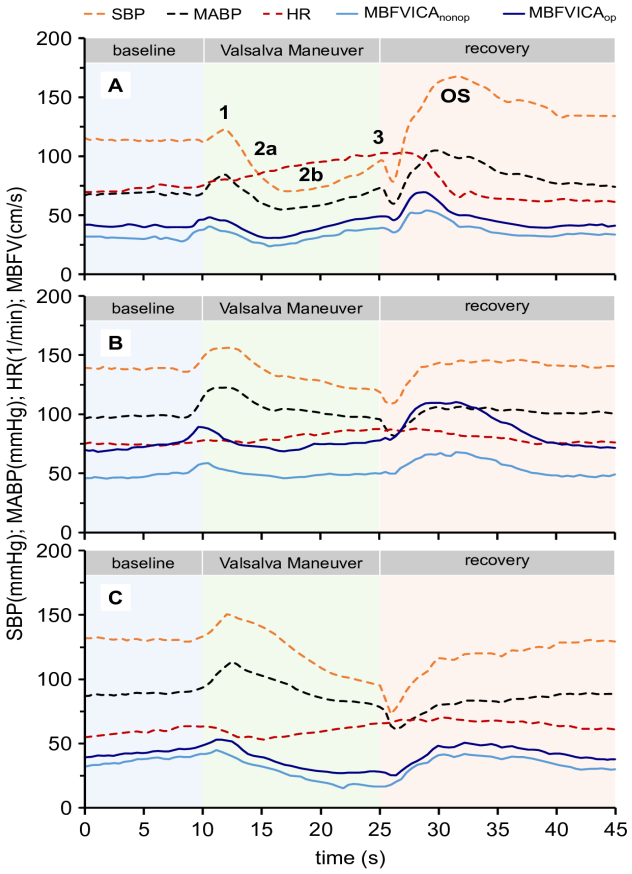


Figure 4: *Original recordings of the Valsalva maneuver of three patients with significant internal carotid artery stenosis (ICAS). (A) The physiologic changes in the middle cerebral artery (MCA) mean blood flow velocity (MBFV), arterial blood pressure (ABP) (systolic and mean), and heart rate (HR) during VM. The first stage (1) is the beginning of the maneuver, where ABP and MBFV increase. In stage 2a, ABP and MBFV progressively decrease. In stage 2b, a compensatory increase in ABP and MBFV begins and continues until stage 3. Stage 3 marks the end of VM with a transient decrease in MABP and MBFV, which is followed by an overshoot (OS) reaction. Regarding heart rate (HR), a continuous increase can be observed until OS. (B,C) Two types of pathological responses. In (B), the increases in stages 2b and OS are minimal regarding the ABP curves. In (C), both ABP and MBFV progressively decrease in stages 2b and OS. The increase in HR is also absent.*

1.4. The clinical relevance of the complex examination of cerebrovascular reactivity and the dysfunction of the cerebrovascular autonomic nervous system

The idea of correlating these parameters arose from the fact that recognizing autonomic dysfunction in atherosclerotic patient population might be relevant, since it can also enhance their cerebral ischemic risk, among other clinical features.

The autonomic dysfunction in patients with significant ICAS might have the following significant clinical relevance:

(1) Based on the results of previous studies, the concomitant impairment of CVR and CANS function should be expected in atherosclerotic patients with ICAS.(43),(67),(68) (69)

(2) Regarding procedural relevance during carotid endarterectomy (CAE), impaired cerebrovascular reactivity combined with cardiovascular instability may result in poor arterial blood pressure control and may enhance the risk of low cerebral perfusion.(70),(71)

(3) Cardiovascular autonomic nervous system dysfunction is associated with cerebral ischemia, carries prognostic significance, may worsen functional outcomes, and may increase cardiovascular mortality.(72),(73),(74),(75)

1.5. The Cardiovascular Autonomic Nervous System Dysfunction as a Risk of Ischemic Stroke

Baroreceptors are an important part of beat-to-beat regulation of ABP via the arterial baroreflex, which maintains ABP within a relatively narrow range despite significant changes in body position or cardiac output.(76) In patients with ICAS of atherosclerotic origin, the vascular stiffening of the vessel wall can decrease the sensitivity of baroreceptors in the carotid bulb due to decreased distensibility, which can lead to reduced baroreflex sensitivity via the impaired afferent signaling of the glossopharyngeal and vagal nerves.(77) The reduced effectiveness of the baroreflex can result in significant oscillations of arterial blood pressure, which, under extreme circumstances (e.g., during surgery), may exceed the pressure limits of cerebral pressure/flow autoregulation, resulting in critical cerebral hyper- or hypoperfusion.(70),(71),(78) The following phenomena are manifestations of impairment of CANS: decreased baroreflex sensitivity, decreased heart rate variability, sympathovagal imbalance, and increased arterial blood pressure variability.(79),(80) Several previous studies have verified the relationship between the impairment of the autonomic nervous system and a higher risk of cerebral ischemic events and myocardial infarcts.(74),(81),(82),(83)

1.5.1. Periprocedural Complications

During the surgical procedure, cerebral perfusion pressure may temporarily decrease due to carotid artery clamping. In the presence of severe hemodynamic instability and insufficient arterial blood pressure control, this may represent an additional significant risk of cerebral ischemia in this patient population. Considering all of this, the preoperative assessment of CANS function is important.(70),(82) Furthermore, the

dilatative and constrictive vasoreactivity capacity of cerebral arterioles may be significantly reduced by prolonged hypoperfusion caused by persistent carotid stenosis. Surgical reconstruction of carotid stenosis eliminates the risk of cerebral hypoperfusion, but the stabilization of vasoreactivity capacity does not occur immediately after the reconstruction procedure. In the case of prolonged stabilization of constrictive capacity, the risk of post-reconstruction hyperperfusion syndrome is clinically significant. This is especially true if, because of cardiovascular autonomic dysfunction, reduced sensitivity, or absence of the baroreflex (bilateral carotid stenosis), the oscillation of arterial blood pressure becomes so significant that it creates the basis for the development of focal cerebral hyperperfusion. These observations are supported by previous studies. (84), (85)

2. Objectives

Recruited patients with atherosclerotic ICAS were examined by TCD before undergoing CEA. The primary focus of our work was driven by the fact that the 2017 Guidelines of the European Society for Vascular Surgery implemented reduced CVR as an independent risk factor for cerebral ischemia. (86), (87) Most of the investigations of evaluating CVR primarily relied on CO_2 reactivity; thus our aim was to provide a more complex and comprehensive assessment of CVR by performing a wide range of vasoactive stimuli in addition to CO_2 reactivity, such as vasoactive tests that elicit pressure-flow-based changes. As detailed above, cerebral autoregulation is a complex process. To assess cerebral vasoreactivity as comprehensively as possible, several tests were applied in this patient population.

The objectives of this study were organized into two main categories:

1) One of the objectives of the work was to assess CVR preoperatively in these patients with TCD. Changes in MCA MBFV were continuously monitored on the side of severe stenosis and on the contralateral side simultaneously. The side of the stenosis was called ICA_{op} (given that this was the to be operated side). In accordance to this, the contralateral side was designated as follows: ICA_{nonop}. Thus, the objective was to compare bilateral velocity values measured in the MCA and to detect differences to quantify the effect of the hemodynamic disturbances caused by ICAS thus affecting CVR. For the comprehensive analysis, a complex evaluation method was applied by performing various vasoactive stimuli.

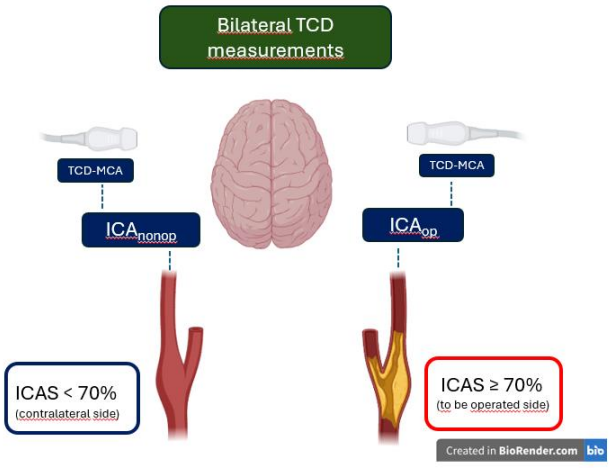


Figure 5. *Schematic Illustration of Objective 1. In this study the MBFV values of the MCA during Hyperventilation and Breath Holding, Common Carotid artery Compression test and Valsalva Maneuver were compared on the to-be-operated side (ICA_{op}) with the not-to-be operated side (ICA_{nonop}). (Original Illustration with BioRender and Microsoft Excel)*

2) A substantial proportion of patients with severe atherosclerosis affecting the aortic arch and the carotid arteries have been shown in previous studies to exhibit cardiovascular autonomic nervous system (CANS) dysfunction. (43),(67), (68), (69) The aim of our second study was to determine whether a statistically significant association can be demonstrated between impaired cerebrovascular reactivity (CVR) resulting from atherosclerotic internal carotid artery stenosis (ICAS) and the accompanying CANS dysfunction.

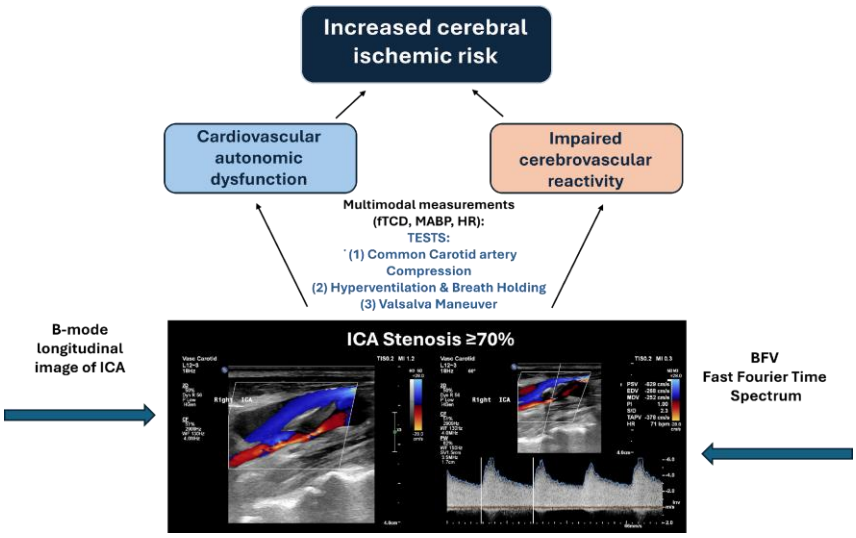


Figure 6.: *Schematic Illustration of Objective 2: Three functional transcranial Doppler (TCD) tests were used to assess cerebrovascular reactivity, and the integrity of the cardiovascular autonomic nervous system was determined based on parameters of the validated autonomic indices of the Valsalva maneuver, namely sympathetic index, Valsalva heart rate ratio, and pressure recovery time. Both reduced cerebrovascular reactivity and cardiovascular autonomic dysfunction may represent serious cerebrovascular risk factors. The image depicting severe internal carotid artery stenosis was obtained using duplex Doppler ultrasound in our laboratory, and Microsoft PowerPoint for Microsoft 365 (Version 2602) was additionally used to prepare this figure.*

3. Methods

This cross sectional, single center, exploratory study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (SE RKEB, Number: 256/2018) and registered at ClinicalTrials.gov (Identifier: NCT03840265). The study was conducted according to the guidelines of the Declaration of Helsinki. Patients admitted to the Department of Vascular Surgery of Semmelweis University for reconstruction of ICAS were enrolled consecutively between 15/01/2019 and 30/09/2021 after providing written consent. The severity of ICAS was determined by Computer Tomography Angiography according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria, with a stenosis of 70% or greater considered hemodynamically significant (i.e. flow limiting stenosis).

In the present study patients were enrolled with significant (≥70%) ICAS. The to be operated side named as ICA_{op}, and regardless of the condition of the contralateral side - not to be operated – as ICA_{nonop}, which was <70% or ≥70%. Exclusion criteria were suboptimal transtemporal insonation window, atrial fibrillation, pacemaker therapy and extensive atherosclerotic plaques in the CCA. The examinations were performed at the Doppler Laboratory of the Department of Neurology at Semmelweis University. Patients were continuously monitored clinically during the investigations. Demographic and medical history data were collected. To achieve reproducibility and best signal-to-noise ratio, the CCC and VM were performed three times, and the largest response was evaluated for each patient.

3.1.Patient population's characteristics

The study group consisted overall of 48 eligible patients (male: 36; mean age ± SD: 68.08 ± 7.23 years; female: 12; mean age ± SD: 70.16 ± 7.35 years). The risk comorbidities of the patients were as follows: 44 patients had hypertension (91.7%), 21 of them had diabetes mellitus (43.8%), 16 were smokers (33.3%) and 14 patients had ischemic heart disease (29.2%). The patients were asymptomatic regarding the ICAS and received antiplatelet therapy in addition to antihypertensives and oral statin medication. The clinical data of patients included are summarized in Table 1.

Table 1.: *Patients' baseline clinical characteristics*

n	Mean Age ± SD:	Sex	Hypertension	Diabetes	Ischemic Heart Disease	Smoking
$n_{(all)} = 48$	male: 68.08 ± 7.23 female: 70.16 ± 7.35	$n_{male} = 36$ $n_{female} = 12$	n = 44 (91.7%)	n = 44 (91.7%)	n = 14 (29.2%)	n = 16 (33.3%)

3.2.Data processing

Digitization of analog signals was performed in parallel on four channels (TCD1, TCD2, ABP tonometry, ECG), with a sampling rate frequency of 500 Hz. Raw data were stored in the European Data Format files. The files were imported, digitally filtered, and segmented using the LabChart software (ADInstruments, LabChart ver. 8, Colorado Springs, CO, USA). The pre-processed data segments describing each cardiac cycle were stored in separate text files. These files were further processed by custom-written Python code (Python version 3.12.2). The program script interpolated cardiac cycle data linearly into 0.5 s equidistant intervals. The interpolated data was exported to Microsoft Excel, and the changes in systolic, mean, and diastolic BFV, ABP, HR, and various vascular reactivity parameters were calculated.

3.3.TCD Examination Protocol

The blood flow velocity (BFV, cm/s) in both MCAs was recorded by TCD probes (2 MHz, DWL Multi-Dop T2, Sipplingen, Germany) in a semi-sitting position through the transtemporal window at a depth of 45–55 mm. During TCD measurements continuous, non-invasive, beat-to-beat arterial blood pressure (ABP) monitoring was performed by radial artery applanation tonometry (Colin-BP508, Hayashi Komaki Aichi, Japan). Heart rate was continuously monitored with electrocardiogram (ECG).

During the examination of patients, 3 vasoactive stimuli were applied in the following order:

1. HV/BH test
2. CCC test
3. VM

3.3.1. Hyperventilation and Breath Holding Tests

A combined stimulus was used to assess the CO_2 reactivity of cerebral vessels, with the aim of evaluating the maximal CO_2-dependent cerebral vascular response; therefore, voluntary hyperventilation and breath-holding was chosen as a simple, safe, and well-tolerated maneuver. In basal conditions (baseline, BL), parameters measured were recorded after 15 min adaptation period. After BL, patients performed the HV test for 30 s in a semi-sitting position and were asked to inhale deeply and exhale dynamically. At the beginning of the HV test, MCA MBFV values started to decrease until they reached their minimum value. Then, 30 s later, patients were asked to inhale and hold their breath as long as they could. During the BH test, MBFV values started to increase and reached their maximum. A detailed graphical illustration of the HV-BH test is shown in Figure 1. Patients raised their hands when they finished the BH test. Each patient had a different BH test time (average 42.3 s, SD: ± 21.67). A total of 31 patients met the best signal-to-noise ratio requirements (74.2% male, age: 68 ± 5.11 years, and 25.8% female, age: 71.5 ± 4.89 years). For the complex evaluation of the HV-BH tests, several parameters were defined. These variables consider not only the MBFV changes during the reactions but also the temporal- and cerebral arterial resistance (CAR) responses. CAR was defined by the ratio of systemic MABP and MBFV. (52),(88) (89) The parameters are defined below (In Table 2-3-4). Figure 7. represents the changes during HV-BH test.

3.3.1.1.Variables defining the Hyperventilation Test

The defined variables of the HV test are presented above in Table 2.

Table 2. *Summary of the variables of the HV test.*

Definition of indicator	Abbreviation	Description
Baseline Mean Blood Flow Velocity	$MBFV_{HVbl}$	average of MBFV values of MCAs for the 10 seconds preceding HV
Mean Blood Flow Velocity – minimum value	$MBFV_{HVmin}$	minimum value of MCA MBFV during HV
Mean Blood Flow Velocity – at the end of 30 sec Hyperventilation	$MBFV_{HV30}$	MCA MBFV value at the end of HV test
difference of Mean Blood Flow Velocity values regarding the HV test	$\Delta MBFV_{HV}$	the difference between MCA $MBFV_{BL}$ and $MBFV_{HVmin}$
Time-to-the minimum value of Mean Blood Flow Velocity during Hyperventilation	time-to-$MBFV_{HVmin}$	is the time passed from the beginning of HV until MCA $MBFV_{HVmin}$

3.3.1.2.Variables defining the Breath Holding Test

The defined variables of the BH test are summarized in Table 3.

Table 3. *Summary of the variables of the BH test.*

Definition of indicator	Abbreviation	Description
Mean Blood Flow Velocity values at the beginning of Breath Holding	$MBFV_{BHstart}$	MCA MBFV value at the beginning of the BH (same as $MBFV_{HV30}$
Mean Blood Flow Velocity values at the end of breath holding	$MBFV_{BHend}$	MCA MBFV value recorded at the end of the BH
difference of the Mean Blood Flow Velocity values regarding Breath Holding test	$\Delta MBFV_{BH}$	$\Delta MBFV_{BH}$ was calculated by the difference between MCA $MBFV_{BHend}$ - $MBFV_{BHstart}$
time to the baseline Mean Blood Flow Velocity values during Breath Holding	time to $MBFV_{BHbl}$	The time passed from MCA $MBFV_{BHstart}$ until it reaches the baseline MBFV value
time of Breath Holding	time of BH	The time passed from $MBFV_{BHstart}$ until $MBFV_{BHend}$
Breath Holding Index(90)	BHI	$BHI = \frac{(MBFVBHend - MBFVBHstart)}{MBFVBHstart} * 100 * \frac{1}{tBH}$.

3.3.1.3. Variables defining the cerebral arterial resistance (CAR) changes during Hyperventilation and Breath Holding Tests

The baseline CAR for HV/BH tests was determined by averaging the CAR values over the 10 seconds preceding the stimulus. Actual CAR values were subtracted from baseline ($CAR_{bl} - CAR_{actual}$), and the cumulative change in CAR until return to baseline CAR was determined by calculating the area under the curve (AUC) using the trapezoidal method.

$$\text{CAR-AUC} = \sum_{i=1}^{n} \frac{CAR_{i-1} + CAR_i}{2} * 0.5$$

The CAR values of these tests are presented in Table 4.

Table 4.: *Summary of the variables of the CAR values of HV-BH test*

Definition of indicator	Abbreviation	Description
Cerebral Arterial Resistance – baseline values	CAR_{bl}	average of CAR values for the 10 seconds preceding HV/BH test
Cerebral Arterial Resistance – maximal values	CAR_{max}	the maximum CAR value registered during the HV/BH test
Cerebral Arterial Resistance – minimal values	CAR_{min}	is the minimum CAR value registered during the HV/BH test
difference of CAR values regarding the maximum and minimum phases	ΔCAR	the difference between CAR_{max} and CAR_{min}
time from the baseline CAR values until the maximal CAR values	$CAR_{time\text{-}to\text{-}max}$	the time passed from CAR_{bl} until CAR_{maxHV}
time from the maximum CAR value until the minimal CAR value	$CAR_{time\text{-}to\text{-}min}$	the time passed from CAR_{max} to CAR_{min}
Cerebral Arterial Resistance Index during Hyperventilation	$CARI_{HV}$	$CARI - HV = \frac{(CARmaxHV - CARbl)}{(CARBL)} * 100 * \frac{1}{30}$
Cerebral Arterial Resistance Index during Breath Holding	$CARI_{BH}$	$CARI - BH = \frac{(CARmin - CARmax)}{(CARmax)} * 100 * \frac{1}{tBH}$

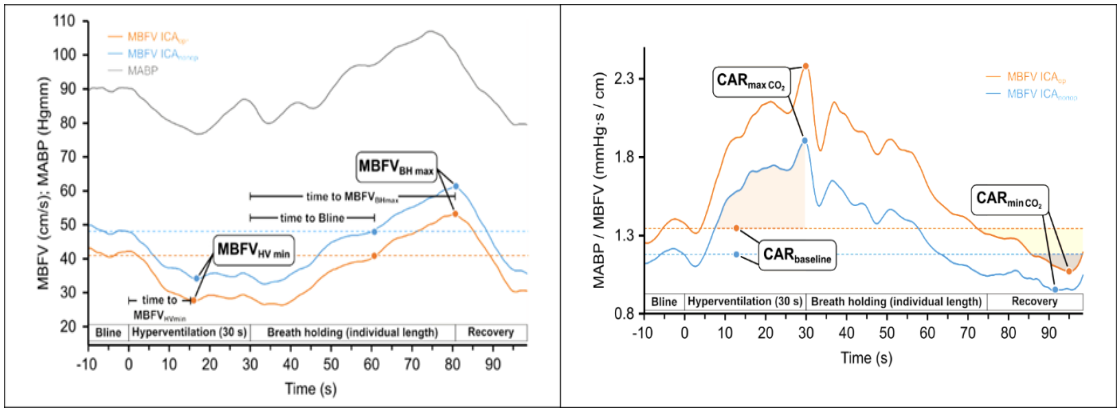


Figure 7: *Original records of changes in the mean blood flow velocities (MBFV) measured in the middle cerebral artery (MCA) in patients with unilateral carotid stenosis during hyperventilation and breath holding tests. The MBFV decreased during hyperventilation, whereas increased during breath holding test, indicating that changes in blood CO_2 level elicited the expected changes in cerebrovascular resistance. The blue line indicates the side of ICA_{nonop}, whereas the yellow line indicates the side ICA_{op}. The minimum and maximum values of MBFV are indicated together with time characteristics. It can be seen, that during the whole response the MBFV was substantially lower (with about 10 cm/sec) in the severe stenotic side compared to the other side and followed the changes in blood pressure. The changes in mean blood pressure are indicated with grey line.*

Changes in the calculated cerebral arterial resistance (CAR: MAPB/MBFV) of both hemisphere during hyperventilation test as a function of time, indicates cerebral vasoconstriction and during breath holding test indicates cerebral vasodilation, respectively. The areas with darker colors represent the Aure Under

3.3.2. Common Carotid artery Compression Test

The CCC test was performed after a carotid duplex ultrasound (CDS) examination. Prior to the examination, high-risk, rupture-prone plaques were screened out to ensure the examination was safe. (91), (92), (93), (94) In case of extensive CCA atherosclerosis, the carotid compression test was omitted due to the risk of cerebral embolization. Since previous publications confirmed that 10 s were required to achieve the maximal MCA BFV response, we used 10 s of manual compression of the CCA. (91) During the maneuver, bilateral MCA blood flow velocity values were measured with TCD. The CCC test was performed on both sides, one by one. Compression was performed 3 times on both CCAs, with a 2 min interval between each maneuver. For the statistical analysis, the technically best and largest amplitude reactions were selected.

3.3.2.1. Variables defining the CCC Test

Table 5. represents the variables characterizing the CCC test:

Table 5. *Variables of the CCC test*

Definition of indicator	Abbreviation	Description
Return to baseline	RTB	the time passed from the release of the carotid compression until the MCA velocity values reached its baseline.
Transient Hyperemic Response Ratio (57)	THRR	.THRR was defined from the MBFV – $THRR_{MBFV}$, and peak systolic velocity (PSV) - $THRR_{PSV}$ values.[15] $THRR=\frac{(F3-F1)}{F1}$, where F1 is the baseline BFV, and F3 means the BFV value just after the release of the compression
Cerebral Arterial Resistance Values – Area Under the Curve during CCC test	CAR_{CCCAUC}:	the cumulative change in CAR until return to baseline CAR was determined by calculating the area under the curve (AUC) using the so-called trapezoidal method mentioned before

CAR_{CCCAUC}: The baseline CAR for CCC tests was determined by averaging the CAR values over the 10 seconds preceding the stimulus. CAR could not be calculated during

carotid compression because the perfusion pressure required to maintain MCA MBFV could not be measured. After carotid compression was released, actual CAR values were subtracted from baseline ($CAR_{BL} - CAR_{actual}$), and the cumulative change in CAR until return to baseline CAR was determined by calculating the area under the curve (AUC) using the so-called trapezoidal method mentioned before (illustrated on Figure 8). The CAR AUC of the ICA_{op} and ICA_{nonop} sides was compared statistically.

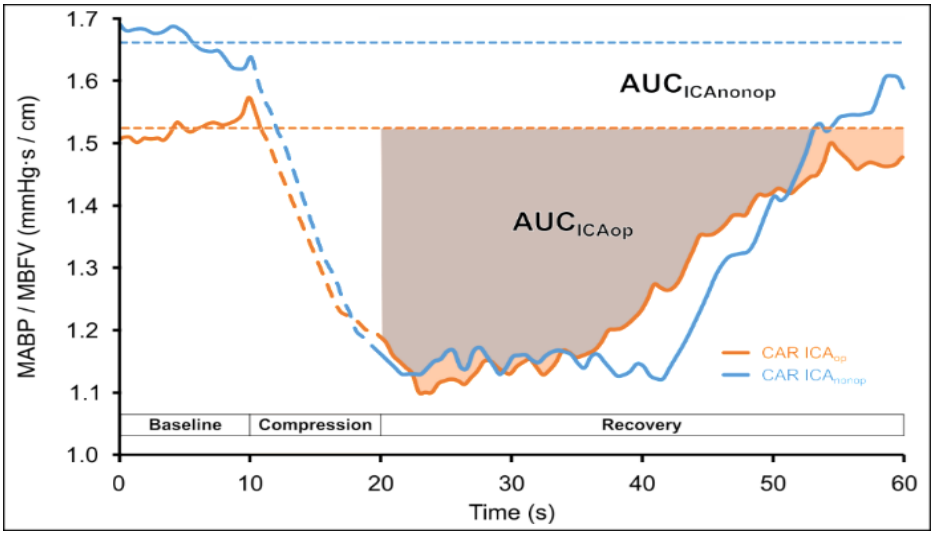


Figure 8: *Changes in the calculated cerebral arterial resistance (CAR: MAPB/MBFV) during common carotid artery compression (CCC) test in a patient with unilateral carotid stenosis as a function of time. Carotid compression resulted in cerebral hypoperfusion, which elicited dilation in the arterial vessels supplied by the middle cerebral artery (MCA), thus CAR decreases. After the compression is released, the CAR gradually returns to baseline. Area under the curve (AUC) was calculated by the trapezoid method until the baseline-subtracted CAR values returned to zero. The areas with darker colors represent the Aure Under the Curve. AUC of ICA_{op} is smaller than the contralateral one.*

3.3.3. Valsalva Maneuver

The duration of the VM was standardized to 15 s. VM was performed with forced exhalation against a sphygmomanometer by maintaining their expiratory pressure at 40 mmHg, which was controlled by the patient and the assistant as well. Recordings of those patients were used, which had an adequate signal-to-noise ratio and were thus suitable for complex signal analysis. As presented above, the VM is a complex reaction which is suitable for the evaluation of CVR and CANS function. VM elicits considerable changes in ABP and BFV in cerebral vessels such as the MCA. The responses to VM can be divided into four phases. Table 6. represents the variables characterizing the VM, while Figure 9. represents the VM.

3.3.3.1. Variables used to characterize cerebrovascular reactivity of the Valsalva Maneuver

Variables of the VM are summarized above in Table 6.

Table 6. *Summary of the CAR variables of the hyperventilation and breath-holding tests.*

Definition of indicator	Abbreviation	Description
Centro-Peripheral Valsalva Ratio	CPRV	reflects the changes regarding the MFBV and MABP between phases 2b and 3 (95) $CPVR=\frac{MBFV3-MBFV2b}{MABP3-MABP2b}$
Centro-Peripheral Overshoot Index	CPOI	based on the CPVR we defined the CPOI which refers to the difference between the OS and BL values of the mean BFV and in the denominator is the difference between the OS and BL values of the MABP $CPOI=\frac{(MBFVos-MBFVbl)}{(MABPos-MABPbl)}$
Cerebrovascular Valsalva Ratio	CVAR	expresses the increase in mean BFV in phase 2b compared to the phase3 (95), (96) $CVAR=\frac{(MBFV3-MBFV2b)}{(t3-t2b)}$
Time to phase 2b of Valsalva Maneuver	time-to-2b	temporal variables refer to the time elapsed until the phase 2b
Time to phase overshoot of Valsalva Maneuver	time-to-OS	temporal variables refer to the time elapsed until the phase OS

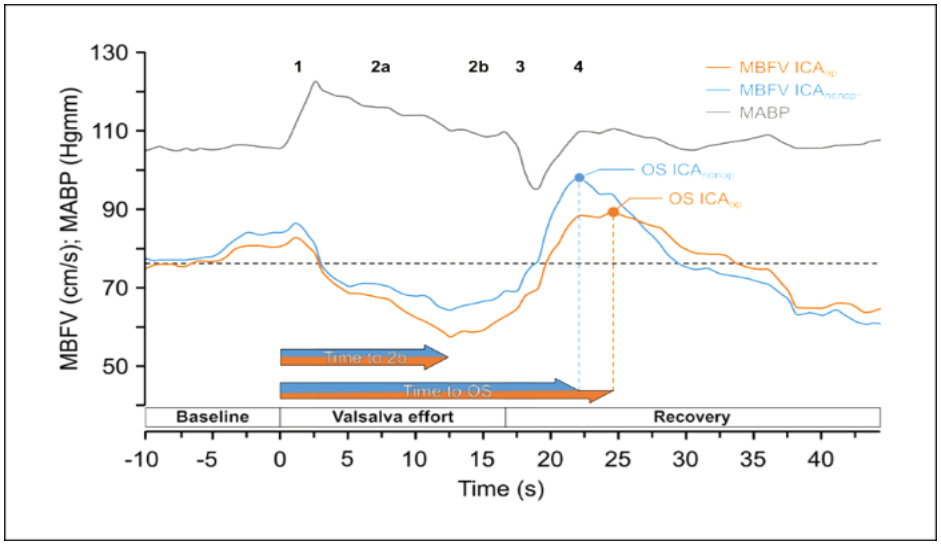


Figure 9.: *Original recording of changes in the mean blood flow velocities (MBFV) measured in the middle cerebral artery (MCA) in patients with unilateral carotid stenosis during Valsalva Maneuver (VM) as a function of time. There were considerable changes in the mean arterial blood pressure (MABP) yet changes in MBFV in the middle cerebral arteries (MCAs) were different in the two sides. Changes were divided into four phases: there was side difference of MBFV values during phase 2a and 2b and a time shift of maximal values between MBFV overshoot (OS) in phase 4. The changes in mean blood pressure are indicated with grey line.*

3.3.3.2. Variables used to characterize cardiovascular autonomic nervous system dysfunction of the Valsalva Maneuver

The variables of CANS dysfunction calculated from the VM are presented in Table 7.

Table 7. *Variables of the CANS dysfunction regarding VM.*

Definition of indicator	Abbreviation	Description
Sympathetic Index	SI	expresses the percentage increase of the MABP measured at the end of phase 2 (MABP 2end) compared to the initial baseline MABP (MABP BL). Normal value of SI is ≥ 0. Based on the SI, patients with cardiovascular ANS dysfunction were defined with a negative SI value. (64), (97) $SI = \frac{(MABP\ IIend - MABP\ BL)}{MBP\ BL} * 100$
Pressure Recovery Time	PRT	is defined as a time interval (in seconds) that starts when the systolic ABP is lowest in phase 3 (t1) and ends when the systolic ABP reaches the BL value again in phase 4 (t2). The normal value of PRT is < 4 seconds. Based on the PRT, patients with a PRT longer than four seconds were considered to have cardiovascular ANS dysfunction. (97), (98) $PRT = t_2 - t_1$
Valsalva Heart Rate Ratio	VHRR	is defined as the maximum heart rate (HRmax) during the maneuver divided by the lowest heart rate (HRmin) obtained within 30 seconds after the peak heart rate. The normal value of VHRR over 60 years is >1.35. Patients with a VHRR less than 1.35 were considered having cardiovascular ANS dysfunction. (65),(97) $VHRR = \frac{HRmax}{HRmin}$

3.4.Statistical analysis

Statistical analysis was performed with TIBCO Statistica® 13.5.0 program and the R programming language version 4.4.1. Data was visualized using Inkscape 1.4. Software and the R programming language version 4.4.1. The significance level was defined as $p<0.05$ for all tests.

3.4.1. Statistical analysis of the comparison of the results of the functional tests

To examine the impact of ICAS on CVR the variables of the four stimuli of the side to be operated on (ICA_{op}) and not to be operated on (ICA_{nonop}) were compared. Additionally, grouped comparisons were also performed by dividing the side not to be operated into two groups:

subgroup 1: where the stenosis of the ICA was estimated to be equal to or above 70%.

subgroup 2: a group where the stenosis was less than 70%.

The Wilcoxon Rank Test was used as the main tool due to the low sample size and non-normal distribution of variables. Since almost all time-to-event variables were free from censoring, they were also compared using the above tests, however, they were evaluated using clustered Cox proportional hazards models too.

3.4.2. Statistical analysis of the correlation of cerebrovascular reactivity and cerebrovascular autonomic nervous system dysfunction

The CVR variables measured on the ICA_{op} side with autonomic variables of the VM were correlated to evaluate the relationship between CVR impairment and cardiovascular autonomic dysfunction in patients with ICAS. The parameters characterizing CVR, calculated based on the CCC, HV-BH, and VM tests, were correlated with the indices indicating cardiovascular autonomic function (SI, PRT, and VHRR). The availability of paired data allowed for correlation of the CVR indices from the CCC test, the HV-BH test, and the VM with the autonomic indices de-rived from the VM. The number of elements of the correlations (n) is shown in Tables 8–10.

Pairwise Spearman's and canonical correlation analyses were used to quantify the relationship between variables. Canonical correlation analysis is a statistical method used for assessing and maximizing correlations between two sets of variables. Only those CVR variables were kept for canonical correlation analysis that had pair-wise correlations above or equal to 0.3 (in absolute value) with at least one autonomic index. In addition, CVR variables with <30 observations were excluded. The statistical significance of the canonical correlation coefficients was tested using the F-approximation (asymptotic) of the Pillai–Bartlett trace.

4. Results

4.1. Results of the complex analysis of the cerebrovascular reactivity – comparison of the results of the functional tests regarding the ICA_{op} and ICA_{nonop} sides.

Table 8-9-10. summarizes the differences of the measured indices of the patients. The graphical illustration of the results is presented in Figure 10-12.

4.1.1. Hyperventilation and Breath Holding Tests

The time to $MBFV_{HVmin}$ was significantly shorter on the ICA_{op} side (Wilcoxon Ranked Test p=0.012) compared to the contralateral side. According to sub-group analysis, the difference was only justified if the stenosis on the ICA_{nonop} side did not reach 70% (Wilcoxon Ranked Test p=0.0088). In response to the BH test original record shows no differences in the response of the two MCA sides except for subgroup-2 analysis regarding the ΔMBFVBH (p=0.042). To assess the changes in the resistance of cerebral arterial vessels during these tests, CAR values were calculated as well. Data included in Table 9. shows no significant differences regarding the CAR values on the two MCA sides.

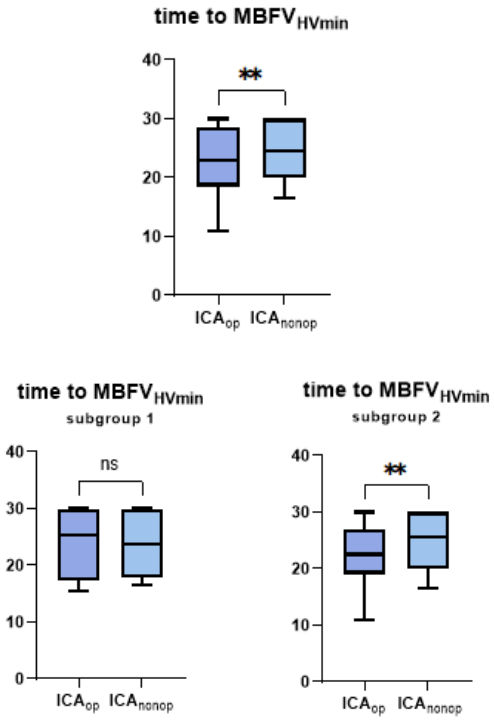


Figure 10: *Illustration of the results of the HV and BH tests. (Wilcoxon Ranked Test).*

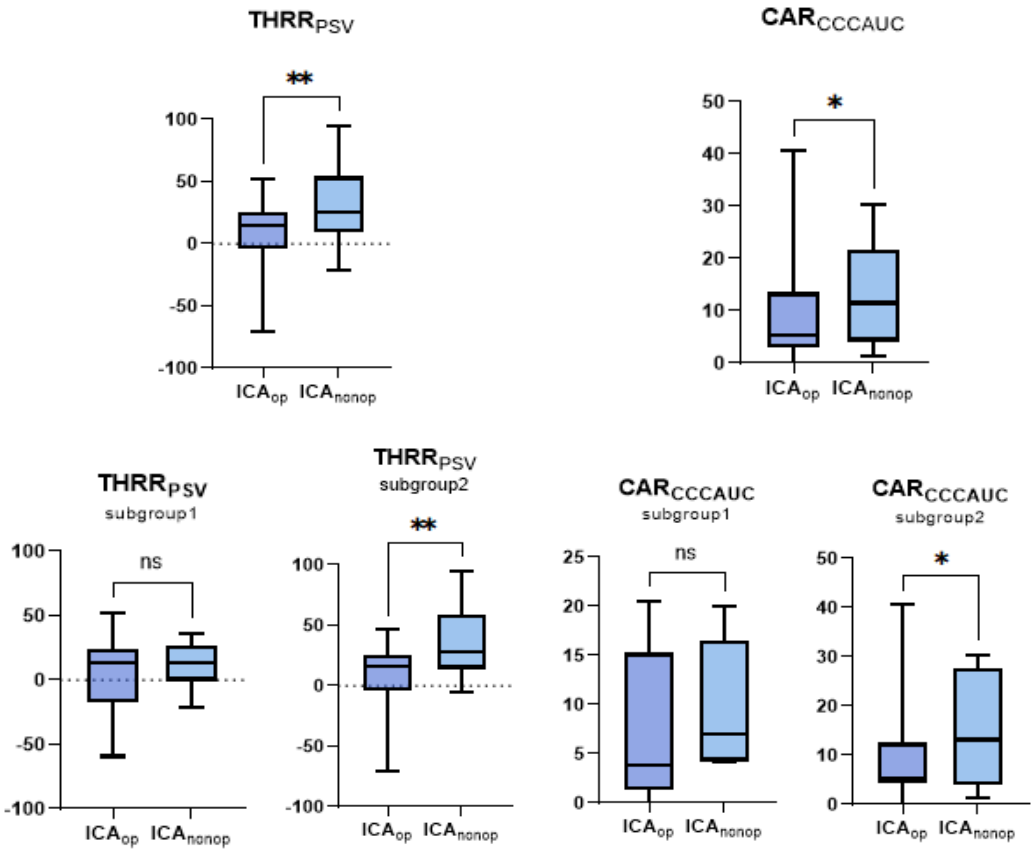
Table 8. *Summary of differences in the measured parameters and calculated indices of the Hyperventilation and Breath Holding test of 31 patients. Values are mean ±SD using Wilcoxon Ranked Test (p<0,05). Significant differences are indicated in bold letter.*

Variables	**ICA_{op} (mean +SD median, IQR)**	**ICA_{nonop} (mean +SD median IQR)**	**p value**
$MBFV_{HVbl}$	52.21 ± 13.86 cm/s 52.72 [42.11, 60.96]	53.54 ± 14.11 cm/s 49.37 [43.39, 63.06]	0.81
$MBFV_{HVmin}$	33.60 ± 9.60 cm/s 34.23 [26.80, 38.99]	33.30 ± 9.80 cm/s 31.52 [27.56, 39.59]	0.69
$\Delta MBFV_{HV}$	18.61 ± 7.74 cm/s 18.49 [12.87, 23.46]	20.24 ± 8.19 cm/s 19.09 [14.30, 24.86]	0.6
$MBFV_{HV30s}$	34.72 ± 9.93 cm/s 34.38 [28.52, 41.69]	35.16 ± 9.44 cm/s 32.83 [28.95, 40.85]	0.94
time to $MBFV_{HVmin}$	**22.98 ± 5.36 s 23.00 [18.75, 27.75]**	**24.66 ± 5.08 s 24.50 [20.25, 30.00]**	**0.012***
HVI	-1.10 ± 0.32 -1.03 [-1.22, -0.89]	-1.12 ± 0.34 -1.13 [-1.25, -0.95]	0.64
$MBFV_{BHstart}$	34.72 ± 9.93 cm/s 34.38 [28.52, 41.69]	35.11 ± 9.48 cm/s 32.83 [28.73, 40.84]	0.97
$MBFV_{BHend}$	60.16 ± 15.99 cm/s 62.60 [50.40, 66.82]	63.75 ± 19.10 cm/s 61.18 [47.71, 70.92]	0.26
$\Delta MBFV_{BH}$	25.44 ± 9.77 cm/s 23.89 [18.64, 29.25]	28.65 ± 13.4 cm/s 25.91 [16.20, 35.84]	0.08
time to $MBFV_{BHbl}$	29.42 ± 14.65 s 24.00 [19.00, 37.00]	30.79 ± 14.64 s 27.50 [20.50, 40.75]	0.85
BHI	2.35 ± 1.87 1.74 [1.32, 2.63]	2.63 ± 2.60 1.84 [1.41, 2.48]	0.32
CAR_{bl}	2.02 ± 0.59 mmHg*s/cm 1.96 [1.64, 2.34]	1.93 ± 0.54 mmHg*s/cm 1.84 [1.51, 2.32]	0.53
CAR_{maxHV}	2.98 ± 1.00 mmHg*s/cm 3.06 [2.33, 3.73]	2.96 ± 1.16 mmHg*s/cm 2.77 [2.21, 3.41]	0.81
CAR_{minBH}	1.75 ± 0.51 mmHg*s/cm 1.74 [1.39, 2.07]	1.66 ± 0.50 mmHg*s/cm 1.65 [1.27, 1.90]	0.31

ΔCAR	1.22 ± 0.63 mmHg*s/cm 1.10 [0.83, 1.68]	1.30 ± 0.92 mmHg*s/cm 1.05 [0.84, 1.36]	0.75
$CAR_{time\text{-}to\text{-}max}$	27.53 ± 11.03 s 29.00 [18.75, 35.25]	27.37 ± 10.19 s 29.00 [17.50, 33.25]	0.43
$CAR_{time\text{-}to\text{-}min}$	50.05 ± 22.40 s 47.00 [34.75, 64.50]	54.05 ± 22.55 s 52.50 [39.50, 72.75]	0.11
$CARI_{HV}$	1.56 ± 0.93 1.39 [1.06, 1.90]	1.72 ± 1.10 1.45 [1.08, 1.90]	0.29
$CARI_{BH}$	-1.19 ± 0.70 -1.03 [-1.34, -0.71]	-1.21 ± 0.70 -0.99 [-1.35, -0.70]	0.43
CAR_{HVAUC}	15.75 ± 12.01 mmHg*s/cm 13.29 [7.21, 21.13]	14.45 ± 9.05 mmHg*s/cm 11.71 [9.40, 17.54]	0.8
CAR_{BHAUC}	4.05 ± 4.89 mmHg*s/cm 2.24 [0.73, 5.43]	5.00 ± 4.86 mmHg*s/cm 3.14 [1.15, 7.45]	0.36

4.1.2. Common Carotid artery Compression Test

The value of $THRR_{PSV}$ was significantly higher on the ICA_{nonop} side (Wilcoxon Ranked Test p=0.0046 and p=0.0033 according to the subgroup-2 analysis). It has also been verified previously that the PSV values might be more sensitive when quantifying the CCC test. The calculated arterial resistance, CCC_{CARAUC}, was significantly lower on the ICA_{op} side (Wilcoxon Ranked Test p=0.021 and p=0.039 according to the subgroup-2 analysis) compared to the ICA_{nonop} side. The BL values of the PSV were significantly different between the two sides; thus, relative values were quantified.



Figre 11: *Graphical Illustration of the resultst of the CCC test (Wilcoxon Ranked Test).*

Table 9. *Summary of differences in the measured parameters and calculated indices in case of 26 patients of the Common Carotid artery compression test. Values are mean ±SD using Wilcoxon Ranked Test (p<0,05). Significant differences are indicated in bold letter.*

Variables	**ICA_{op} (mean +SD Median, IQR)**	**ICA_{nonop} (mean +SD Median, IQR)**	p value
RTB	19. 35 ± 14.72 s 14.50 [8.00, 33.50]	18.15 ± 12.22 s 17.00 [9.88, 22.25]	0.94
$THRR_{MBFV}$	0.12 ± 0.17 0.12 [-0.02, 0.25]	0.20 ± 0.25 0.22 [0.02, 0.35]	0.13
$THRR_{PSV}$	**8.80 ± 28.17** **14.43 [-3.53, 24.96]**	**31.07 ± 29.04** **25.00 [10.89, 49.34]**	**0.0046***
CAR_{CCCAUC}	**4.46 ± 4.64** **2.65 [1.84, 5.99]**	**6.46 ± 5.13** **4.97 [2.05, 10.22]**	**0.021***

4.1.3. Valsalva Maneuver

CVAR had a significantly lower value on the ICA_{op} side. (Wilcoxon Ranked Test p=0.002 and p=0.014 according to the subgroup-2 analysis) and time-to-OS was significantly longer on the ICA_{op} side (both according to Wilcoxon Ranked Test with p=0.015; p=0.038 according to the subgroup-2 analysis) and the Wald test of the Cox proportional hazards model with p<0.0001. Evaluating CANS values, we found that 15 patients had a VHRR value less than 1.35, 8 patients had a longer PRT than 4 sec and 23 patients had an SI below 0.

Figure 12: *Graphical Illustration of the Results of the CCC test (Wilcoxon Ranked Test).*

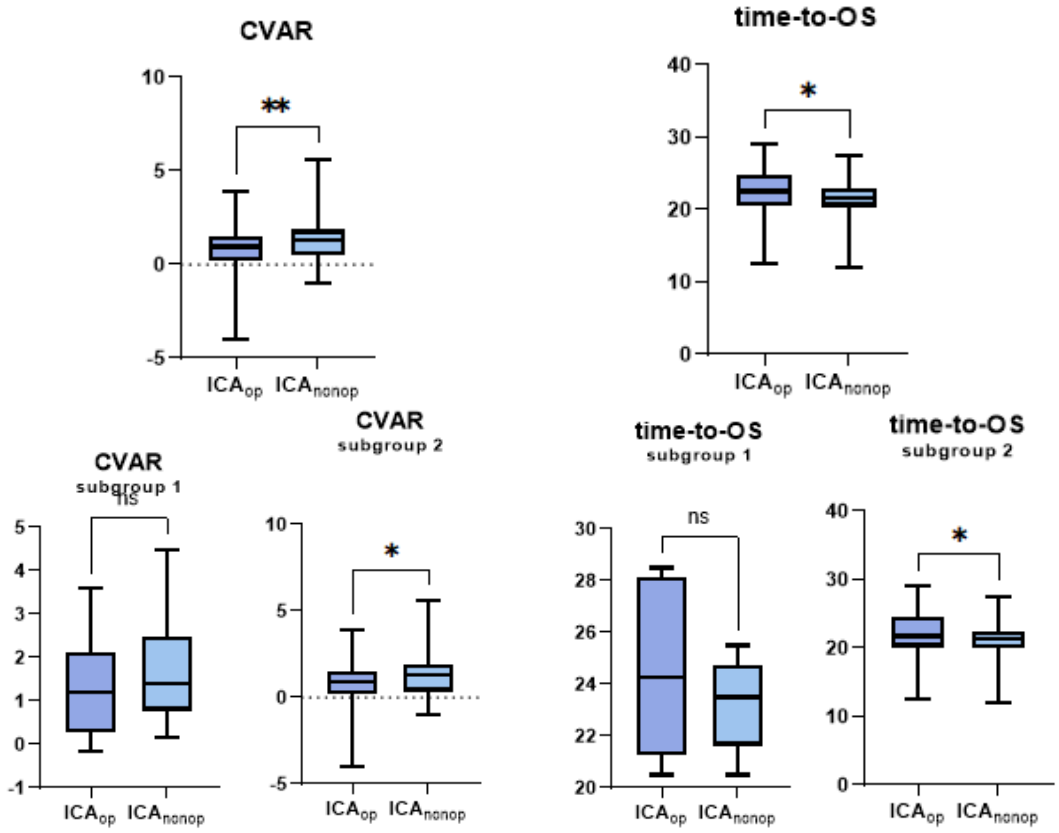


Table 10. *Summary of differences in the measured parameters and calculated indices of the Valsalva Maneuver (VM) in case of 34 patients. Values are mean ±SD using Wilcoxon Ranked Test (p<0,05). Significant differences are indicated in bold letter.*

Variables	ICA_{op} (mean +SD)	ICA_{nonop} (mean +SD)	p value
CPRV	0.20 ± 7.84 -0.05 [-0.10, -0.01]	-1.57 ± 5.93 -0.03 [-0.06, 0.00]	0.5
CVAR	**0.91 ± 1.33** **0.10 [-1.08, 1.58]**	**1.47 ± 1.43** **-0.24 [-2.13, 1.51]**	**0.002***
CPOI	1.57 ± 6.94 -0.08 [-0.11, -0.03]	1.42 ± 6.87 -0.03 [-0.11, 0.01]	0.72
time-to-2b	8.18 ± 2.69 s 0.92 [0.45, 2.22]	8.15 ± 2.70 s 1.13 [0.50, 3.14]	0.86
time-to-OS	**22.68 ± 3.54 s** **0.97 [0.41, 1.51]**	**21.49 ± 2.80 s** **1.28 [0.60, 1.87]**	**0.015***

4.2. Results of the correlation of cerebrovascular reactivity and cerebrovascular autonomic nervous system dysfunction

4.2.1. Pairwise Spearman's Correlations

Using the established cut-off values for the cardiovascular autonomic indices from the VM, an abnormal SI was found in 23 patients, VHRR in 15 patients, and PRT in 8 patients. Several CVR indices of the three functional tests have been described previously. The results of Spearman's pairwise correlation analysis of the CVR in-dices, which showed significant correlation with the cardiovascular autonomic indices of VM, are summarized in Table 11.

4.2.1.1. Correlation Between Changes in CVR in Response to CCC Test and CANS Indices

The indices of the CCC test described previously were correlated with the CANS indices of the VM. There was no significant correlation observed with any of them.

Table 11. *Results of Spearman's pairwise correlation test ($p < 0.05$) between CANS and CVR indices of CCC. The "n" columns list the number of patients available for the given pairwise correlation analysis.*

CVR Indices	VHRR	n	SI	n	PRT	n
	(Spearman's Correlation Coefficient; *p*-Value)					
RTB_{ICAop}	0.046; 0.876	14	0.307; 0.265	15	0.169; 0.564	14
$THRR_{PSVICAop}$	−0.279; 0.277	17	0.121; 0.633	18	−0.028; 0.918	17
$THRR_{MBFVICAop}$	−0.125; 0.633	17	−0.176; 0.484	18	0.092; 0.735	16

4.2.1.2.Correlation Between HV-BH Test CVR and CANS Indices

A significant correlation was found between $CAR_{timetomaxICAop}$ and VHRR ($p = 0.041$), while a positive, significant correlation was observed between $CAR_{timetominICAop}$ and SI ($p = 0.019$).

Table 12. *Results of Spearman's pairwise correlation test ($p < 0.05$) between CANS and CVR indices of HV-BH. Significant differences ($p < 0.05$) are indicated in bold numbers. The "n" columns list the number of patients available for the given pairwise correlation analysis.*

CVR Indices	VHRR	n	SI	n	PRT	n
	(Spearman's Correlation Coefficient; *p*-Value)					
HVI_{ICAop}	−0.192; 0.404	21	0.045; 0.834	24	−0.285; 0.211	21
$timetoMBFV_{HVminICAop}$	0.163; 0.481	21	−0.032; 0.881	24	−0.013; 0.954	21
BHI_{ICAop}	0.081; 0.729	21	−0.369; 0.076	24	0.108; 0.640	21
$CAR_{timetomaxICAop}$	**0.450; 0.041**	21	−0.027; 0.900	24	−0.243; 0.288	21
$CAR_{timetominICAop}$	0.081; 0.729	21	**0.474; 0.019**	24	0.025; 0.913	21

4.2.1.3.Correlation Between VM CVR and CANS Indices

A significant correlation was found between $CVAR_{ICAop}$ and PRT ($p = 0.002$).

Table 13. *Results of Spearman's pairwise correlation test ($p < 0.05$) between CANS and CVR indices of the VM. Significant differences ($p < 0.05$) are indicated in bold numbers. The "n" columns list the number of patients available for the given pairwise correlation analysis.*

CVR Indices	VHRR	n	SI	n	PRT	n
	(Spearman's Correlation Coefficient; *p*-Value)					
time to $2b_{ICAop}$	−0.109; 0.565	30	0.187; 0.297	33	0.144; 0.439	31
time to OS_{ICAop}	−0.219; 0.244	30	0.026; 0.886	33	0.244; 0.185	31
$CPOI_{ICAop}$	−0.008; 0.966	29	0.133; 0.467	32	0.340; 0.061	31
$CPVR_{ICAop}$	0.046; 0.807	30	0.105; 0.562	33	−0.164; 0.379	31
$CVAR_{ICAop}$	−0.172; 0.373	29	0.230; 0.205	32	**−0.529; 0.002**	31

4.2.2. Canonical Correlation Analysis

The following eight reactivity indices satisfied the pairwise correlation and sample size requirements: time to $_{MBFVBHbl\text{-}ICAop}$, BHI_{ICAop}, $CAR_{max\text{-}ICAop}$, $CAR_{timetomax\text{-}ICAop}$, $CAR_{timetomin\text{-}ICAop}$, $CAR_{AUCHV\text{-}ICAop}$, $CPOI_{ICAop}$, and $CVAR_{ICAop}$. In total, 17 patients were included in the canonical correlation analysis, as complete cases were required across all variables in both the CVR and autonomic domains. Table 5 lists the canonical correlation coefficients and related test results for all three canonical variates (in each set of variables—CVR or CANS indices). The first line shows the coefficient for the first canonical variate and the significance of all three canonical variates taken together; the second shows the coefficient for the second variate and the combined significance for the second and third variates; the last line shows the third variate alone.

The first canonical variate for the reactive set was mostly determined by $CAR_{time\text{-}tomin\text{-}ICAop}$ and $CAR_{timetomax\text{-}ICAop}$. The second canonical variate was mostly determined by the same two indices in the opposite direction and by $CAR_{HVAUC\text{-}ICAop}$. The first variate from the autonomic set was mostly determined by PRT and SI. For the second variate, SI was dominant. The canonical correlation coefficient was 0.782 between the first sets of autonomic and reactive variates. For the second canonical variate, the

canonical correlation was somewhat lower at 0.725. While relatively strong correlations were observed for these (above 0.7), none were significant.

Table 14. Canonical Correlation Coefficients

Canonical variates	Correlation	Test statistic	p-value
1-3	0.782	1.585	0.392
2-3	0.725	0.973	0.454
3	0.668	0.447	0.410

5. Discussion

In the present thesis the findings of two separate yet closely interrelated studies were presented, building upon these established insights:

5.1. Interpretation of the comprehensive characterization of cerebrovascular reactivity

To assess cerebral vascular reactivity in a complex manner, well-known vasoactive stimuli were used that are based on vascular reactivity induced by CO_2 and pressure-flow changes. Comprehensive protocol was used to assess cerebrovascular regulatory impairments.

The main findings of the first examination in patients with significant internal carotid artery stenosis are the following:

1) First, the CO_2 changes induced by hyperventilation and breath-holding elicited similar responses in MBFV on both carotid/MCA sides (i.e., ICA_{op} and ICA_{nonop}). The only significant differences were found between the two sides in the time to reach the minimum MBFV of the ipsilateral MCA induced by hyperventilation and $\Delta MBFV_{BH}$ in subgroup 2.

2) The reactive hyperemic blood flow velocity responses elicited by CCC tests were significantly different in the two carotid/MCA vascular regions. Two indices of response to CCC were significantly lower on the planned operated side (ICA_{op}) compared to the other side (ICA_{nonop}).

    a. the Transient Hyperemic Response Ratio calculated from MCA peak systolic blood flow velocity values
    b. the cumulative change in cerebral arterial resistance (CAR_{AUC}) after cessation of common carotid artery compression.

3) Valsalva Maneuver also induced different flow changes in the two carotid/MCA sides. Cerebrovascular Valsalva Ratio, and the time to overshoot phase of the

Valsalva Maneuver were significantly reduced on the side to be operated on (ICA_{op}) compared to the other side (ICA_{nonop}).

5.1.1. The Clinical Importance of Comprehensive Analysis

5.1.1.1. CO_2 Reactivity (Hyperventilation and Breath-Holding)

In the cerebral vasoreactivity studies included in a metaanalysis presented in the approved Guideline of the European Society of Vascular Surgery (ESVS) CVR was determined based on changes in cerebral blood flow velocity induced by increasing blood CO_2 concentration or intravenous administration of acetazolamide, evaluating the maximal cerebral vasodilation capacity.(99)

In the protocol of the present study, using hyperventilation (HV) and breath-holding (BH) stimuli the total CO_2 reactivity was calculated, which represents a novel approach for evaluating the overall CO_2 reactivity of cerebral resistance vessels.

In the patient population studied the only difference was found in the magnitude of CO_2-decrease–induced reactivity between the ICA_{op} and ICA_{nonop} sides during hyperventilation: the minimum value of the MBFV occurred earlier on the ICA_{op} side. This difference can indicate reduced cerebral vasoconstriction capacity on the ICA_{op} side.

It is of note that the BH index calculated from the MBFV data of patients showed a higher value than that of healthy individuals of the same age reported in a previous publication.(100) This is probably due to the fact that during the BH test, the arterial blood pressure of patients involved in our study significantly increased, and in parallel, their cerebral blood flow velocity increased partially, as a function of systemic blood pressure, indicating an impairment of the CVR. Thus, we recommend that the simultaneous change in arterial blood pressure should be considered when establishing the BH index and that the CO_2 vasodilation reactivity be characterized by the resistance index calculated in this way.

5.1.1.2. Pressure–Flow induced Cerebral Vasoreactivity

The response of cerebral arterial vessels to changes in cerebral perfusion pressure/flow was assessed by the CCC test and the Valsalva Maneuver.

5.1.1.2.1. Common Carotid artery Compression Test

The CCC test estimates the effectiveness of cerebral pressure–flow regulation by temporarily reducing the ipsilateral MCA perfusion.(56) If, during CCC, the 10 s hemispheric cerebral low perfusion reaches a critical level, focal deficit symptoms may appear(93), but we did not observe this in the present study, confirming the safety of the CCC test.

In response to CCC, the calculated $THRR_{PSV}$ index was significantly lower on the ICA_{op} side. During compression, ipsilateral MCA perfusion was reduced which was indicated by the decreased MBFV. This elicited (passive and active) vasomotor response of cerebral resistance vessels, indicated by the cerebral arterial resistance change. After the compression was released, a transient increase in MCA MBFV was observed, and then CAR was lower than the baseline value preceding CCC, which may be due to the dilation of cerebral resistance vessels of MCA territory. The cumulative change in the cerebral arterial resistance (CAR_{CCCAUC}) was significantly smaller on the side of the ICA_{op} side, suggesting that the dilation capacity of resistance vessels was reduced. Thus, the index which was introduced in the present study seems to be sensitive to hemodynamic disturbances resulting from severe carotid stenosis.

5.1.1.2.2. Valsalva Maneuver

Several indices have previously proposed to evaluate cerebrovascular reactivity induced by the VM. (65), (95),(96), (98) In our examinations besides the defined parameters in previous studies new indices were also defined, such as time-to-2b and time-to-OS. As a result, CVAR was significantly lower on the ICA_{op} side. In addition, there was a difference in the OS time, because OS occurred later the ICA_{op} than on the contralateral side; thus, this new temporal variable may also be a suitable index for characterizing the dynamics of cerebrovascular reactivity.

5.1.2. The importance of a complex TCD Protocol

In clinical practice, the most widely used and proven methods for determining cerebrovascular reactivity are the CO_2 reactivity tests and the breath-holding index. However, when this index is calculated, changes in arterial blood pressure are not taken into consideration, since, based on the prevailing assumption, no significant

hemodynamic changes occur during the test. However, the data of the present study showed that changes in arterial blood pressure are not negligible during these tests. Thus, changes in cerebral blood flow velocity must be "normalized" to changes in arterial blood pressure to obtain a correct evaluation of changes in CVR, which would be very important to develop a more proper preoperative ischemic risk assessment.

The patients involved in the present study had severe carotid artery stenosis, which can cause reduced cerebral vasoreactivity, and were awaiting reconstructive carotid vascular surgery. During the operation, the carotid arteries are clamped, eliciting a reduction in brain perfusion (intraoperative cerebral hypoperfusion). That is why we added hemodynamic stimuli to the preoperative assessment of cerebrovascular reactivity tests.

The results of the measurements and detailed analysis of data confirmed that the CCC and VM tests, which induce significant changes in pressure/flow in the cerebral arteries, were different between the two sides, providing important information regarding the preoperative cerebral ischemic risk.

The VM and CCC tests are eliciting fast and considerable hemodynamic changes, i.e., the levels of blood pressure and flow to the brain. In contrast, during the HV/BH test, cerebral hemodynamic conditions remain relatively stable; thus, changes in cerebrovascular reactivity are predominantly due to changes in arterial CO_2 concentration. As we have shown, almost all members of our patient population were hypertensive; in addition, they had other diseases, such as diabetes, and harmful habits like smoking, which may have also impaired cerebrovascular function. In the present study, according to the data of comparative statistical analysis, we conclude that CO_2 sensitivity of cerebral vessels is maintained even in severe ICAS: thus, an assessment based only on CO_2 tests does not provide sufficient information about the hemodynamic effects of severe carotid stenosis.

In the present study, however, the responses to hemodynamic changes elicited by CCC and VM tests were prolonged on the ICA_{op} side compared to the ICA_{nonop} side. Thus, the inclusion of CCC and VM tests in the evaluation of patients might be important to provide a more complex assessment of cerebrovascular reactivity in patients with ICAS.

Overall, a complex testing protocol that includes tests based on pressure–flow changes in addition to CO_2 reactivity tests might be beneficial.

5.2. Interpretation of the results of the correlation of CVR and CANS impairment

These results of the first study suggested that most patients studied also had cardiovascular autonomic dysfunction, which, in addition to impaired cerebral vasomotor regulation, may further increase the risk of cerebral ischemia, especially during potential hypotension during carotid reconstruction surgery/intervention. To evaluate the pre-operative status of the patients before undergoing CEA, we correlated the results of the autonomic indices calculated from the Valsalva Maneuver with the parameters defining the previously detailed vasoactive stimuli.

5.2.1. Results of the Common Carotid Artery Compression Test

Correlation tests with the variables of cerebral vasoreactivity of the common carotid artery compression (CCC) test and the autonomic indices of the VM did not demonstrate significant correlation. This finding may be explained by the fact that the response to the CCC stimulus is significantly influenced by the number and capacity of the anastomoses of the circle of Willis. Thus, the cerebrovascular reactivity (CVR) indices derived from the CCC carry complex information, including the modifying effect of the available anastomoses of the circle of Willis, in addition to the regional carotid cerebral vasoreactivity.

5.2.2. Results of the Hyperventilation, Breath-Holding Tests, and Valsalva Maneuver

The HV-BH and VM tests affect the entire cerebral vasculature; thus, the anastomotic role of the circle of Willis is negligible. The significant relationship between HV-BH and VM CVR indices and CANS functions likely indicates valid mechanistic connections.

5.2.3. Interpretation of Canonical Correlation

The canonical correlation evaluates the strongest possible linear relationship between the CVR and CANS indices. Based on the canonical variates, the strongest relationship is observed between the $CAR_{timetomax}$ variable of the CVR indices and the PRT and SI variables of the CANS indices. In other words, out of the many defined CVR variables of

the three vasoactive tests, only a few are sensitive to the hemodynamic disturbance caused by ICAS. In addition, the autonomic indices of VM do not designate identical, matching patient groups, so neither index is likely to be suitable to adequately verify vasoreactivity disorder and associated CANS dysfunction. The current clinical significance of the canonical correlation results lies in the relatively strong relationship observed between the indices. Therefore, the impairment of CVR and CANS in advanced atherosclerosis is not independent of each other in many cases, and both aspects have clinical significance in assessing the risk of cerebral ischemia.

5.2.4. Clinical Significance of Cardiovascular Autonomic Dysfunction Associated with Carotid Stenosis

The patient population and the method in our work differ from those of previous studies that have already drawn attention to the possibility of atherosclerosis and the often associated cardiovascular autonomic dysfunction.(67) We assessed cardiovascular autonomic function based on the validated autonomic indices of the Valsalva maneuver, which is reproducible and suitable for the examination of cerebral vasoreactivity. The significant correlation of dysfunction-vasoreactivity disorder was also confirmed using this approach. We consider the VM to be a highly informative test that is easy to perform, standardized, and extremely valuable in everyday clinical practice; according to our experience, the technique is reproducible even in elderly patients. Moreover, due to the frequent concomitant dysfunctions of cerebral vasoreactivity and arterial blood pressure regulation—as demonstrated by the present findings—the development of postoperative focal hyperperfusion syndrome should be expected in this group of operated patients.(84) Thus, prolonged postoperative monitoring of arterial blood pressure and MCA blood flow velocity should be an essential aspect for optimal patient care. As presented above many factors influence the integrity of CVR.(3) For example, aging leads to the stiffening of large arteries and the degradation of elastin fibers - these changes then also reduce the vascular compliance.(101) Among other factors, atherosclerosis, dysfunction of the cardiovascular autonomic nervous system, endothelial dysfunction, and changes of cardiac output can also lead to the impairment of CVR. (3),(102)

One of the major consequences is cerebral hypoperfusion and consequent cerebral ischemia. If blood pressure falls in patients with impaired CVR, then cerebral vessels will

not be able to dilate sufficiently to maintain CBF. This may lead to inadequate oxygen levels, especially in the watershed regions between the major vascular territories. Impaired CVR and cerebral hypoperfusion can be an additional risk during surgical procedures.(70)Another major disadvantage is global cerebral hyperperfusion in case of elevated arterial blood pressure. In this case, the vessels cannot constrict appropriately, thus leading to excessive blood flow, disruption of the brain-blood barrier, which results in vasogenic edema and intracerebral hemorrhage.(84) This can be a serious local complication after carotid revascularization procedures, such as carotid endarterectomy or stent implantation, as cerebral resistance vessels, which had previously lost their ability to contract, are suddenly confronted with the restoration of normal perfusion, which can lead to local cerebral edema and, in some cases, fatal intracerebral hemorrhage. Furthermore, impairment of CVR in patients with severe ICAS is associated with increased risk of ischemic events.(103)

5.2.5. The Significance of Multimodal Functional Methods, Like TCD with ABP Monitoring: Relationship Between Cardiovascular Autonomic Dysfunction and Cerebrovascular Reactivity

Monitoring of cerebral blood flow velocity, arterial blood pressure, and heart rate allows simultaneous assessment of cerebral and systemic circulatory changes and their interactions in response to vasoactive stimuli. Measuring BFV with the transcranial Doppler method is widely accepted for testing the effects of autonomic dysregulation on cerebral perfusion.(68),(104) This method is applicable in the diagnosis of vasovagal syncope, orthostatic intolerance, chronic autonomic failure, and baroreflex failure. The estimation of the risk of cerebral ischemia in patients with carotid stenosis is of great importance, especially in the preoperative period. It has been proven that impaired cerebrovascular reactivity is associated with a higher risk of cerebral ischemia, cognitive decline, and worse clinical outcome in ischemic stroke. (72), (81)

5.3. Strength of the Studies presented above

In these studies, vasoactive tests were summarized which were previously considered suitable for assessing CVR and presents it on a relevant patient population. The combination of the vasoreactivity tests utilized allows complex hemodynamic

characterization and even more personalized decision-making. This aspect could have implications for ICA-stenotic patients' preoperative risk assessment in future studies.

Furthermore, we were able to evaluate cardiovascular and cerebrovascular responses in a complex manner in response to the Valsalva maneuver and other vasoactive stimuli, which were easily and safely performed even in relatively elderly patients with significant carotid artery stenosis. From a neurological point of view, it is known that reduced cerebral vasoreactivity is an independent risk factor for cerebral ischemia. Based on our results, which are also in line with the findings of previous publications – including the assessment of cardiovascular autonomic dysfunction as a risk factor for cerebral ischemic events, especially before CEA—it may be useful in preventing perioperative neurological complications caused by intraoperative clamping due to the depletion of cerebrovascular reserve capacity.

5.4. Limitations

A limitation of the present work is the relatively small number of patients included, which was due to the following reasons:

1) the absence of an adequate bilateral temporal acoustic window;
2) strict TCD protocol requirements combined with the selection criteria: only those registrations were evaluated in which the signal-to-noise ratio was excellent. Due to the low number of patients examined, it was not possible to include additional control variables enabling subgroup analyses.
3) Another disadvantage is that the duration of the BH test cannot be fully standardized,

   because not all patients were able to complete the task in accordance with the instructions. In the future, we will consider standardizing the duration of the BH test. We were unable to calculate the CAR AUC value of the HV test in one patient due to movement artifact, and similarly, in 11 cases, the CAR AUC value of the BH test could not be calculated because the CAR value did not exceed the BL level.
4) The method for assessing cerebral vascular CO_2 reactivity lacked end-tidal CO_2 measurement, which would have facilitated comparative analysis of the data despite different breath-holding times. This may explain why, in contrast to

previous publications, we did not observe a significant decrease in CO_2 reactivity on the side of carotid stenosis.

6. Conclusion

Overall, the salient findings of the present work regarding the impairment of cerebrovascular reactivity and cardiovascular autonomic nervous system disfunction in patients with significant internal carotid artery stenosis are the following:

1) First, patients with significant internal carotid artery stenosis CO_2 reactivity tests were not sufficiently sensitive to hemodynamic disturbances caused by significant carotid artery stenosis.
2) Second, other tests, such as hemodynamic tests (CCC and VM), which elicit changes in cerebral perfusion pressure and flow velocity, should be used to assess cerebrovascular reactivity and quantify cerebral ischemic risk.
3) Third, it might be important to assess cerebrovascular reactivity in a complex manner. The findings of the present studies suggest that in patients with severe internal carotid artery stenosis, a significant correlation exists between the reduced capacity of cerebral resistance vessels and the impairment of the cardiovascular autonomic nervous system. One potential underlying mechanism is impaired baroreceptor signaling due to remodeling (stiffening) of large extracranial arteries, resulting in blood pressure instability, which leads to increased risk of cerebral hypoperfusion and ischemia, especially during endovascular surgical procedures.

7. Summary

In this study, patients aged between 60-70 years with significant atherosclerotic internal carotid artery stenosis were examined and scheduled for carotid endarterectomy by using a multimodal Transcranial Doppler Ultrasound Protocol.

The primary aim of the study was to evaluate cerebral vasoreactivity as comprehensively as possible. In addition to the widely used CO_2 reactivity assessment, further tests were applied based on pressure-flow changes, such as the Common Carotid artery Compression test and the Valsalva Maneuver.

Our results demonstrated that the pressure-flow based tests appeared to be more sensitive compared to the conventional CO_2 reactivity tests such as Hyperventilation and Breath Holding tests. Furthermore, by applying the Valsalva Maneuver a substantial proportion of patients were found with concomitant cardiovascular autonomic nervous system dysfunction. Therefore, parameters suitable for assessing the autonomic dysfunction were correlated with parameters evaluating cerebral vasoreactivity. Based on our findings, severe cardiovascular autonomic dysfunction and impaired cerebral vasoreactivity are not independent from each other.

These findings may have important clinical implications. Both impaired cerebral vasoreactivity and cardiovascular autonomic dysfunction can be regarded as potential cerebral ischemic risk factors in this patient population. Therefore, the combined assessment of cerebrovascular reserve and autonomic cardiovascular regulation may help identify patients with high ischemic risk and perioperative cerebrovascular complications.

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9. Bibliography of the candidate's publications

9.1. Publications relevant to the dissertation:

Pál H, Magyar-Stang R, Csányi B, Gaál A, Mihály Z, Czinege Z, Sótonyi P, Horváth T, Dobi B, Bereczki D, Koller A, Debreczeni R. Complex Characterization of Cerebral Vasoreactivity in Internal Carotid Artery Stenotic Patients with Transcranial Doppler Sonography. Life (Basel). 2025 Oct 30;15(11):1692. doi: 10.3390/life15111692. PMID: 41302117; PMCID: PMC12653681.

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9.2. Publications unrelated to the dissertation:

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Presentations, posters:

1) **Complex Transcranial Doppler Ultrasound examinations in patients with significant internal carotid artery stenosis**
Hanga Pál, Rita Magyar-Stang, Borbála Csányi, Anna Gaál, Zsuzsanna Mihály, Zsófia Czinege, Péter Sótonyi, Tamás Horváth, Dániel Bereczki, Akos Koller, Róbert Debreczeni
2024. November, Semmelweis Symposium

2) **Arteria carotis interna stenosisban szenvedõ betegek agyi vazoreaktivitásának komplex elemzése funkcionális Transzkraniális Doppler Ultrahang vizsgálatok alapján**

Nagy Kevin, Kovács Ágnes Rita

Témavezető: Dr. Debreczeni Róbert, Dr. Pál Hanga

SE TDK Konferencia 2025. előadás, II. helyezett

3) **Complex Functional TCD Examinations in Patients with Significant Internal Carotid Artery Stenosis**

ePoster session at the 11th EAN Congress, Helsinki, June 21 – 24.

Hanga Pál, Rita Magyar-Stang, Borbála Csányi, Anna Gaál, Zsuzsanna Mihály, Zsófia Czinege, Péter Sótonyi, Tamás Horváth, Balázs Dobi, Dániel Bereczki, Akos Koller, Róbert Debreczeni

4) **Arteria carotis interna stenosisban szenvedő betegek agyi vazoreaktivitásának komplex elemzése funkcionális Transzkraniális Doppler Ultrahang vizsgálatok alapján**

Magyar Neuroszonológiai Társaság XVI. Konferenciája (Győr)2025. szeptember 11-13.

Pál Hanga, Magyar-Stang Rita, Csányi Borbála, Gaál Anna, Mihály Zsuzsanna, Sótonyi Péter, Horváth Tamás, Koller Ákos, Bereczki Dániel, Debreczeni Róbert

5) **A cerebrális vazoreaktivitás és a kardiovaszkuláris autonóm diszfunkció egyidejű fennállása szignifikáns carotis stenosisban szenvedő betegeknél – funkcionális TCD vizsgálat**

Magyar Neurológiai Társaság 40. Kongresszusa, 2026. május 14-16. Kodály Központ, Pécs

Pál Hanga, Magyar-Stang Rita, Csányi Borbála, Gaál Anna, Mihály Zsuzsanna, Czinege Zsófia, Sótonyi Péter, Horváth Tamás, Dobi Balázs, Bereczki Dániel

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