EARLY NEUROLOGICAL IMPROVEMENT FOLLOWING REPERFUSION THERAPY IN ACUTE ISCHAEMIC STROKE

PhD THESIS

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Publications directly related to the thesis

I. **Annus Á**, Gera FZ, Sztriha L, Klivényi P. DWI-FLAIR mismatch guided thrombolysis in patients without large-vessel occlusion: real-world data from a comprehensive stroke centre. *Heliyon.* 2022 Dec 5;8(12):e12069. DOI: 10.1016/j.heliyon.2022.e12069. (IF: 3.776, [2021])

II. **Annus Á,** Tömösi F, Rárosi F, Fehér E, Janáky T, Kecskeméti G, Toldi J, Klivényi P, Sztriha L, Vécsei L. Kynurenic acid and kynurenine aminotransferase are potential biomarkers of early neurological improvement after thrombolytic therapy: a pilot study. *Advances in Clinical and Experimental Medicine.* 2021 Dec;30(12):1225-1232. DOI: 10.17219/acem/141646. (IF: 1.736)

III. **Annus Á,** Nagy A, Vécsei L, Klivényi P. 24-hour near-infrared spectroscopy monitoring of acute ischaemic stroke patients undergoing thrombolysis or thrombectomy: a pilot study. *Journal of Stroke and Cerebrovasc Diseases.* 2019 Aug;28(8):2337-2342. DOI: 10.1016/j.jstrokecerebrovasdis.2019.05.026. (IF: 1.787)

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Publications not directly related to the thesis

I. **Annus Á**, Juhász LÁ, Szabó E, Rárosi F, Szpisjak L, Vécsei L, Klivényi P. Connection between small vessel disease related stroke and the MTHFR C677T polymorphism in a Hungarian population. *Heliyon.* 2020 Nov 2;6(11):e05305. DOI: 10.1016/j.heliyon.2020.e05305. eCollection 2020 Nov.

II. **Annus Á**, Bencsik K, Járdánházy T, Vécsei L, Klivényi P. Unilateral thalamic infarction causing downward gaze palsy in a patient with uncorrected tetralogy of Fallot: a case report. *Ideggyógyászati Szemle.* 2016 Nov 30;69(11-12):415-419. DOI: 10.18071/isz.69.0415. (IF: 0.322)

III. **Annus Á**, Csáti A, Vécsei L. Prion diseases: New considerations. *Clinical Neurology and Neurosurgery.* 2016 Nov;150:125-132. DOI: 10.1016/j.clineuro.2016.09.006. (IF: 1.381)

IV. **Annus Á**, Vécsei L. Spotlight on opicapone as an adjunct to levodopa in Parkinson's disease: design, development and potential place in therapy. *Drug Design Development and Therapy*. 2017 Jan 9;11:143-151. DOI: 10.2147/DDDT.S104227. (IF: 2.935)

V. **Annus Á,** Bencsik K, Obál I, Kincses ZT, Tiszlavicz L, Höftberger R, Vécsei L. Paraneoplastic neuromyelitis optica spectrum disorder: A case report and review of the literature. *Journal of Clinical Neuroscience.* 2018 Feb;48:7-10. DOI: 10.1016/j.jocn.2017.10.030. (IF: 1.593)

VI. Boros FA, Maszlag-Török R, Szűcs M, **Annus Á**, Klivényi P, Vécsei L. Relationships of ischemic stroke occurrence and outcome with gene variants encoding enzymes of tryptophan metabolism. *Biomedicines.* 2021 Oct 11;9(10):1441. DOI: 10.3390/biomedicines9101441. (IF: 4.757)

VII. Kalmár PJ, Tárkányi G, Nagy CB, Csécséi P, Lenzser G, Bosnyák E, Karádi ZN, **Annus Á**, Szegedi I, Büki A, Szapáry L. Comparing endovascular treatment methods

in acute ischemic stroke due to tandem occlusion focusing on clinical aspects. *Life (Basel)*. 2021 May 20;11(5):458. DOI: 10.3390/life11050458. (IF: 3.253)

VIII. Tárkányi G, Csécsei P, Szegedi I, Fehér E, **Annus Á**, Molnár T, Szapáry L. Detailed severity assessment of Cincinnati Prehospital Stroke Scale to detect large vessel occlusion in acute ischemic stroke. *BMC Emergency Medicine*. 2020 Aug 24;20(1):64. DOI: 10.1186/s12873-020-00360-9. (IF: 2.119)

IX. Majláth Z, **Annus Á**, Vécsei L. Kynurenine system and multiple sclerosis, pathomechanism and drug targets with an emphasis on laquinimod. *Current Drug Targets.* 2018;19(7):805-814. DOI: 10.2174/1389450117666161223125417. (IF: 2.642)

X. Juhász A, Aschermann Z, Ács P, Janszky J, Kovács M, Makkos A, Harmat M, Tényi D, Karádi K, Komoly S, Takáts A, Tóth A, Nagy H, Klivényi P, Dibó G, Dézsi L, Zádori D, **Annus Á**, Vécsei L, Varannai L, Kovács N. Levodopa/carbidopa intestinal gel can improve both motor and non-motor experiences of daily living in Parkinson's disease: An open-label study. *Parkinsonism & Related Disorders.* 2017 Apr;37:79-86. DOI: 10.1016/j.parkreldis.2017.02.001. (IF: 4.721)

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List of abbreviations

3-HANA: 3-hydroxyanthranilic acid

3-HK: 3-hydroxy-kynurenine

5-HIAA: 5-hydroxy-3-indoleacetic acid

ACA: anterior cerebral artery

AhR: aryl hydrocarbon receptor

ANA: anthranilic acid

AUC: area under the curve

ASITN: American Society of Interventional and Therapeutic Neuroradiology Collateral Flow Grading

BBB: blood-brain barrier

CBF: cerebral blood flow

CBV: cerebral blood volume

CE: cardioembolic

CI: confidence interval

CRP: C-reactive protein

CTA: computer tomography angiography

DWI: diffusion-weighted imaging

ECASS II: European Cooperative Acute Stroke Study II

ED: Emergency Department

ENI: early neurological improvement

EXTEND: Extending the Time for Thrombolysis in Emergency Neurological Deficits

FA: formic acid

FLAIR: fluid-attenuated inversion recovery

Hb_{deoxy}: deoxyhaemoglobin

Hb_{oxy}: oxyhaemoglobin

Hb_T: total haemoglobin

IDO: indoleamine 2,3-dioxygenase

ICA: internal carotid artery

ICH: intracerebral haemorrhage

ICU: intensive care unit

$IH\Delta rSO_{2:}$ interhemispheric rSO_2 difference

IL: interleukin

IM: internal medicine

IQR: interquartile range

IVT: intravenous thrombolysis

KAT: kynurenine aminotransferase

KMO: kynurenine monooxygenase

KYN: kynurenine

KYNA: kynurenic acid

LAA: large-artery atherosclerosis

LVO: large-vessel occlusion

L-KYNs: L-kynurenine sulfate

MAO: monoamine oxidase

MCA: middle cerebral artery

MCAO: middle cerebral artery occlusion

MeOH: methanol

MMSE: Mini-Mental State Examination

MS/MS: tandem mass spectrometry

MRA-TOF: magnetic resonance angiography – time of flight

mRS: modified Rankin Scale

MT: mechanical thrombectomy

NCCT: non-contrast computer tomography

NIHSS: National Institutes of Health Stroke Scale

NINDS: Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study

NIRS: near-infrared spectroscopy

NMDA: N-methyl-D-aspartate

NMDAR: N-methyl-D-aspartate receptor

NRU: neurorehabilitation unit

OR: odds ratio

PH: parenchymal haemorrhage

PICA: picolinic acid

PRE-FLAIR: Identification of Stroke Patients ≤ 3 and ≤ 4.5 Hours of Symptom Onset by Fluid Attenuated Inversion Recovery Imaging and Diffusion-Weighted Imaging

PRISMS: A Study of the Efficacy and Safety of Alteplase in Participants With Mild Stroke

QC: quality control

QIUN: quinolinic acid

rSO_2: regional O_2 saturation

ROC: receiver operating characteristic

ROS: reactive oxygen species

SERO: serotonin

SD: standard deviation

sICH: symptomatic intracerebral haemorrhage

SN: sensitivity

SP: specificity

SpO_2: peripheral oxygen saturation

SVD: small vessel disease

SWAN: susceptibility-weighted angiography

TDO: tryptophan-2,3-dioxygenase

TOAST: Trial of Org 10172 in Acute Stroke Treatment

TRP: tryptophan

UHPLC: ultra-high-performance liquid chromatography

VA: vertebral artery

WAKE-UP: Efficacy and Safety of MRI-based Thrombolysis in Wake-up Stroke

XA: xanthurenic acid

1. Introduction

According to the recent Global Burden of Disease estimates, stroke is the second leading cause of death and the third leading cause of combined death and disability worldwide.[1] The lifetime risk of stroke has increased by approximately 50% in the last 20 years, and affects one in four people.[2] Ischaemic events account for the majority of stroke cases.[3] Acute treatment of ischaemic stroke has developed significantly in recent years. Intravenous thrombolysis (IVT) with alteplase or tenecteplase (recombinant tissue-plasminogen activators) is the first-line treatment in patients who present within 4.5 hours of symptom onset.[4] In selected cases, the time window for treatment can be extended to 9 hours based on the results of the Extending the Time for Thrombolysis in Emergency Neurological Deficits (EXTEND) trial.[5] For patients with large-vessel occlusion (LVO), mechanical thrombectomy (MT) is an effective endovascular procedure for recanalization. Meta-analyses indicate a number needed to treat value of 2.6 to reduce disability in patients with anterior circulation LVO.[6] In selected cases, MT can be performed up to 24 hours after symptom onset.[7]

Most randomized clinical trials in ischaemic stroke assess the efficacy of treatment or intervention with the modified Rankin Scale (mRS). The mRS is a 7-point scale (from 0 to 6) that indicates the degree of disability in patients' daily activities, with an emphasis on mobility.[8] Higher scores indicate more severe disability. It is usually measured approximately three months after stroke. The mRS is brief, simple and has high inter-rater reliability.[9] However, the mRS does not account for comorbidities, polypharmacy, socioeconomic factors, availability and quality of rehabilitation, and support provided by family. For example, a patient with an LVO who has successful recanalization and is discharged home with an mRS score of 1 will have a three-month mRS of 6 if dying of a cardiac arrest two months after discharge. In a clinical trial where mRS at three months is the primary outcome to assess treatment efficacy, treatment would be deemed unsuccessful in this patient, although the cause of the unfavourable outcome was not ineffective treatment of the stroke itself.

Therefore, we believe that the treatment effect of reperfusion therapies may be better reflected by short-term changes in the National Institutes of Health Stroke Scale (NIHSS) score rather than the 90-day mRS score. The NIHSS measures impairment rather than disability, with scores ranging from 0 to 42, with higher scores indicating a more severe deficit. The short-term improvement in NIHSS score is usually termed early neurological improvement (ENI). Unfortunately, the definition of ENI varies significantly among studies.

In the National Institute of Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study (NINDS trial), it was defined as complete resolution of symptoms or at least a 4-point decrease in the NIHSS score 24 hours after treatment.[10] Another study analysed the absolute change between baseline and 24-hour NIHSS scores.[11] Percentage changes in NIHSS score were also reported.[12,13] Despite the various definitions of ENI, several studies have shown that ENI correlates with clinical outcomes at later time points.[14,15] Jantasri et al. demonstrated that a 2-point difference in the NIHSS score 24 hours after IVT predicts three-month functional outcomes.[16] In another study, a reduction in NIHSS score by 10, or an absolute score of 4 or less 2 hours after IVT, was an independent predictor of a favourable outcome at three months.[17] Yaghi et al. found that the percentage change in NIHSS score at 24 hours correlated better with mRS 0-1 at three months than the absolute change in NIHSS score.[18]

The thesis will focus on ENI after reperfusion therapies (IVT and MT). The definition of ENI varied among the studies included in this thesis. We aimed to tailor the definition of ENI to suit the investigated stroke population and to best correlate with functional outcomes.

1.1. DWI-FLAIR mismatch guided thrombolysis

The determination of the exact time of symptom onset in acute ischaemic stroke is often difficult. Approximately 14-27% of stroke patients present to the emergency department with unknown symptom onset time.[19] A commonly encountered scenario is a wake-up stroke. Furthermore, agitated or aphasic patients and those with neurocognitive deficits might be unable to tell when their symptoms started, making treatment decisions challenging. Current guidelines recommend systemic IVT within 4.5 hours after symptom onset.[4,20] If the onset time is unknown and the patient was last seen well more than 4.5 hours ago, specific imaging modalities can help to establish eligibility for thrombolytic therapy. One option is the MRI-based diffusion-weighted imaging (DWI) – fluid-attenuated inversion recovery (FLAIR) mismatch concept. DWI detects ischaemia-induced changes in cellular water diffusion within minutes.[21,22] In comparison, T2 weighted imaging and, consequently, FLAIR imaging are sensitive to only detect net water increase and vasogenic oedema that follows cytotoxic oedema.[23,24] Therefore, it takes at least 1-4 hours for the ischaemic stroke to become visible on FLAIR imaging. The PRE-FLAIR (Identification of Stroke Patients ≤ 3 and ≤ 4.5 Hours of Symptom Onset by Fluid Attenuated Inversion Recovery Imaging and Diffusion-Weighted

Imaging) study demonstrated that the DWI-FLAIR mismatch pattern identifies ischaemic lesions within 4.5 hours after symptom onset with 78% specificity and 83% positive predictive value.[25] In a multicentre, randomised, double-blind, placebo-controlled trial (Efficacy and Safety of MRI-based Thrombolysis in Wake-up Stroke, i.e. the WAKE-UP trial), alteplase treatment was administered for patients with unknown stroke onset times who were last seen well more than 4.5 hours before symptoms were noticed, and had DWI-FLAIR mismatch.[26] Mismatch was defined as a DWI lesion without corresponding marked FLAIR hyperintensity. Patients treated with alteplase had significantly better functional outcomes at 90 days than those who received placebo. However, severe parenchymal haemorrhages were also significantly more common in the treatment arm. Nevertheless, the WAKE-UP trial provided high-quality evidence on the benefit of alteplase treatment in patients with unknown symptom onset times and a DWI-FLAIR mismatch on MRI.

1.2. Prognostic biomarkers in acute ischaemic stroke

Biomarkers are objective indicators of physiological or pathological processes and have valuable applications in predicting and monitoring clinical responses to therapeutic interventions.[27] Despite a significant increase in the number of blood-based biomarkers reported, prognostic biomarkers that could aid in predicting the outcome of ischaemic stroke or response to reperfusion therapy are still lacking in routine clinical practice.[28,29] A few blood biomarkers have shown promise: copeptin, a fragment of vasopressin, increases the prognostic accuracy of the NIHSS in predicting functional outcome and mortality.[30] In other reports, matrix-metalloproteinase-9 levels correlated with hemorrhagic transformation after thrombolytic therapy, and S100B was elevated in patients with malignant middle cerebral artery (MCA) syndrome.[31,32] Faille et al. showed that low soluble thrombomodulin and endothelial protein C receptor admission levels in patients with arterial occlusion are associated with higher recanalization rates after thrombolytic therapy.[33] In another study, pre-IVT low endogenous thrombin potential was an independent predictor of both short- and long-term mortality following treatment.[34] Although its prognostic value has not been elaborated yet, animal models and clinical studies have unequivocally proved that the KYN pathway is activated in acute ischemic stroke.[35-41]

1.3. Kynurenine pathway

The kynurenine (KYN) pathway is the main route of tryptophan (TRP) metabolism. It plays a vital role in N-methyl-D-aspartate (NMDA) receptor (NMDAR) mediated excitotoxicity, reactive oxygen species (ROS) production and inflammation. More than 95% of TRP is metabolised through the KYN pathway.[42] The first and rate-limiting step is the metabolism of TRP to L-KYN by indoleamine-2,3-dioxygenase (IDO) or tryptophan-2,3-dioxygenase (TDO). Inflammatory cytokines (e.g., IL-1β, TNF-α and INF-γ) increased IDO activation.[36,43,44] The expression of TDO is induced by TRP, glucocorticoids and nicotinamide deficit.[41] L-KYN (which has a vasodilatory effect) can be further metabolised into three distinct molecules.[45] Kynurenine aminotransferase (KAT), kynurenine-3-monooxygenase (KMO), and kynureninase enzymes catalyse the production of kynurenic acid (KYNA), 3-hydroxy-L-kynurenine (3-HK) and anthranilic acid (AA), respectively. 3-HK and AA can be metabolised to 3-hydroxyanthranilic acid (3-HAA) and quinolinic acid (QUIN). The molecules mentioned above are collectively called kynurenines.[46] The most significant neuroactive compounds of the KYN pathway are KYNA, 3-HK and QUIN. KYNA is a known endogenous, competitive inhibitor of NMDAR.[47]. Also, it is an antagonist of the α7 nicotinic acetylcholine receptor, which is involved in enhanced presynaptic glutamate release in the human neocortex.[48] Therefore, it is believed that KYNA has neuroprotective properties by limiting NMDA-mediated excitotoxicity. In addition, it has been postulated that KYNA also functions as a ROS scavenger and endogenous antioxidant.[49] 3-HK is a neurotoxic compound that mediates the production of free radicals.[50] However, growing evidence shows that it can also have antioxidant effects.[51] QUIN is also a neurotoxic metabolite, which acts as a potent NMDAR agonist and leads to glutamatergic excitotoxicity.[52,53] QUIN causes oxidative stress, lipid peroxidation, and the release of inflammatory cytokines in astrocytes, promoting a local inflammatory response.[54] **Figure 1** summarizes the main metabolites of the KYN pathway that were analysed in our biomarker study. A summary of the most relevant animal and human studies of the KYN pathway in ischaemic stroke is shown in **Tables 1-2**.

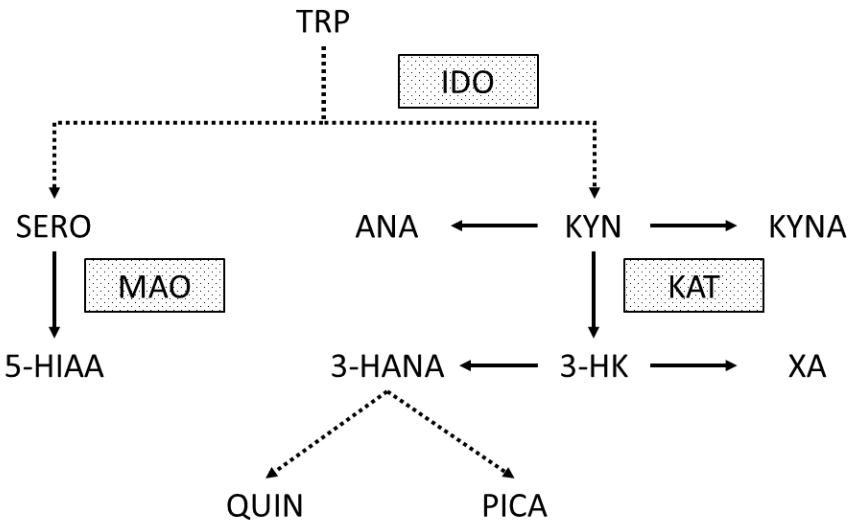


Figure 1. Kynurenine pathway and other tryptophan metabolites measured in the biomarker pilot study. Dotted lines indicate more than one enzymatic step. Dotted boxes highlight the most relevant enzymes of the pathway.

Reference	Study design	Study results
Cuartero et al.[55]	permanent or transient middle cerebral artery occlusion (MCAO) by ligation in mice	ischaemia induces TDO-mediated L-KYN production, which activates the aryl hydrocarbon receptor (AhR) pathway
Nozaki and Beal[56]	pre-ischaemic, intraperitoneal administration of L-KYN in a neonatal rat model	dose-dependent reduction in infarct volume and dose-dependent increase in cortical KYNA levels
Gigler et al.[57]	intraperitoneal administration of L-kynurenine sulfate (L-KYNs) before unilateral MCAO in mice	decreased cortical infarct surface area
Robotka et al.[39]	systemic administration of L-KYNs in 4 vessel occlusion model in Wistar rats	reduced cortical neuronal loss

Gellert et al.[58]	systemic administration of L-KYNs after transient MCAO and reperfusion in Wistar rats	L-KYNs exacerbated the neuronal damage following reperfusion
Jackman et al.[59]	intraluminal occlusion of the MCA in IDO wild–type and IDO gene-deficient mice	significantly increased IDO expression in the endothelium of cerebral arterioles
		markedly increased IDO activity

Table 1. Summary of animal studies investigating the role of the KYN pathway in ischaemic stroke.

Reference	**Study design**	**Study results**
Brouns et al.[36]	kinetics of KYN metabolites in the serum of ischaemic stroke patients	KYN/TRP ratio correlated with C-reactive protein (CRP) levels, severity of ischaemic stroke and long-term outcome
	blood samples were taken at admission, 24, 48 hours and 7 days after stroke	KYN pathway was more active in severe cases
Mo et al.[38]	kynurenines in the serum of acute ischaemic stroke patients	elevated CRP and increased IDO activity in stroke patients
	blood samples were taken within 24 hours after the ischaemic event	decreased concentrations of TRP, KYNA and KAT activity
		positive correlations between NIHSS score, CRP and IDO activity
Darlington et al.[37]	levels of kynurenines in acute stroke patient	elevated KYN:TRP ratio in stroke patients, which correlated with neopterin levels
		3-HAA:AA ratio significantly correlated with infarct volume

	blood samples were taken 24 h, 2, 3, 4, 7 and 14 days after stroke for most patients	KYNA was higher in those patients who died within 21 days after stroke
Ormstad et al.[41]	metabolites of the KYN pathway within 72 hours in 45 acute ischaemic stroke patients	significantly higher QUIN concentrations and QUIN/KYNA ratios in female stroke patients
		positive correlation between interleukin 6 (IL-6) and QUIN/KYNA ratio
		positive correlation between IL-10 and KYNA
Gold et al.[60]	correlation between cognitive impairment and IDO activity in ischaemic stroke patients within 30 days	higher KYN/TRP ratios among patients with lower scores on Mini-Mental State Examination (MMSE)
Hajsl M et al.[61]	serum KYN metabolites and enzymes of ischaemic stroke patients with LVO who underwent endovascular treatment within 6 hours and controls	significant differences in concentrations of TRP, 3-HK, 3-HAA and AA
		difference in activity of IDO and KMO

Table 2. Summary of human studies investigating serum concentrations of kynurenines in ischaemic stroke.

1.4. Near-infrared spectroscopy (NIRS)

Monitoring acute ischaemic stroke patients during reperfusion therapies (IVT or MT) is based mainly on frequent neurological and physical examinations. No objective measurement of cerebrovascular haemodynamics is currently used in routine clinical practice. Near-infrared spectroscopy (NIRS) is a bedside, noninvasive, continuous, real-time tool that can be used to monitor acute stroke patients. It is commonly applied during cardiac surgery and carotid endarterectomy to detect and prevent cortical desaturations and permanent

neurological sequelae.[62] To our knowledge, only a few observational and pilot studies have reported the potential of NIRS monitoring during acute ischaemic stroke.[63-66]

NIRS utilizes a light source that emits photons in the near-infrared range (700-1100 nm). These photons penetrate the skull a few centimetres deep into the brain parenchyma. The emitted light is partly redirected, scattered and absorbed. The absorption spectrum of oxyhaemoglobin (Hb_{oxy}) and deoxyhaemoglobin ($Hb_{deoxy)}$ varies at different wavelengths.[67] This difference allows for the calculation of Hb_{oxy} and Hb_{deoxy} concentrations based on the difference in intensity of emitted and received light, using the Beer-Lambert equation:

$$A = \lg \frac{I_0}{I} = \varepsilon \times c \times l$$

(A: absorption, I_0: intensity of emitted light, I: intensity of received light, ε: absorption coefficient, c: concentration, l: photon pathlength).

Total haemoglobin (Hb_T) concentration is the sum of Hb_{oxy} and Hb_{deoxy} concentrations and is proportional to cerebral blood volume (CBV).[68] Therefore, NIRS can measure cortical blood oxygenation/saturation, i.e. the fraction of Hb_{oxy} relative to Hb_T.[62,69] Mean cortical saturation measured with NIRS comprises approximately 70% venous and 30% arterial blood.[67] A marker of cerebral haemodynamics is the relative change in regional O_2 saturation (rSO_2) and not the absolute rSO_2 since absolute values show significant interindividual variability.[70,71] Environmental and individual features that influence absolute rSO_2 values are contamination from hair and skin, sweating, skull thickness, extracranial circulation, O2 extraction of brain parenchyma (e.g., reduced O_2 extraction of infarcted or oedematous territory), blood pressure, peripheral oxygen saturation (SpO_2), Hb concentration in blood and level of consciousness.[63,72] The combined effect of these factors can make the interpretation of absolute rSO_2 values uncertain.

It is well known that the collateral circulation plays a pivotal role in reducing the progression of ischaemic brain damage.[73] Patients with good collaterals develop smaller infarcts, respond better to MT, show better clinical outcomes, and have a lesser chance of haemorrhagic transformation after reperfusion than patients with poor collaterals.[74-76] However, a real-time assessment tool of collateral circulation in acute ischaemic stroke is lacking. Multimodal MRI studies have shown that augmented CBV, preserved cerebral blood flow (CBF) and delayed mean transit time implies the presence of collateral flow.[77] Taussky

et al. used CT perfusion to demonstrate a linear correlation between rSO_2 and CBF.[78] Therefore, both CBV and CBF correlate with rSO2 values, which can be measured in real-time at the bedside, non-invasively with NIRS.

2. Aims

The aims of this thesis were:

I. Analyse ENI after alteplase treatment in patients presenting with DWI-FLAIR mismatch. Furthermore, we compared treated patients' outcomes to those not eligible for IVT due to no DWI-FLAIR mismatch or other contraindications. Our analysis included patients with unknown symptom onset times and cases where MRI was performed because of diagnostic uncertainty within 4.5 hours after symptom onset. Patients with LVO were excluded.

II. Perform a pilot study to investigate whether metabolites of the KYN pathway and activity of relevant enzymes measured before and 12 hours after IVT could be potential biomarkers for predicting ENI.

III. Explore if NIRS is suitable for monitoring anterior watershed territory leptomeningeal collateral circulation, and analyse the correlation between NIRS readings (during IVT and MTI) and ENI.

3. Methods

3.1. Patients

3.1.1. *Patients in the DWI-FLAIR mismatch guided thrombolysis study*

A retrospective single-centre observational study was conducted between. January 2017 and April 2020. We identified patients with a suspected clinical diagnosis of acute ischaemic stroke, where an MRI showed DWI hyperintensity. Patients with LVO, who were candidates for MT, were excluded because our main goal was to analyse the effects of IVT alone. Based on the DWI-FLAIR mismatch pattern, an experienced attending stroke neurologist established the indication for IVT. Alteplase was given per the Hungarian Acute Ischaemic Stroke Diagnostic and Treatment Recommendations.[79] We used a more permissive protocol than the WAKE-UP trial: patients with partial DWI-FLAIR mismatch were also eligible for treatment, and pre-stroke functional dependence or age were not contraindications

for IVT. Patients who received alteplase had repeat imaging (CT or MRI) approximately 24 hours after IVT. All patients or their legal representatives gave informed consent before treatment. The Ethics Committee of the University of Szeged, Albert Szent-Györgyi Health Centre, approved our study (ID: 6/2017-SZTE), which was conducted according to the revised Declaration of Helsinki. Parameters of patients treated with alteplase were compared to those who did not receive this treatment due to a matched DWI-FLAIR pattern or other contraindications. For each patient, we recorded detailed demographic characteristics and vascular risk profiles. Blood glucose was measured from serum samples taken upon arrival at the Emergency Department (ED). Blood pressure readings were recorded upon arrival at the ED.

3.1.2. Patients in the biomarker pilot study

Our study on prognostic biomarkers included acute ischemic stroke patients who underwent IVT with alteplase between January and December 2018. Alteplase was administered according to the Hungarian Acute Ischaemic Stroke Diagnostic and Treatment Recommendations.[79] We excluded patients who had MT and those with a baseline mRS score >2. Patient data were collected from the Stay Alive Acute Stroke Registry, a national, multicentric database. Participating centres included the University of Debrecen, the University of Pécs and the University of Szeged. The Registry contains clinical, investigational and outcome data on patients who received reperfusion therapy. All patients or their legal representatives gave informed written consent before storing their data in the Registry. The biomarker pilot study was conducted in accordance with the revised Declaration of Helsinki, and the Ethics Committee of the University of Szeged, Albert Szent-Györgyi Clinical Centre approved the protocol (GINOP 2.3.2-15- 2016-00048). All patients or their relatives gave informed consent for inclusion before participation in the biomarker study.

3.2. Imaging protocol in the DWI-FLAIR mismatch guided thrombolysis study

Each patient underwent an acute multimodal brain MRI with a 1.5 T GE Signa Excite MRI scanner. The acute stroke MRI protocol included DWI, FLAIR, and susceptibility-weighted angiography (SWAN). DWI-FLAIR mismatch was defined as an ischaemic DWI lesion with no corresponding signal change on the FLAIR sequences. Partial mismatch was defined as a corresponding FLAIR signal change more diminutive than the DWI hyperintensity. **Figures 2-4** show examples of DWI-FLAIR complete mismatch, matched

pattern and partial mismatch. The attending radiologist and neurologist rated the mismatch patterns visually.

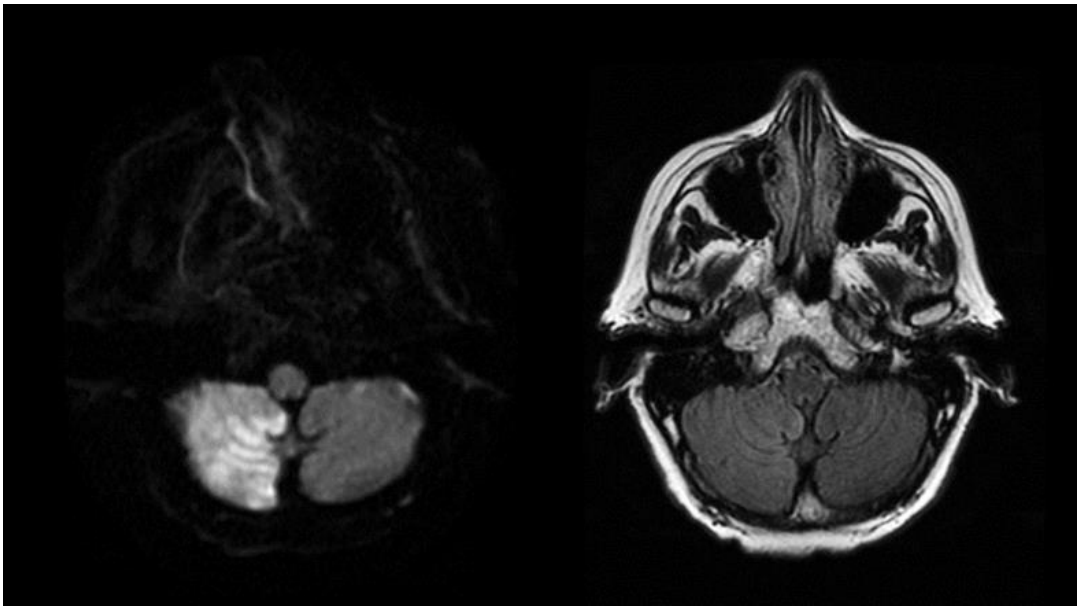


Figure 2. Complete DWI-FLAIR mismatch of a right cerebellar infarct.

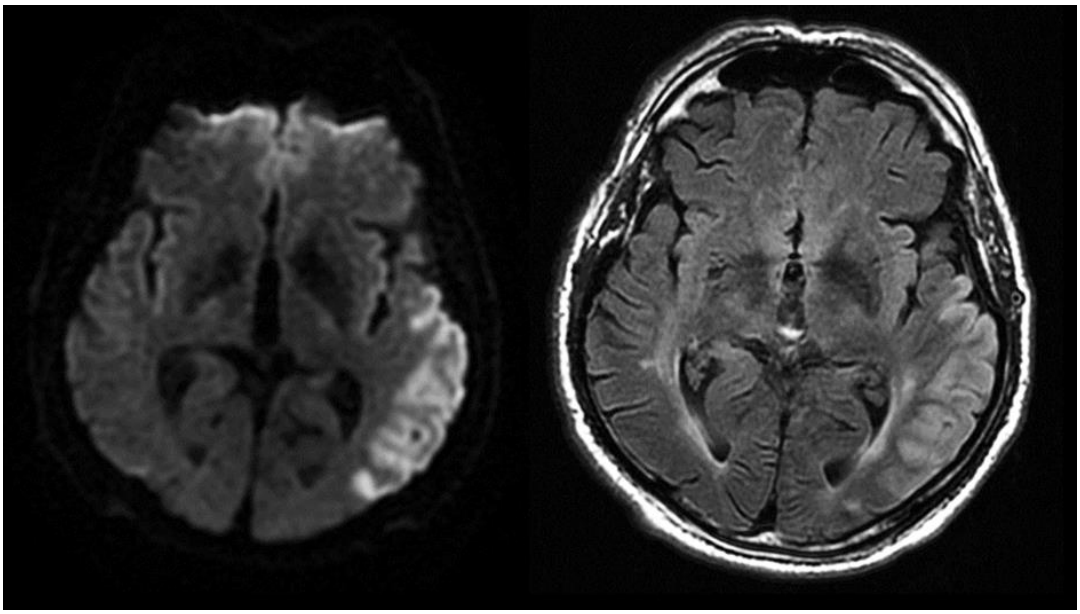


Figure 3. Matched DWI-FLAIR pattern of a left temporal infarct.

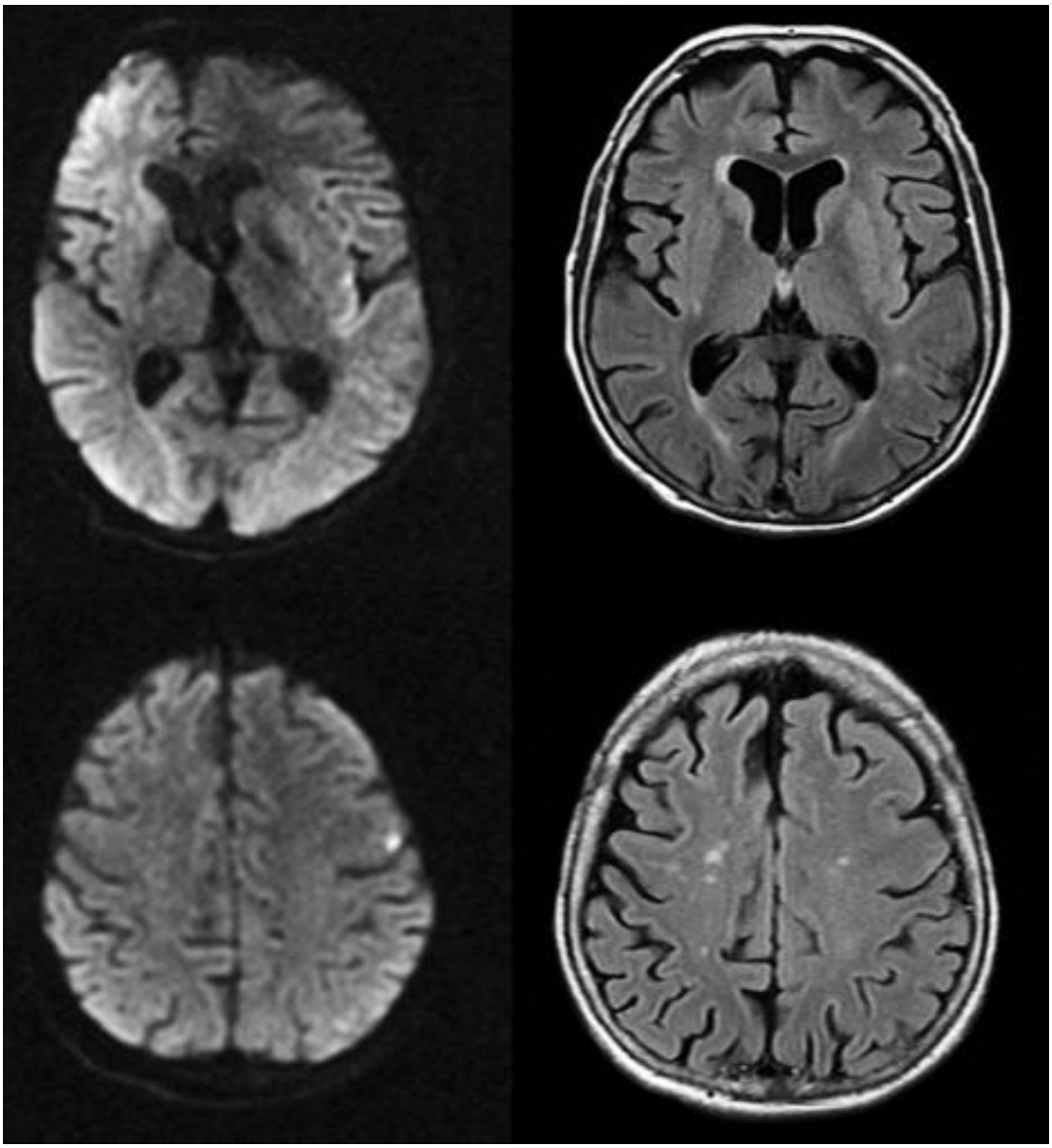


Figure 4. Example for partial mismatch. Superior row: left insular DWI hyperintensity with corresponding FLAIR signal change. Inferior row: the left frontal cortical DWI lesion is not yet visible on FLAIR.

3.3. Sampling in the biomarker pilot study

Peripheral venous blood samples for the biomarker study were taken just before IVT, and 12 hours after the initiation of treatment (samples A and B, respectively). Blood samples were centrifuged at 3000/min for 13 min, and sera were stored at -80 °C until further analysis.

3.4. Measurement of kynurenines by ultra-high-performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS)

3.4.1. Reagents and chemicals

All reagents and chemicals were analytical or LC-MS grade. TRP and its metabolites and d4-picolinic acid (PICA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). d3-3-HK was obtained from Buchem B. V. (Apeldoorn, The Netherlands). The other deuterated internal standards (ISs; d4-serotonin [SERO], d4-KYN, d3-3-3-HAA, d5-TRP, d5-5-hydroxy-3-indoleacetic acid [5-HIAA], d5-KYNA, d4- XA, and d3-QUIN) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Acetone, methanol (MeOH) and water were obtained from VWR Chemicals (Monroeville, PA, USA). Formic acid (FA) was purchased from Fisher Scientific (Portsmouth, NH, USA).

3.4.2. Preparation of standard, IS, and quality control (QC) solutions

Stock solutions, calibration standards and QC samples were prepared as described by Tömösi et al.[80] Calibration standards consisted of 100 µL of "blank" serum, 10 µL of standard solution mix (156.25–5000 nM SERO, 312.5–10000 nM KYN, 7.8–250 nM 3-HAA, 6.25–200 µM TRP, 7.8–250 nM 5-HIAA, 6.25–200 nM AA, 4.7–150 nM KYNA, 6.25–200 nM 3-HK, 1.5–50 nM XA, 3.125–100 nM PICA, and 62.5–2000 nM QUIN in 0.1% [v/v] aqueous FA), were treated with 370 µL of ice-cold acetone:MeOH (1:1, v/v) containing 10 µL of the SIL-IS mix (1500 nM d4-SERO, 1000 nM d4-KYN, 65 nM d3-3-HAA, 5250 nM d5-TRP, 200 nM d5-5-HIAA, 50 nM d5-KYNA, 90 nM d3-3-HK, 25 nM d4-XA, 80 nM d4-PICA, and 300 nM d3-QUIN) to precipitate proteins. After centrifugation, 400 µL supernatant was transferred to a new tube, spun for 15 s, and divided into two equal parts. After concentration under vacuum (Savant SC 110 A Speed Vac Plus, Savant, USA), half of the sample was treated with 70 µL of derivatising reagent (n-butanol-acetyl chloride, 9:1, v/v) and was incubated for 60 minutes at 60 °C. The mixture was dried under nitrogen before reconstitution. Both sample parts were dissolved in 100-100 µL of the starting eluent, vortexed, centrifuged and combined.

3.4.3. Preparation of human serum samples for analysis

Human serum samples were prepared as described by Tömösi et al.[80] Briefly, to 100 µL of each serum sample, 10 µL 0.1% (v/v) of aqueous FA and 370 µL of ice-cold acetone–

MeOH (1:1, v/v) containing 10 µL of the SIL-IS mix (the same as used in the preparation of the calibration standards) were added, and 400 µL supernatant was treated as mentioned above.

3.4.4. Instrumentation and UHPLC–MS/MS analysis

UHPLC separation of TRP and its metabolites was performed on pentafluorophenyl column (Phenomenex, 100 Å, 100 mm × 2.1 mm, particle size 2.6 µm) connected to an ACQUITY I-Class UPLC™ liquid chromatography system (Waters, Manchester, UK) using 0.1% (v/v) aqueous FA as solvent A and MeOH containing 0.1% (v/v) FA as solvent B. All mass spectrometric measurements were carried out on an online connected Q Exactive™ Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) operating in the positive electrospray ionisation mode. The parallel reaction monitoring data acquisition mode was selected for quantitative mass spectrometric analysis via MS/MS. The optimised parameters and the UHPLC-MS/MS analysis validation for human serum were determined previously.[80]

3.5. Methodology of the NIRS pilot study

The Ethics Committee of the University of Szeged, Albert Szent-Györgyi Clinical Centre approved our study (ID: 211/2016-SZTE). All participating patients or first-degree relatives gave written informed consent prior to NIRS monitoring. We used the INVOS™ 5100C Cerebral/Somatic Oximeter (Medtronic, Minneapolis, MN, USA) for 24-hour continuous monitoring. The NIRS sensors were placed over bilateral frontal areas corresponding to the anterior watershed territories. The application of the sensors did not delay the start of IVT or MT. Baseline rSO_2 was measured before IVT was started. rSO_2 measurements were made approximately every 30 seconds. We analysed the 5-minute average rSO_2 values registered at the start of IVT and at 1 hour, 6 hours, 12 hours, 18 hours and 24 hours after the initiation of treatment. Interhemispheric rSO_2 difference ($IH\Delta rSO_2$) was calculated as rSO_2 on the affected side minus rSO_2 measured above the contralateral side. Based on previous articles, a 4% change in rSO_2 value and a 2% change in $IH\Delta rSO_2$ were considered significant.[65,81]. Stroke subtypes were determined based on the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.[82] If LVO was present, collateral circulation on imaging was determined by a neuroradiologist using a 3-grade scale (good-intermediate-poor).

3.6. Outcome measures

3.6.1. Outcome measures in the DWI-FLAIR mismatch guided thrombolysis study

We analysed baseline and discharge NIHSS scores as outcome of efficacy. We defined ENI as any neurological improvement, as indicated by a lower NIHSS score at discharge compared to baseline. Regarding safety outcomes, we analysed the occurrence of intracerebral haemorrhage (ICH) on 24-hour repeat imaging after IVT. The extent of the haemorrhage was graded according to the European Cooperative Acute Stroke Study II (ECASS II).[83] Symptomatic ICH (sICH) was also defined according to the ECASS II trial criteria (i.e., any haemorrhage leading to death or neurological deterioration causing at least 4 point increase in the NIHSS score compared to baseline). Hypersensitivity reaction to alteplase, transfer to intensive care unit (ICU), and mortality within 90 days after stroke were recorded.

3.6.2. Outcome measures in the biomarker pilot study

Efficacy endpoints in our biomarker study were ENI and good functional outcome at 30 and 90 days after the stroke. ENI was defined according to the Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study (NINDS) criteria: at least a 4-point decrease in the NIHSS score or the patient became symptom free. However, contrary to the NINDS trial, we used the NIHSS score at discharge, not at 24 hours after treatment. The criterion for good functional outcome was a mRS score ≤ 2.

3.6.3. Outcome measures in the NIRS pilot study

Clinical outcomes were assessed with NIHSS and mRS scores. ENI was defined as per the NINDS criteria at 24 hours. Good functional outcome was a mRS score ≤ 2 at three months.

3.7. Statistical analysis

ENI and good outcomes at 30 and 90 days were categorical variables in the DWI-FLAIR mismatch guided thrombolysis and biomarker studies.

Continuous clinical variables were expressed as mean ± SD in the biomarker pilot study. The distribution of KYN metabolites and enzymatic activities were analysed with the Shapiro-Wilk test. Normally distributed variables were expressed as mean ± SD. Non-parametric data were expressed as median and IQR. Pre-thrombolysis and 12-hour KYN metabolite concentrations and enzymatic activities were compared with either paired sample

t-test or Wilcoxon matched-pairs signed-ranks test (depending on data distribution). To compare means of concentrations and enzymatic activities between groups with and without ENI or good functional outcome, we used the independent sample t-test or Mann-Whitney U test (depending on the distribution of data). Boxplots were drawn to allow for better visualisation of statistically significant findings. Furthermore, we performed receiver operating characteristic (ROC) analysis if statistical significance was met. We calculated area under the curve (AUC), as well as sensitivity (SN) and specificity (SP) for different cut-off values. Logistic regression was not performed due to the small sample size.

In the DWI-FLAIR mismatch guided thrombolysis study, the Shapiro-Wilk test was applied to analyse the distribution of continuous variables. Parametric data were expressed as mean ± SD, whereas non-parametric data were expressed as median and IQR. Between-group comparisons were made with independent samples t-test. Pearson's chi-squared test of independence was applied to compare categorical variables. Fisher's exact test was used where sample sizes were small (i.e. equal to or less than 5). To compare ENI and mortality between the groups, 95% confidence intervals (CI) and odds ratios (OR) were calculated by standard approaches. We also performed multivariable logistic regression to analyse the correlation between thrombolysis and ENI. We applied a backward likelihood ratio model selection method. Variables included in the analysis were age, gender, admission blood glucose, admission systolic and diastolic blood pressure, baseline NIHSS and thrombolysis. OR and 95% CI were again calculated.

In both studies, a p-value of <0.05 was regarded as statistically significant. 95% confidence intervals (CI) were presented where appropriate. All analyses were carried out with IBM SPSS (version 22, IBM Corp., Armonk, USA) statistical software.

Due to the small sample size of the NIRS pilot study, we did not draw statistical conclusions. Instead, we set out to describe the five NIRS recordings, and explain how these findings could correlate with cerebrovascular haemodynamics and clinical outcome.

4. Results

4.1. Results of the DWI-FLAIR mismatch guided thrombolysis study

We identified 121 patients with a clinical diagnosis of acute ischaemic stroke and DWI hyperintensity on their MRI. Patients with known and unknown stroke onset times were both included. MRI images were unavailable for review in 17 patients due to technical reasons. Twenty-five patients with LVOs were also excluded. Furthermore, eight patients were excluded because their symptom onset was confirmed as beyond the 4.5-hour time window. Eventually, 71 patients were included in our final analysis. **Figure 5** shows the flowchart of patient selection.

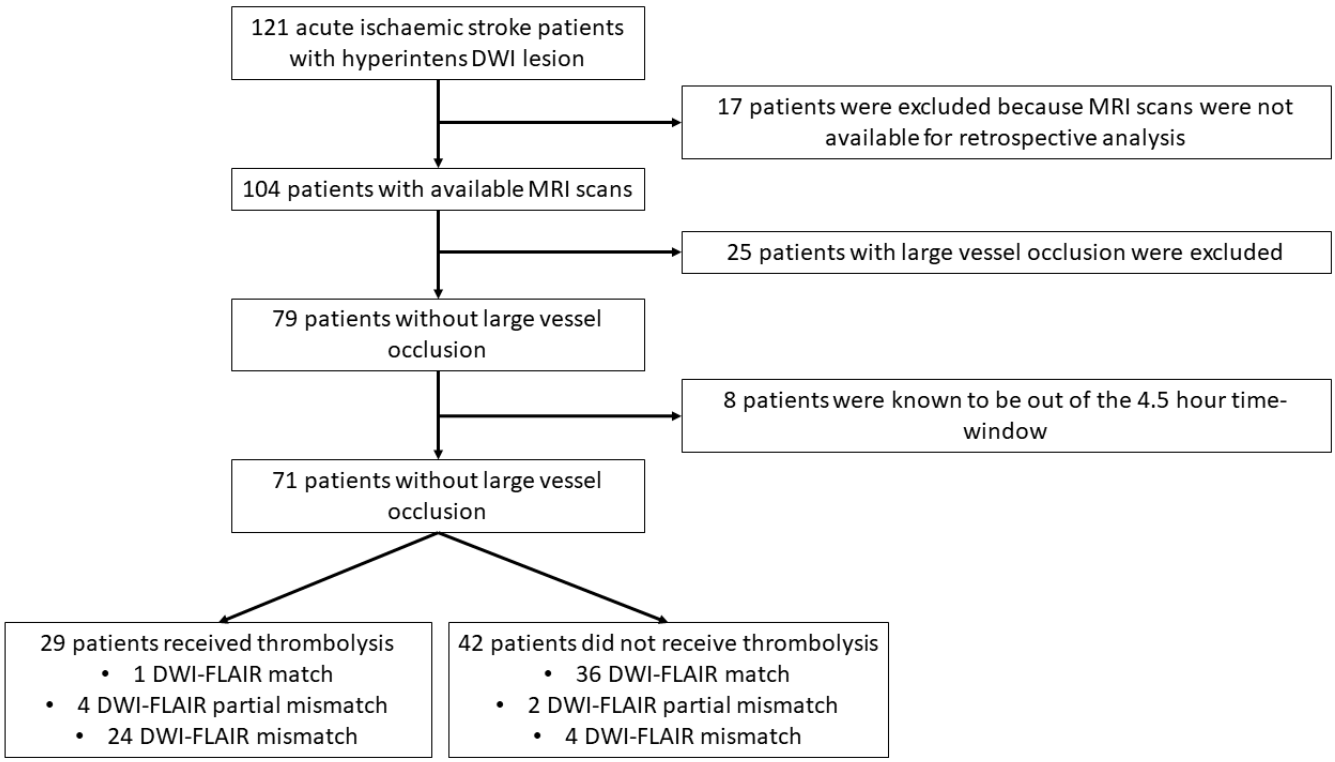


Figure 5. Flowchart demonstrating patient selection in the DWI-FLAIR mismatch guided thrombolysis study.

Twenty-nine patients received IVT. One patient received alteplase despite having a matched DWI-FLAIR pattern because the onset of symptoms was known to be within 4.5 hours. In this particular case, MRI was ordered due to diagnostic uncertainty. Six patients had

partial DWI-FLAIR mismatch on MRI. Two of these patients were not thrombolysed due to sulcal siderosis in one and a previous intracerebral haemorrhage in the other case, as detected on SWAN. Four patients had DWI-FLAIR mismatch but did not receive alteplase due to contraindications: two had signs of previous clinically significant intracerebral haemorrhage on SWAN, one had a haemorrhagic transformation of the culprit infarct, and one had pancreatic cancer and more than ten cortical microbleeds on SWAN. **Table 3** presents the demographics and clinical characteristics of our study population. One patient in the non-thrombolysed group suffered a stroke in the emergency department; therefore, a door-to-imaging time is unavailable for this case. We detected significantly more lacunar infarcts in non-thrombolysed patients (p=0.042). Otherwise, the two groups were well balanced.

	Thrombolysis (n=29)	**No thrombolysis (n=42)**	**p value**
Mean age ± SD	73.34 ± 8.66	71.52 ± 9.40	0.404
Male sex – no. (%)	16 (55.17%)	22 (52.38%)	0.817
Medical history – no. (%)			
Hypertension	26 (89.66%)	40 (95.24%)	0.393
Hyperlipidaemia	19 (65.52%)	34 (80.95%)	0.142
Diabetes mellitus	11 (37.93%)	14 (33.33%)	0.690
Smoking	8 (27.59%)	12 (28.57%)	0.928
Excess alcohol consumption	6 (20.69%)	8 (19.05%)	0.864
Atrial fibrillation	8 (27.59%)	12 (28.57%)	0.928
Carotid stenosis > 50%	4 (13.79%)	5 (11.90%)	1.000
Symptomatic carotid stenosis	4 (13.79%)	3 (7.14%)	0.433
Previous carotid endarterectomy or stenting	2 (6.90%)	1 (2.38%)	0.563
Coronary artery disease	3 (10.34%)	5 (11.90%)	1.000
Peripheral artery disease	1 (3.45%)	1 (2.38%)	1.000
Clinical parameters			
Median blood glucose (IQR) – mmol/l	7.70 (6.55-9.75)	6.70 (n=41, 6.10-9.30)	0.233

Mean systolic blood pressure ± SD - mmHg	167.93 ± 24.09	168.05 ± 30.20	0.986
Mean diastolic blood pressure ± SD - mmHg	90.14 ± 16.33	90.74 ± 18.04	0.885
Median NIHSS score at baseline (IQR)	5.00 (3.00-9.00)	4.50 (3.00-7.00)	0.337
Median NIHSS score at discharge (IQR)	3.00 (2.00-7.50)	4.00 (3.00-6.00)	0.855
Lacunar stroke	5 (17.24%)	17 (40.48%)	**0.042**
Time intervals			
Median door-to-imaging time (IQR) - min	31.00 (24.50-60.50)	36.00 (n=41, 22.00-74.50)	0.672
Median imaging-to-needle time (IQR) – min	34.00 (20.50-42.50)	-	-
Median door-to-needle time (IQR) – min	70.00 (50.00-109.00)	-	-
Median length of hospital stay (IQR) -days	5.00 (4.00-5.50)	5.00 (3.00-6.25)	0.785
Outcome measures			
Early neurological improvement	17 (58.62%)	13 (30.95%)	**0.020**
Haemorrhagic transformation	2 (6.90%)	-	-
sICH	1 (3.45%)	-	-
Transfer to intensive care unit	0 (0.00%)	0 (0.00%)	-
Allergic reaction to alteplase	0 (0.00%)	-	-
Mortality at 90 days	2 (n=20, 10.00%)	4 (n=33, 12.12%)	1.000

Table 3. Demographics and clinical characteristics of patients in the DWI-FLAIR mismatch guided thrombolysis study. Some data were not available for all patients. These are highlighted at each criterion accordingly.

4.1.1. Efficacy and safety outcomes

Significantly more patients had ENI in the thrombolysed group (OR, 3.16; 95% CI, 1.178-8.479; p=0.020). In the multivariable logistic regression analysis, IVT was the only variable correlated with ENI (OR, 3.051; 95% CI, 1.135-8.206; p=0.027).

Only patients in the thrombolysis arm had follow-up imaging. There were no clinical indications for a repeat scan in any non-thrombolysed patients. Two thrombolysed patients had ICH on follow-up imaging (6.90%). One patient had parenchymal haemorrhage type I (PH 1), and another had a remote PH 2. Only the patient with PH 2 had an sICH (3.45%). He died five days after IVT. The initial MRI scan showed a complete DWI-FLAIR mismatch for a right hemispheric lacunar infarct without hypointense signal changes on SWAN. The follow-up CT (**Figure 6[A-D]**) showed a large left parieto-temporo-occipital haemorrhage with perifocal oedema, mass effect, and propagation into the ventricles.

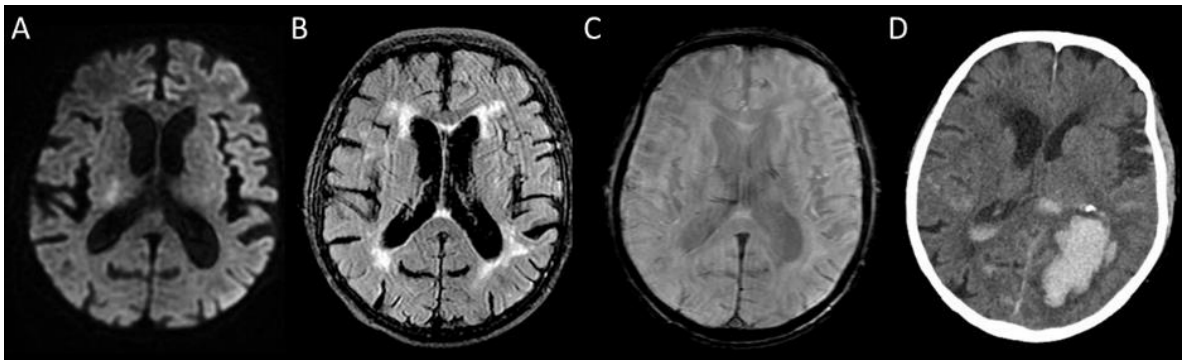


Figure 6. Pre-thrombolysis MRI and 24-hour post-treatment CT of the patient with sICH. A) DWI demonstrating a right hemispheric lacunar infarct. B) No corresponding hyperintensity is visible on FLAIR. Periventricular white matter hyperintensities indicate small vessel disease. C) No microhaemorrhage was detected on SWAN. D) 24-hour repeat CT shows a remote, left parieto-temporo-occipital haemorrhage with perifocal oedema, mass effect, and propagation into the lateral ventricles.

There was no hypersensitivity reaction to alteplase, and there was no need for ICU transfer for ventilation or vasopressor support in either group.

Eighteen patients were lost to long-term follow-up. Of the remaining 53 individuals, six died within 90 days after stroke (11.32%): two in the thrombolysed (n=20, 10.00%) and

four in the non-thrombolysed group (n=33, 12.12%), with statistically non-significant odds (OR, 0.81, 95% CI, 0.134-4.856; p=1.000). Autopsies were not performed, and the cause of death was determined on clinical grounds. In the thrombolysed group, one patient died five days after stroke due to the previously mentioned sICH, and another died 18 days after stroke due to complications from a sacral pressure sore. In the non-thrombolysed group, one patient died 34 days post-stroke from decompensation of heart failure, and two patients died due to infections at 18 (pneumonia) and 63 (Clostridium difficile) days after stroke, respectively. One patient died at 25 days from complications of a posterior circulation stroke.

4.2. Results of the biomarker pilot study

Our pilot study included 48 patients. Thirty-nine were known to be within the 4.5-hour thrombolysis time window. In the remaining nine patients with unknown stroke onset time, IVT was carried out based on DWI-FLAIR mismatch on acute brain MRI, as per the WAKE-UP trial.[26] The flowchart of patient selection is shown in **Figure 7**.

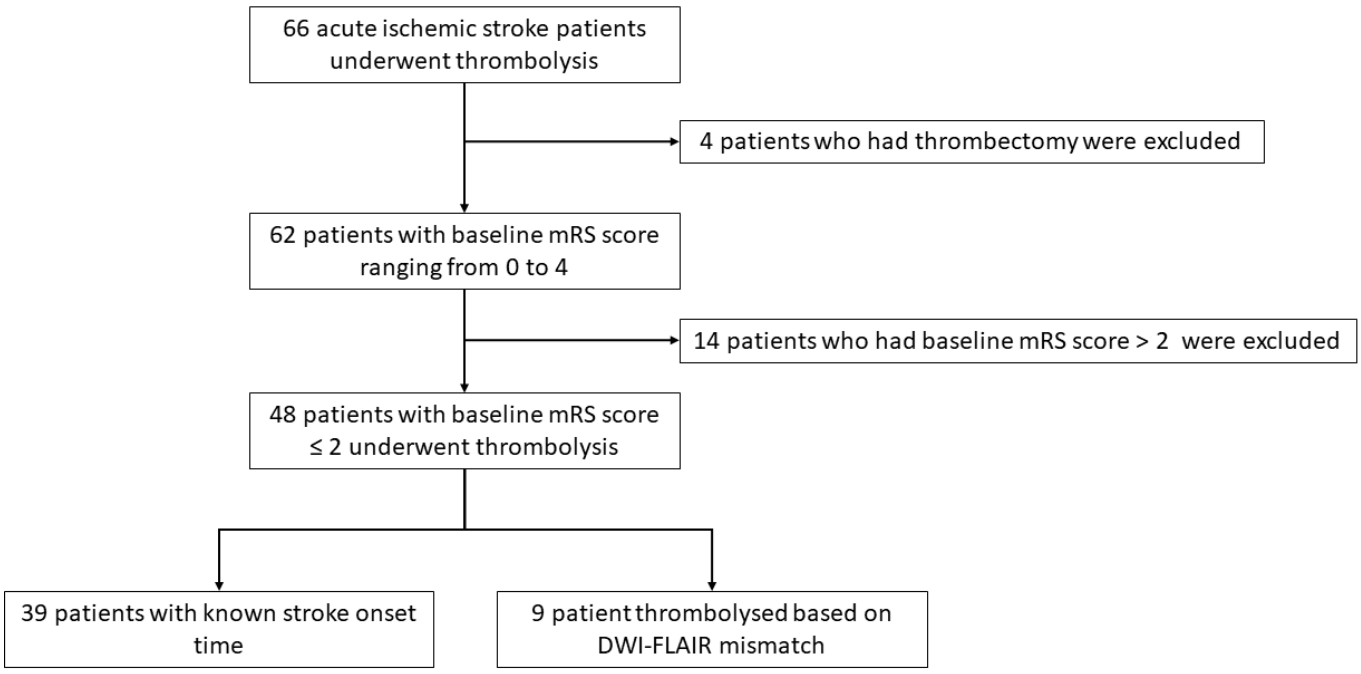


Figure 7. Flowchart showing patient enrolment in the biomarker pilot study.

The clinical characteristics of the biomarker study population are presented in **Table 4**. Seventeen patients had LVO, but MT was not performed due to the limited availability of this service in our centre at the time. We collected 32 blood samples before thrombolysis and 36 samples 12 hours after treatment. Twenty-three patients had samples taken at both time points.

Mean age ± SD (years)	67.33 ± 12.04
Female	24 (50%)
Male	24 (50%)
Hypertension	41 (85.42%)
Diabetes mellitus	13 (27.08%)
Hyperlipidemia	39 (81.25%)
Smoking	14 (29.17%)
Atrial fibrillation	7 (14.58%)
Coronary artery disease	10 (20.83%)
Mean baseline NIHSS score ± SD	8.81 ± 4.29
Mean baseline mRS score ± SD	0.79 ± 0.77
Large-vessel occlusion	17 (35.42%)
Mean stroke onset-to-needle time ± SD (min, n=39)	136.59 ± 53.9
Mean door-to-needle time ± SD (min, n=47)	57.45 ± 35.72
Mean length of stay in Stroke Unit (days)	4.91 ± 2.05
Intracerebral haemorrhage after treatment	4 (8.33%)
Mean C-reactive protein ± SD (mg/l, n=47)	10.44 ± 18.78
Mean white cell count ± SD (G/l)	8.07 ± 2.31
Mean discharge NIHSS score ± SD	6.71 ± 7.89
Early neurological improvement	19 (39.58%)
Mean mRS score at 30 days ± SD (n=45)	2.47 ± 1.84
Mean mRS score at 90 days ± SD (n=40)	2.38 ± 1.9
Good functional outcome at day 30 (n=45)	27 (60%)
Good functional outcome at day 90 (n=40)	24 (60%)

Table 4. Demographic and clinical data of our biomarker study population (n=48). Some data were not available for all patients. These are highlighted after each criterion accordingly.

We simultaneously quantified TRP and its ten essential metabolites (SERO, KYN, 3-HAA, 5-HIAA, AA, KYNA, 3-HK, XA, PICA, and QUIN) with the UHPLC–MS/MS method.[80] Concentration of the measured KYN metabolites of the 23 patients who had sampling at both time points is shown in **Table 5**. Significant changes in paired serum levels were observed for KYN, AA, KYNA, XA, PICA and QUIN. Enzymatic activity of IDO, MAO and KAT was calculated by the following ratios: KYN/TRP, 5-HIAA/SERO and KYNA/KYN, respectively. The changes in enzymatic activities were significant for IDO and MAO, which is also indicated in **Table 5**.

	Sample A (nM)	**Sample B (nM)**	**p value**
SERO	655.34 ± 301.3	680.97 ± 263.54	0.44
KYN	3669.52 ± 1044.79	3413.1 ± 1114.47	**0.03**
3-HAA	53.07 (40.9-79.74)	43.33 (35.58-62.01)	0.05
TRP	46371.75 (41588.69-54644.26)	50599.91 (41798.89-53961.54)	0.56
5-HIAA	96.58 ± 35.47	88.15 ± 36.29	0.18
AA	53.75 (38.73-68.44)	43.8 (27.07-60.2)	**0.01**
KYNA	45.44 ± 20.02	37.69 ± 14.55	**0.004**
XA	11.27 (5.8-16.51)	4.58 (2.56-7.93)	**0.001**
3-HK	125.68 (79.33-175.98)	115.73 (87.04-161.72)	0.26
PICA	43.33 (34.09-52.22)	29.47 (24.12-39.6)	**<0.001**
QUIN	673.69 ± 230.82	620.26 ± 236.66	**0.001**
IDO	0.08 ± 0.02	0.07 ± 0.02	**0.02**
MAO	0.16 (0.11-0.21)	0.12 (0.08-0.2)	**0.02**
KAT	0.01 (0.01-0.02)	0.01 (0.01-0.01)	0.14

Table 5. Mean and median concentrations of KYN metabolites taken before IVT (sample A), and 12 hours after treatment (sample B). Twenty-three patients were sampled. The unit of measurement for all metabolites was nanomole (nM). Enzymatic activities are also presented (these ratios do not have units of measurement).

Patients with ENI had significantly lower concentrations of KYNA and lower KAT activity in sample A (independent sample t-test, p=0.01, and Mann-Whitney U test, p=0.002, respectively, **Figure 8**). There were no statistically significant correlations between sample B concentrations and ENI. We also did not find any correlation regarding good outcomes at 30 and 90 days with concentrations or enzymatic activities in samples A or B.

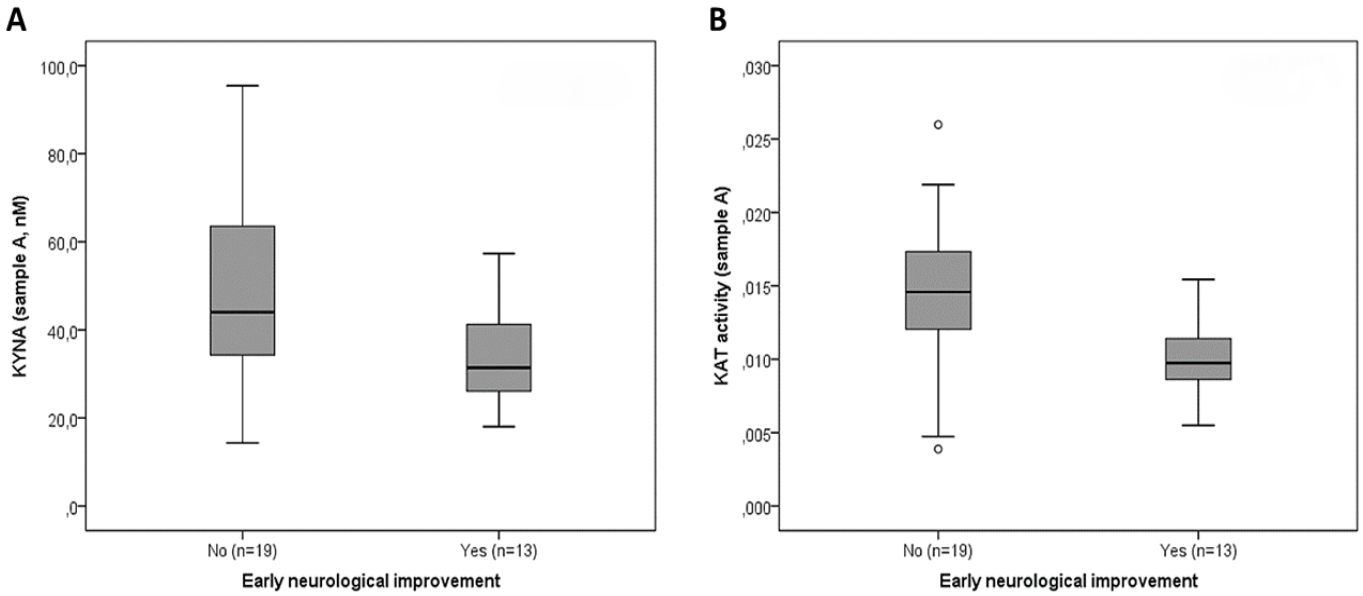


Figure 8. Boxplots highlight the significant difference between pre-treatment KYNA levels (A) and KAT activity (B) between patients without and with ENI. For each box, the horizontal line inside the box shows the median. The ends of the boxes represent the first and third quartiles. The whiskers extend to the highest and lowest values not considered outliers (defined as 1.5 times the IQR). Outliers are shown as circles.

We performed ROC analysis for ENI with KYNA levels and KAT activity measured before treatment (**Figure 9**). AUC for KYNA concentrations was 0.74, 95% CI 0.57-0.91, p=0.02. The optimal cut-off concentration to predict ENI was 37.8 nM (SN 69.2%, SP 68.4%). Similarly, AUC for KAT activity was 0.82, 95% CI 0.67-0.98, p=0.002, and the optimal cut-off activity was 0.0127 (SN 92.3%, SP 73.7%).

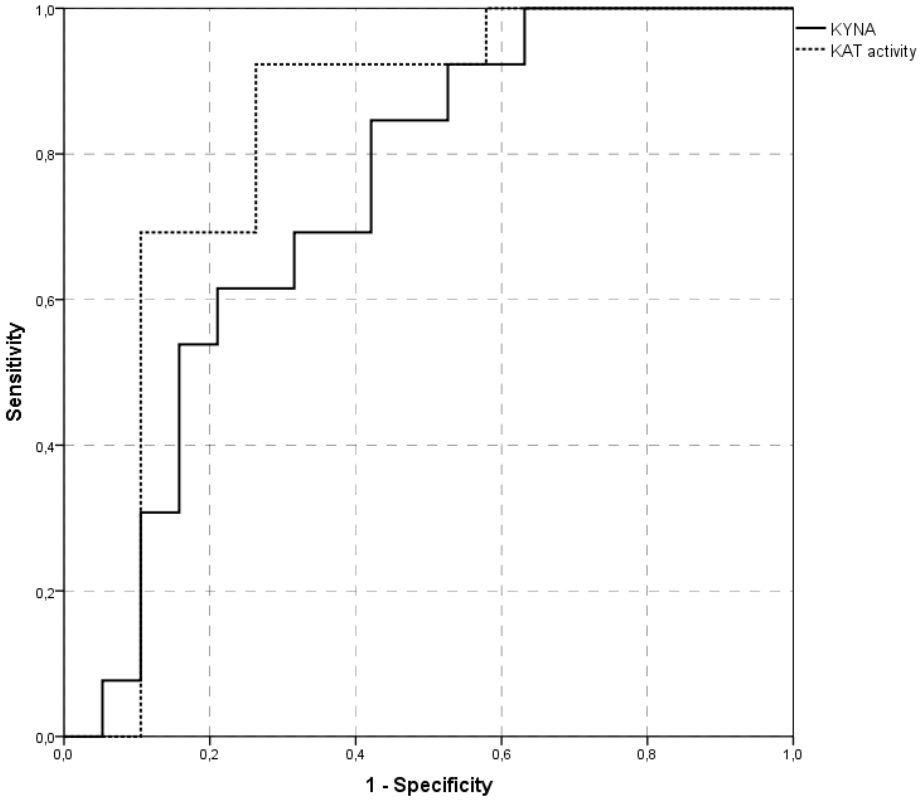


Figure 9. ROC curve showing the accuracy of pre-thrombolysis KYNA concentration and KAT activity in predicting ENI.

4.3. Results of the NIRS pilot study

Our study population included five acute stroke patients with left-sided anterior circulation infarcts. Patient 3 had MT due to M1 segment occlusion. Detailed patient characteristics are shown in **Table 6**. All participants received alteplase according to the Hungarian Acute Ischaemic Stroke Diagnostic and Treatment Recommendations.[79] Before IVT, Patients 1-3 had CT angiography (CTA), and Patients 4-5 had MR angiography – time of flight (MRA-TOF) imaging. **Figure 10** shows the CT and MRI scans approximately 24 hours after thrombolysis. Blood pressure, SpO_2, heart rate and electrocardiography were monitored for all patients throughout the study period. SpO_2 was above 92% in all but one case while patients were breathing ambient air; therefore, O_2 supplementation was not required. The only exception was Patient 3, who had MT under general anaesthesia because he could not cooperate during the procedure due to severe aphasia.

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**
Sex	female	male	male	female	female
Age	80	67	63	66	78
Vessel territory	left MCA	left ICA	left MCA	left MCA	left MCA
Last known well-to-treatment time (min)	128	97	200	245	228
Hypertension	yes	yes	yes	yes	yes
Hyperlipidaemia	no	yes	yes	yes	yes
Diabetes mellitus	no	no	no	no	no
Atrial fibrillation	no	no	no	no	no
Ischaemic heart disease	yes	no	yes	no	yes
Smoking	no	no	yes	yes	yes
Haemoglobin (g/l)	142	140	139	153	121
Large-vessel occlusion	left M2	left ICA	left M1	no	chronic right ICA
Collateral score	good	good	intermediate	not applicable	not applicable
Stroke subtype (TOAST)	CE	LAA	CE	SVD	CE
NIHSS baseline	14	15	17	7	9
NIHSS at 24-hours	9	12	12	10	4
ENI (according to NINDS criteria)	yes	no	yes	no	yes
NIHSS at discharge	4	9	8	10	1
Length of hospital stay (days)	8	4	4	6	5
Discharge destination	home	NRU	NRU	NRU	IM
mRS at 90 days	2	2	1	3	1

Table 6. Patient characteristics in the NIRS pilot study

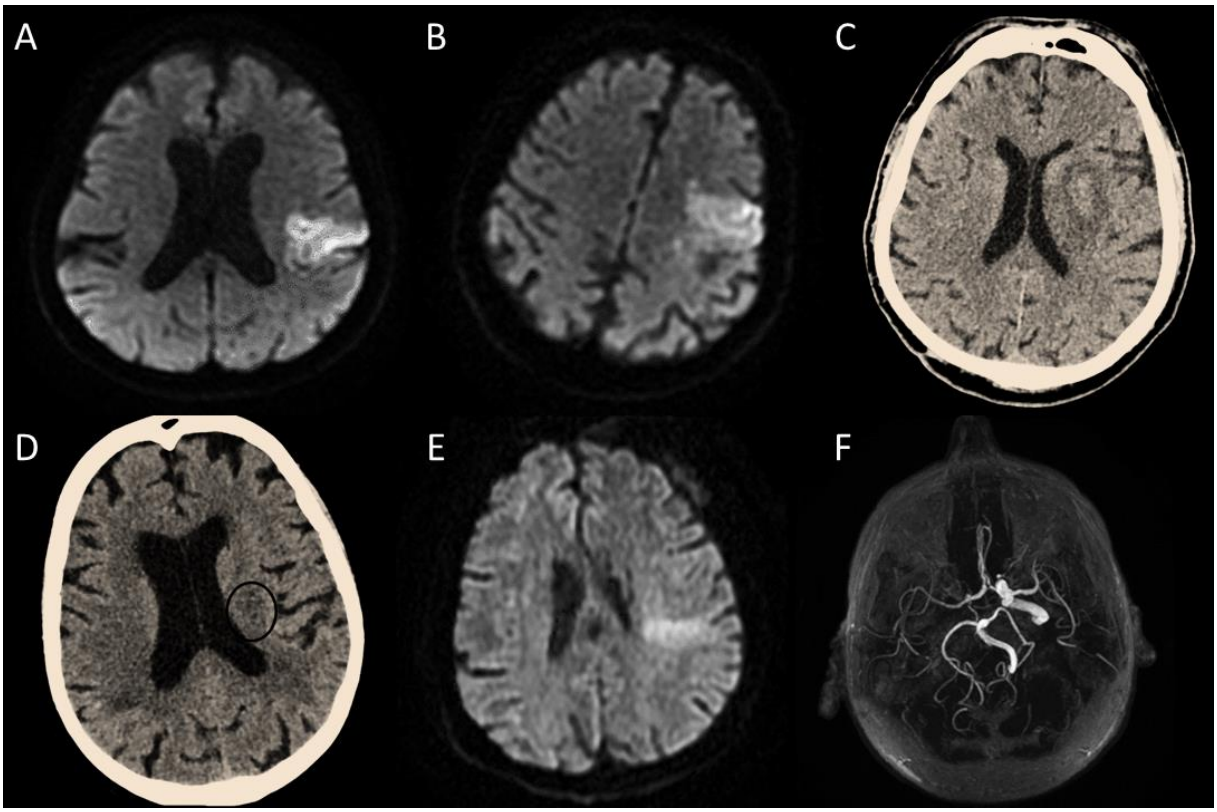
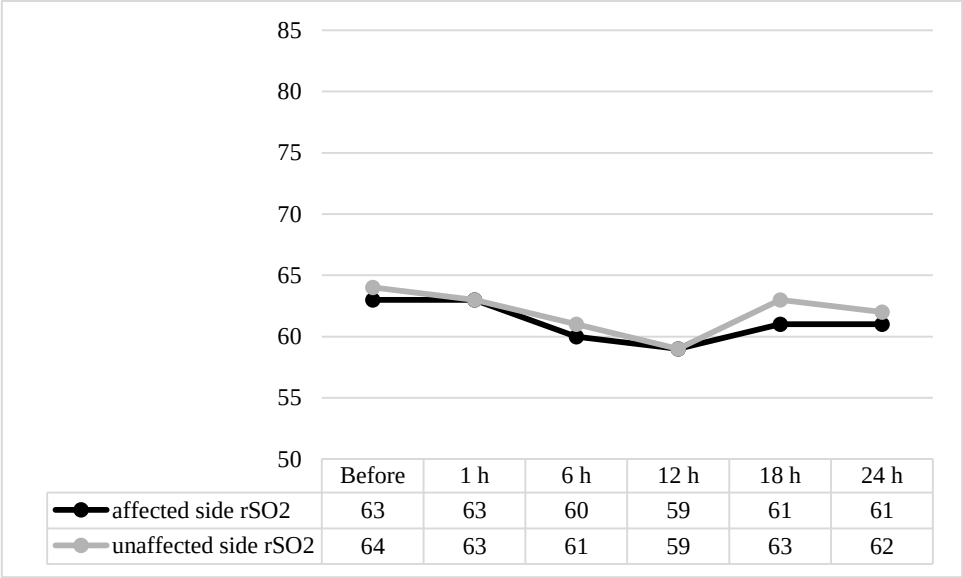


Figure 10. CT and MRI scans of patients approximately 24 hours after reperfusion treatment. A) DWI scan of Patient 1, showing left MCA territory cortical hyperintensity. B) DWI scan of Patient 2 shows a similar, embolic-appearing acute brain infarct. C) Non-contrast CT (NCCT) scan of Patient 3 after thrombectomy. The infarct involves the left basal ganglia, internal capsule and corona radiata. D) NCCT of Patient 4 shows slight hypodensity in the left corona radiata. E) DWI scan of Patient 5 shows a left MCA territory infarct. F) Patient 5's MRA-TOF reconstruction demonstrates enlarged left ICA and MCA.

4.3.1. Descriptive analysis of the NIRS recordings

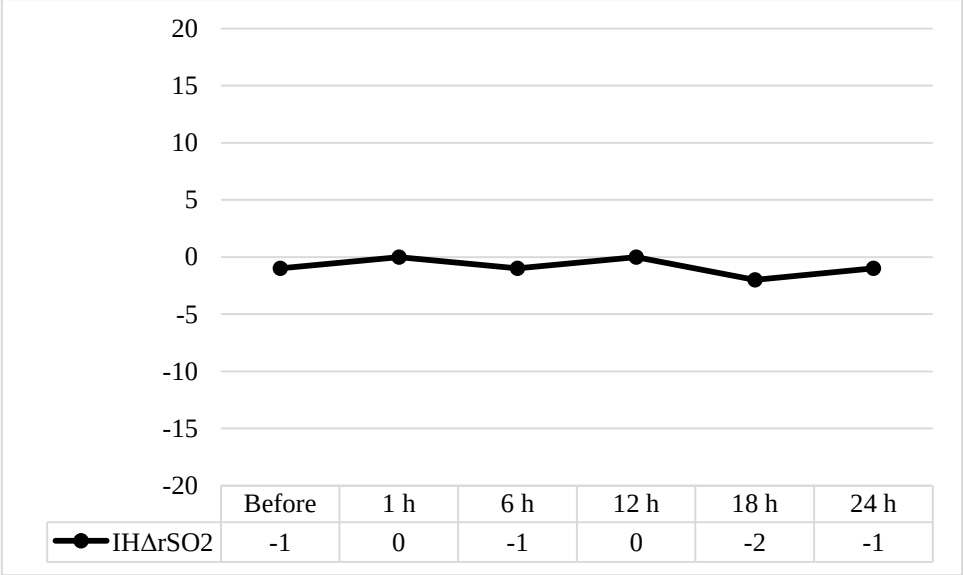
4.3.1.1. Patient 1

Patient 1 suffered a left MCA infarct due to an M2 occlusion. CTA showed good collateral circulation. We did not observe any relevant rSO_2 difference between the two hemispheres ($IH\Delta rSO_2$ was between -2% and 0%). rSO_2 values were relatively stable on both sides. The patient achieved ENI defined according to the NINDS criteria at 24 hours and had a good clinical outcome at three months.



	Before	1 h	6 h	12 h	18 h	24 h
affected side rSO2	63	63	60	59	61	61
unaffected side rSO2	64	63	61	59	63	62

Figure 11. rSO_2 (%) trends of Patient 1.



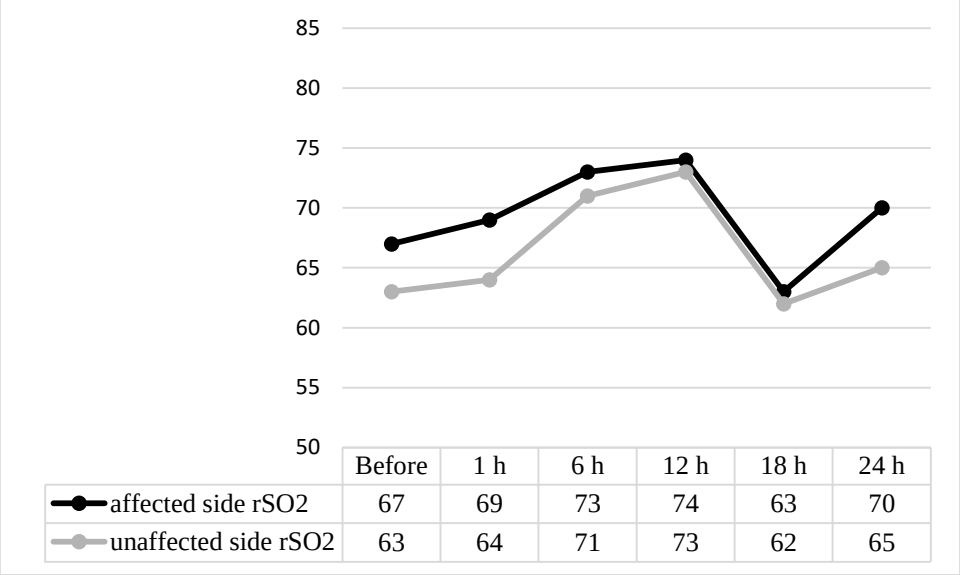
	Before	1 h	6 h	12 h	18 h	24 h
IHΔrSO2	-1	0	-1	0	-2	-1

Figure 12. $IH\Delta rSO_2$ (%) trend of Patient 1.

4.3.1.2. Patient 2

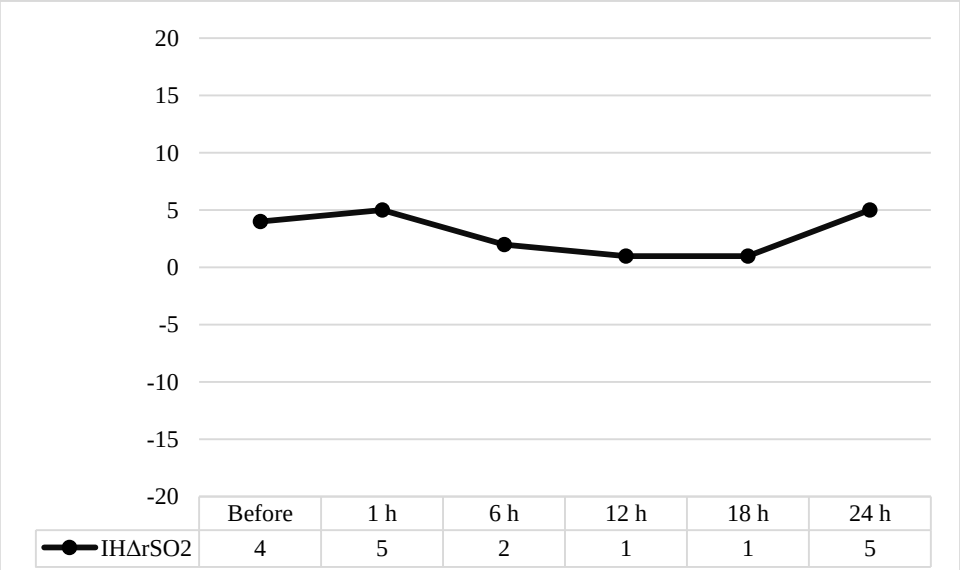
Patient 2 had a left internal carotid artery (ICA) occlusion above the bifurcation due to severe atherosclerosis. CTA showed good collaterals, and rSO_2 absolute values were higher above the ipsilateral side (average $IH\Delta rSO_2$ was 3%). rSO2 levels gradually rose on both sides in the first 12 hours, possibly indicating a subtle increase in CBV and CBF in the leptomeningeal collaterals. Although he did not fulfil the criteria for ENI, the patient's NIHSS

score decreased in the first few days, and eventually, he had a favourable clinical outcome at three months.



	Before	1 h	6 h	12 h	18 h	24 h
affected side rSO2	67	69	73	74	63	70
unaffected side rSO2	63	64	71	73	62	65

Figure 13. rSO_2 (%) trends of Patient 2.



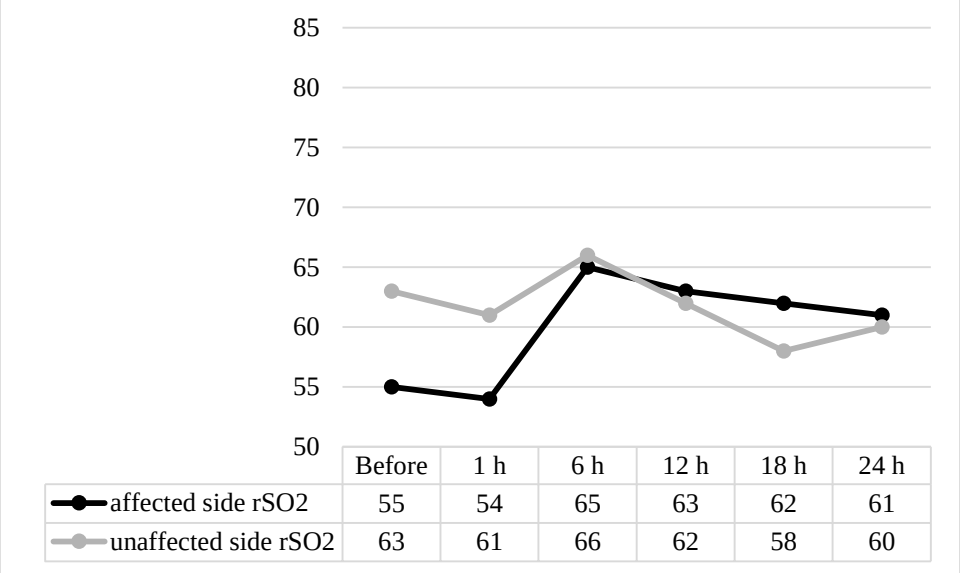
	Before	1 h	6 h	12 h	18 h	24 h
IHΔrSO2	4	5	2	1	1	5

Figure 14. $IH\Delta rSO_2$ (%) trend of Patient 2.

4.3.1.3. Patient 3

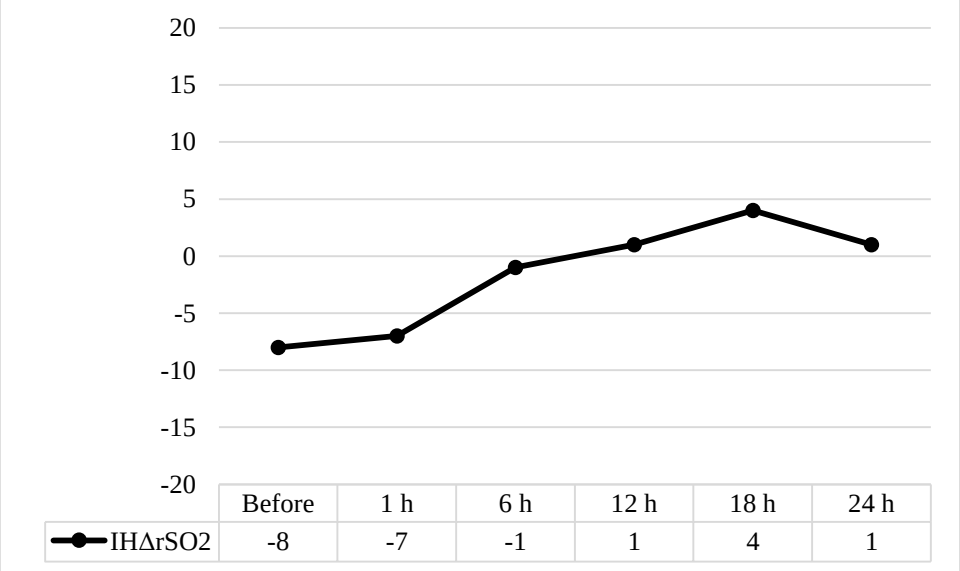
Patient 3 had a left M1 occlusion, and he underwent MT after IVT. CTA showed intermediate collaterals. Initially, the affected side had a lower absolute rSO_2 value (55% vs 63%). After IVT, this difference did not change (1 h post thrombolysis $IH\Delta rSO_2$ was -7%).

However, after MT, there was a significant increase in rSO_2 on the ipsilateral side; consequently, $IH\Delta rSO_2$ substantially decreased. $IH\Delta rSO_2$ absolute values even became positive after 12 hours. These findings possibly imply that NIRS sensors were placed above ischaemic territory or that the leptomeningeal collateral circulation was insufficient. As expected from the NIRS recording of the first 24 hours, the patient recovered well (the definition of ENI was met, and mRS was 1 at 90 days).



	Before	1 h	6 h	12 h	18 h	24 h
affected side rSO2	55	54	65	63	62	61
unaffected side rSO2	63	61	66	62	58	60

Figure 15. rSO_2 (%) trends of Patient 3.



	Before	1 h	6 h	12 h	18 h	24 h
IHΔrSO2	-8	-7	-1	1	4	1

Figure 16. $IH\Delta rSO_2$ (%) trend of Patient 3.

4.3.1.4. Patient 4

Patient 4 did not meet the criteria for ENI, and she was the only individual who did not achieve a good functional outcome at 90 days. She suffered a left MCA territory infarction without LVO. Baseline absolute rSO_2 was significantly higher on the ipsilateral side (69% vs 61%). After one hour, a marked increase of rSO_2 was observed above both hemispheres (+9%, $IH\Delta rSO_2$ remained at 8%). $IH\Delta rSO_2$ then steeply decreased to -2% at 12 hours. The patient's NIHSS score worsened. A follow-up CT scan demonstrated a left striatocapsular infarct. The striatocapsular territory is supplied by perforator arteries stemming from the proximal part of M1 and does not have collateral circulation.[84] Since rSO_2 increased similarly above both hemispheres in the first hour, the ischaemic insult possibly provoked an increase in global cerebral perfusion.

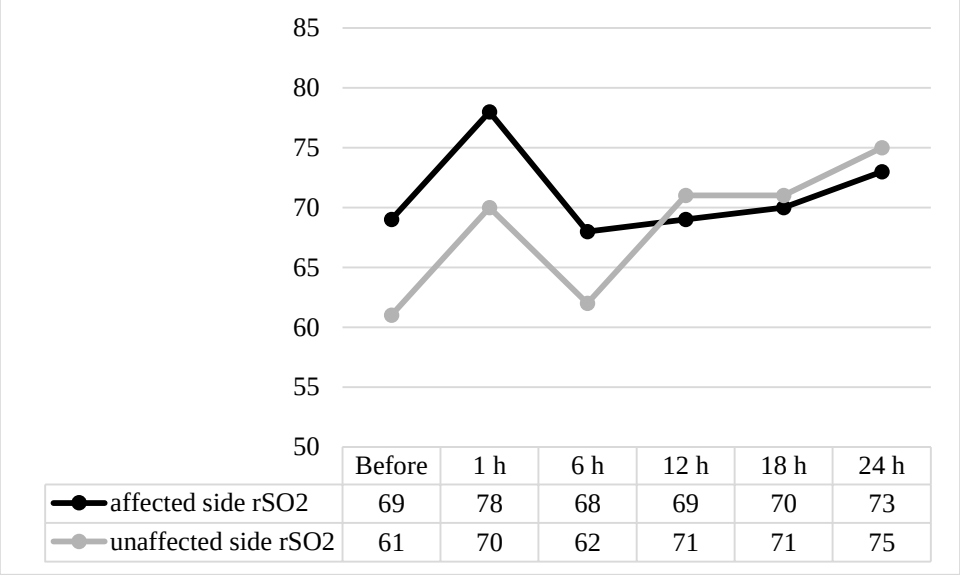


Figure 17. rSO_2 (%) trends of Patient 4.

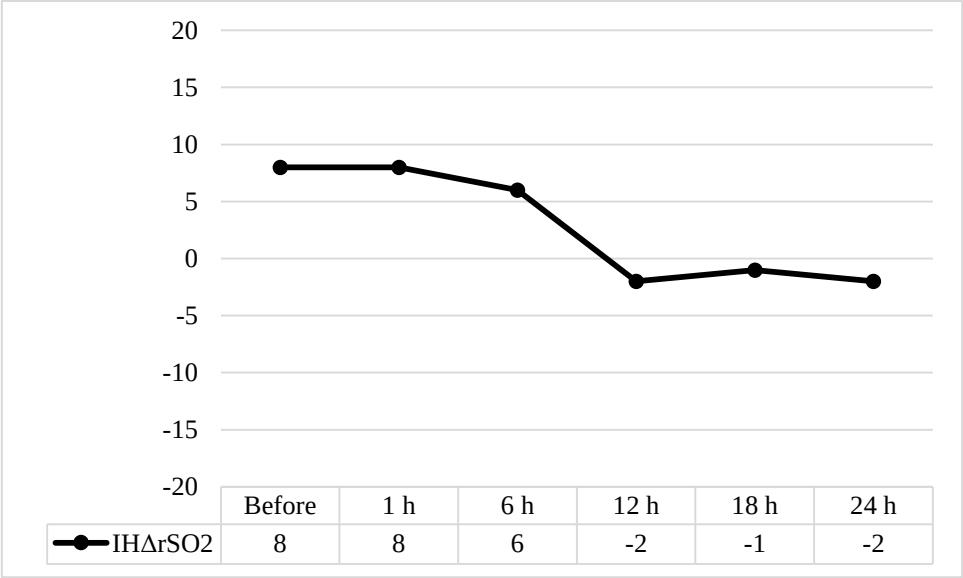
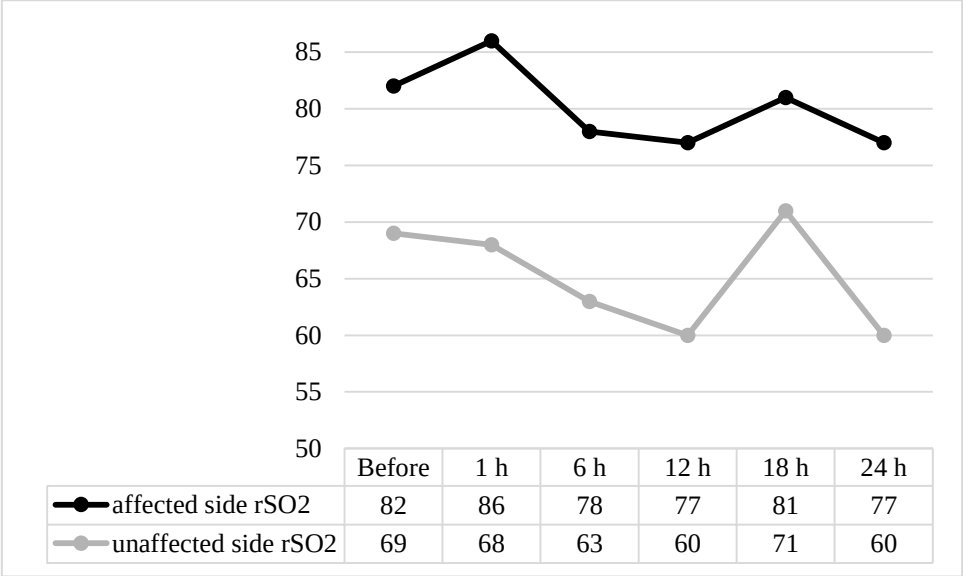


Figure 18. IHΔrSO$_2$ (%) trend of Patient 4.

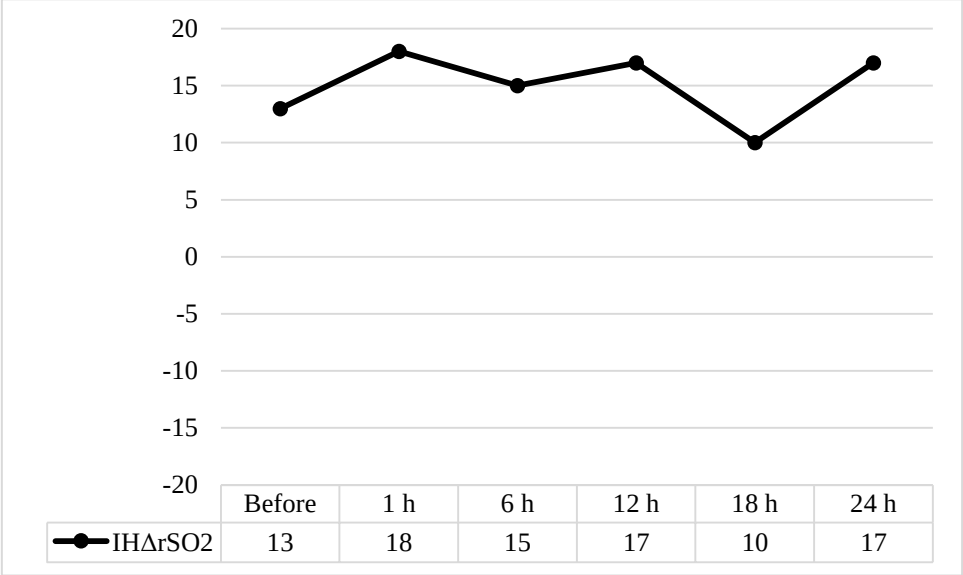
4.3.1.5. Patient 5

Patient 5 had elevated rSO$_2$ values above the ipsilateral hemisphere (82% vs 69%). The significantly high IHΔrSO$_2$ was possibly a consequence of chronic right ICA occlusion, leading to collateralization via the circle of Willis, and consequent enlargement of left ICA, MCA, and anterior cerebral artery (ACA) (**Figure 10/F**). Increased blood flow in the left MCA and ACA explains the high rSO$_2$ values above the ipsilateral watershed area, implying well-developed leptomeningeal collaterals. The IHΔrSO$_2$ value remained high throughout the 24-hour monitoring, and the patient achieved ENI and good functional outcome at three months.



	Before	1 h	6 h	12 h	18 h	24 h
affected side rSO2	82	86	78	77	81	77
unaffected side rSO2	69	68	63	60	71	60

Figure 19. rSO_2 (%) trends of Patient 5.



	Before	1 h	6 h	12 h	18 h	24 h
IHΔrSO2	13	18	15	17	10	17

Figure 20. $IH\Delta rSO_2$ (%) trend of Patient 5.

5. Discussion

5.1. Discussion of the DWI-FLAIR mismatch guided thrombolysis study

Our real-world data support using the DWI-FLAIR mismatch concept for alteplase treatment in acute ischaemic stroke patients without LVO. We analysed ENI to investigate the short-term response to alteplase because the beneficial effect of IVT could be better reflected by the change in NIHSS score rather than the 90-day mRS score. The latter depends on

various other factors, such as comorbidities, the availability and quality of rehabilitation, and socioeconomic status. As the baseline NIHSS scores were relatively low in our study (a median of 5 and 4.5 points in the thrombolysed and non-thrombolysed arms, respectively), we felt that defining ENI similar to the NINDS trial was not practical. Data was not available for 24-hour NIHSS scores. Therefore, we measured NIHSS scores at discharge, which in our opinion, is reasonable practice considering that the median length of hospital stay was only five days in both groups. Saver and Altman demonstrated that the NIHSS score at 24 hours showed a similar correlation with the 90-day functional outcome as the NIHSS score taken 7-10 days after stroke.[85]

Due to the relatively low baseline NIHSS scores, we felt that any short-term improvement in the NIHSS could be considered a favourable response to treatment that would lead to a good functional outcome at 90 days. Unfortunately, 90-day outcome data was scarce. Consequently, we could not correlate our definition of ENI with 90-day mRS scores.

One might argue that ENI, as defined in our study, could result from spontaneous recovery and not a consequence of IVT. 16-24% of patients with a disabling stroke reportedly achieve good functional outcomes at one week or upon discharge, without thrombolysis.[86,87] Lacunar syndromes were significantly more common in patients with neurological recovery. Lacunar strokes result from lipohyalinosis of small perforating arteries, branch atheromatous disease or microembolisms, and they usually have milder clinical presentations compared to large-artery atherosclerosis or cardioembolic strokes.[88] An interesting observation in our study was the significant difference in the proportion of lacunar strokes, which was significantly more common in the non-thrombolysed group. Conversely, ENI was more frequent among thrombolysed patients, which supports the short-term effectiveness of IVT over spontaneous recovery. Multivariable regression analysis also demonstrated a significant association between ENI and IVT. Furthermore, the efficacy of IVT in lacunar stroke has been proven in several clinical studies and the post hoc secondary analysis of the WAKE-UP trial.[89-91]

Regarding safety outcomes, we compared our results with those of the WAKE-UP trial. It is worth mentioning that 33.7% of patients in the treated arm of the WAKE-UP trial had occlusion of a large intracranial artery. As discussed earlier in the Methods section, we excluded these patients from our retrospective analysis. The number of patients with PH1 was not reported in the WAKE-UP trial. The percentage of PH2 according to ECASS II was similar: 3.45% in our study compared to 4.0% in the randomised clinical trial. In a pooled

analysis of thrombolysis trials, the rate of severe ICH was 5.2%. In the included studies, symptom onset times were known, and alteplase could be given up to 6 hours after symptom onset. The percentage of sICH was also similar: 3.45% in our population vs 2.8% in the WAKE-UP trial.

In patients who received alteplase, the 10% mortality at 90 days in our population was higher than the 4.1% in the WAKE-UP study. Potential explanations are that our patients were older with more vascular risk factors, and were more disabled at baseline. We did not contraindicate alteplase treatment based on mRS, whereas only patients with mRS 0-1 were included in the WAKE-UP trial.

Our study included only four patients who had thrombolysis with DWI-FLAIR partial mismatch. Therefore, meaningful conclusions about the safety and efficacy of alteplase treatment in patients with such imaging patterns cannot be drawn. Jakubicek et al. reported that 27 thrombolysed patients with partial mismatch did not have higher rates of sICH than 37 patients without FLAIR signal change.[92] The beneficial effects of alteplase were similar in the two groups. They implied that the mismatch pattern used in the WAKE-UP trial might be over-selective. Similarly, a report from the Bernese stroke registry found no association between FLAIR hyperintensities and sICH after IVT and MT.[93] Treatment in that study was indicated based on an "eyeball" assessment of whether the corresponding FLAIR signal change exceeded 50% of the DWI lesion. Among 159 patients included, 89 had partial DWI-FLAIR mismatch, in whom the rate of sICH was 6.7%. We believe that IVT in partial DWI-FLAIR mismatch should be tested in large, prospective, randomised clinical trials to assess whether a broader population of patients could benefit from thrombolysis.

Limitations of our study include the small sample size and the retrospective observational nature of data collection. However, the two groups in our study were similar regarding vascular risk factor profile and medical management pre-stroke. Based on their DWI-FLAIR pattern, the non-thrombolysed group comprised patients with onset times most probably beyond 4.5 hours. In addition to comparing the rates of ENI between the two groups, we also performed a logistic regression analysis to investigate the predictors of ENI. This regression analysis identified IVT as the only variable associated with ENI.

5.2. Discussion of the biomarker pilot study

To our knowledge, we performed the first study that analysed changes in KYN metabolite serum levels and enzymatic activities in acute ischemic stroke patients who received IVT. The main finding of our pilot study is that patients with ENI have significantly lower concentrations of KYNA and lower KAT activity before alteplase treatment. We therefore propose that pre-thrombolysis KYNA levels and KAT activity are potential biomarkers of ENI. Another study by our workgroup supports this hypothesis: Boros et al. found that NIHSS improvement after thrombolysis was more frequent in patients with the rs10988134 variant of the KAT1 enzyme.[94] KAT1 is the most relevant of four isoforms of KAT in the peripheral blood of humans.[46] It is a 422 amino acid protein encoded by the KYAT1 gene on the long arm of chromosome 9. The rs10988134 single nucleotide polymorphism (C/T) within this gene is proposed to affect the stability of the KAT1 transcript.[95]

It should be highlighted that similarly to the DWI-FLAIR mismatch guided thrombolysis study, ENI was assessed based on the difference between the admission and discharge NIHSS scores (24-hour NIHSS scores were unavailable). The mean length of hospital stay was 4.91 days. We believe that the approximately four-day difference between 24-hour and discharge NIHSS scores does not confound the beneficial effect of intravenous alteplase.[85] Since it suited our population, we applied the NINDS criteria to define ENI (mean baseline NIHSS score was 8.81). About 40% of patients achieved ENI, and 60% had a favourable outcome at 30 and 90 days after IVT. In univariate analysis, patients with ENI had significantly higher odds of achieving mRS ≤ 2 at 30 days (OR 5.385, 95%CI 1.261-22.987, p=0.23) compared to patients who did not show ENI. The same was true for 90-day functional outcomes, but this trend did not meet statistical significance in the univariate model (OR 4.333, 95%CI 0.978-19.202, p=0.054).

Based on previous research in animal stroke models, we hypothesized that neuroprotective KYNA would be higher in patients with better outcomes.[39,57] On the contrary, lower concentrations of KYNA and lower KAT activity were found to predict good treatment response with good sensitivity and specificity. Our findings are similar to previous observations by Darlington et al., who detected higher KYNA levels in patients who died within 21 days after ischemic stroke, compared to those who survived.[37] It is unclear why

increased levels of KYNA are associated with worse clinical outcomes in ischemic stroke. One possible explanation is that KYNA is an endogenous antagonist of the NMDAR. Therefore, it could further decrease synaptic activity in brain ischemia and worsen brain function.[37,47] It has also been reported that KYNA can interfere with mitochondrial respiration, resulting in reduced ATP synthesis and increased levels of oxidative stress.[96]

We found no correlation between KYN metabolites or enzymatic activities and good functional outcomes measured 30 and 90 days after stroke. This finding was somewhat surprising, given the results of the ROC analysis. Brouns et al. reported that the KYN/TRP ratio correlated with the 3-month mRS score.[36] Possible explanations behind the lack of correlation in our study are the small sample size and the effect of thrombolytic treatment. Apart from the small sample size, the main limitation of our pilot study is the absence of a control group. An ideal control group would have comprised acute ischemic stroke patients who did not receive alteplase treatment. However, this would have been unethical, given the evidence behind alteplase treatment.[20] We could not obtain blood samples from every patient at both time points due to the occasional limited availability of our biobank facility, and we lost a few patients to follow-up. Logistic regression analysis was not performed due to the small number of individuals in our study.

It is important to note that KYN metabolites were measured from the serum of patients. Therefore, our findings do not reflect the intracerebral changes in KYN metabolites and enzymes in acute ischemic stroke. It was reported that approximately 40% of KYN is produced locally in the central nervous system, and the remaining 60% is taken up from the circulation. [97] Metabolites that can cross the blood-brain barrier (BBB) via large neutral amino acid transporters are TRP, KYN and 3-HK.[46,98] KYNA only has a limited ability to traverse across the BBB.

5.3. Discussion of the NIRS pilot study

Our NIRS monitoring study investigated whether NIRS is feasible to evaluate leptomeningeal collaterals at the anterior watershed areas and analyse the correlation between NIRS readings and ENI. Although our findings are exploratory because of the small sample size, we believe an excellent example is the case of Patient 5. Due to a chronic right ICA occlusion, significantly higher rSO_2 values were measured above the left hemisphere. A possible explanation for this finding is an increased blood flow in the left carotid system,

which provides adequate perfusion to both hemispheres through the circle of Willis. Long-standing increase of blood flow has led to the enlargement of these vessels and a well-developed leptomeningeal collateral circulation. The collateral circulation remained stable (the IHΔrSO$_2$ was stable and high throughout the 24-hour monitoring), and the patient achieved ENI and good functional outcome at three months.

According to the NINDS criteria, three out of five patients achieved ENI at 24 hours. At three months, four patients had favourable functional outcomes. If we apply the NINDS criteria for the discharge NIHSS scores, all four patients with mRS ≤ 2 at three months had ENI. However, NIHSS scores taken after 24 hours post-IVT or MT also reflect other therapeutic interventions: stabilization of blood pressure and blood glucose, antithrombotic or anticoagulant treatment for secondary prevention, lipid-lowering treatment, recognition and management of dysphagia, early mobilization, preventive measures for infections, thrombosis prophylaxis, psychological support, and speech therapy, to name a few.[20]

Ritzenthaler et al. performed 24-hour NIRS monitoring in 17 stroke patients who had MT.[64] All patients had lower absolute rSO$_2$ values above the affected hemispheres, but good collateral flow as assessed with the American Society of Interventional and Therapeutic Neuroradiology (ASITN) collateral flow grading scale. They found no significant relationship between initial ipsilateral rSO$_2$ and collateral circulation. A possible explanation behind their finding might be that the NIRS sensors were above the ischaemic territory. In our study, Patient 3 demonstrated a similar NIRS trend to those published by Ritzenthaler et al. After successful recanalization, IHΔrSO$_2$ significantly decreased. Patient 2 had left ICA occlusion but had higher rSO$_2$ on the ipsilateral side, possibly due to well-developed leptomeningeal collaterals.

Hametner et al. used NIRS monitoring during MT in 43 cases.[63] They reported that the median of IHΔrSO$_2$, measured at the end of MT, was significantly lower in patients who died within 90 days. In addition, patients with lower rSO2 variability showed significantly worse 90-day outcomes (mRS score 3-6). In another study, Damian and Schlosser investigated patients with MCA occlusions with consequent brain oedema.[81] NIRS monitoring was performed in the subacute phase of stroke (at least 12 hours after, but within four days after symptom onset). Interestingly, 22 out of 24 patients had higher absolute rSO$_2$ values above the ipsilateral frontal area. These data are contrary to the results published by Ritzenthaler et al. We hypothesize this contradiction is due to the different time points when the measurements were made (subacute vs acute phase of stroke). The positive absolute IHΔrSO2

values in Damien and Schlosser's study may reflect increased compensatory leptomeningeal collateral circulation, which developed on the ipsilateral side a few days after the cerebrovascular event. The article reported good clinical outcomes 6-24 weeks after rehabilitation in cases where average IHΔrSO_2 values increased over time. All 5 cases where the initial IHΔrSO_2 decreased were fatal. Another relevant finding of the study was that clinical signs of progressing brain oedema and unfavourable rSO_2 changes were reversible in some cases by hemicraniectomy, hyperventilation, hypothermia or improved systemic perfusion.[81] Therefore, the correct interpretation of NIRS monitoring could guide therapeutic interventions. Previous studies showed that a decrease in systemic blood pressure or SpO_2 correlates well with a drop in rSO_2.[63,65] NIRS parameters could guide clinicians in finding individually tailored target blood pressure and SpO_2 ranges. For instance, patients with acute ICA occlusion could benefit from increased systemic blood pressure to maintain adequate collateral circulation until recanalization. A pre-clinical study investigated this concept and found that mild induced hypertension increased cortical collateral blood flow and significantly reduced infarct volume in mice with transient distal MCA occlusion.[99]

We performed NIRS monitoring with two channels placed over the two frontal areas. It would provide additional information if more sensors were placed over the cerebral hemispheres. Rummel et al. used multichannel NIRS monitoring during transient balloon occlusion of cerebral arteries, and demonstrated different rSO_2 changes over the ischaemic core and watershed areas due to haemodynamic changes in collateral flow.[68] Moreau et al. also used multichannel NIRS monitoring in five acute ischaemic stroke patients with LVO. The sensors were placed over the frontal, parasagittal frontal, Rolandic sulcus, Broca and Wernicke areas.[72] The symptom onset to monitoring time was within 9 hours. They reported that at least one region of the affected hemisphere showed reduced rSO_2 values compared to the contralateral side. One patient suffered a haemorrhagic transformation a few days after the ischaemic event. Not surprisingly, rSO_2 values were significantly higher above the affected hemisphere than on the contralateral side. Highly oxygenated blood within the haematoma accounts for this finding.[72] Therefore, NIRS could also help readily identify haemorrhagic transformation.

5.4. Proposal for a new criterion of early neurological improvement

There are various definitions for ENI after reperfusion therapy in the literature.[10-18] Despite the different definitions, ENI correlates well with long-term functional outcomes measured with the mRS. The actual treatment effect of IVT and MT is probably best reflected in the 24-hour change in the NIHSS score. Although discharge NIHSS scores have been shown to correlate with functional outcomes at three months similarly, it is also dependent on other therapeutic interventions and preventive measures.[20,83]

How the change in NIHSS scores should be measured is still controversial. **Table 7** proposes new criteria to define ENI, based on previous studies and clinical experience. We categorise patients into three groups based on their initial NIHSS score. In the Study of the Efficacy and Safety of Alteplase in Participants With Mild Stroke (PRISMS) trial, approximately 90% of patients with minor, non-disabling strokes (defined as NIHSS score of 0 to 5, and the deficits were judged not to be clearly disabling) had an mRS 0-2 at 90 days.[100] The percentage change in the criterion was based on the work of Agarwal et al.[18]

Stroke severity	Definition for ENI
Minor stroke (NIHSS ≤ 5)	at least 1 point decrease in NIHSS score at 24 hours
Moderate stroke (5 < NIHSS < 15)	NIHSS becomes ≤ 5 (i.e. minor stroke) or at least 40% improvement in NIHSS score compared to baseline at 24 hours
Severe stroke (16 ≤ NIHSS)	at least 40% improvement in NIHSS score compared to baseline at 24 hours

Table 7. Proposed new criteria for ENI

6. Conclusions

Investigation of ENI is essential in predicting prognosis after IVT and MT. Good collateral circulation is pivotal in reducing the progression of ischaemic brain damage and achieving ENI. Based on our work, NIRS monitoring provides valuable real-time information on the state of leptomeningeal collaterals, and helps to assess the effects of reperfusion therapies. Changes in rSO_2 values and interhemispheric rSO_2 differences could guide individualised blood pressure management and oxygen supplementation. Future studies with

NIRS should use multichannel monitoring to gain further insight into the relationship between haemodynamic changes in leptomeningeal collaterals and their association with ENI.

The thesis contains the first study that has analysed the changes in KYN metabolite serum levels and enzymatic activities in acute ischemic stroke patients who received IVT. We propose that baseline serum KYNA concentration and KAT activity are potential biomarkers of ENI.

Furthermore, the thesis provides real-world data on the association between ENI and IVT in acute ischaemic stroke patients with a DWI-FLAIR mismatch in the absence of LVO. The rate of haemorrhagic complications was similar to those published in large clinical thrombolysis trials with known onset times. Randomised studies are warranted to test the efficacy and safety of alteplase treatment in patients with partial DWI-FLAIR mismatch.

The definition of ENI varied among the studies included in this thesis. To avoid future controversies, new criteria for ENI have been proposed based on previous definitions and clinical expertise. ENI established using our proposed new criteria could serve as a helpful efficacy outcome measure for future clinical trials assessing new treatments for acute stroke.

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Research article

DWI-FLAIR mismatch guided thrombolysis in patients without large-vessel occlusion: real-world data from a comprehensive stroke centre

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HIGHLIGHTS

- Real-world data on DWI-FLAIR mismatch based thrombolysis.
- Only patients without large vessel occlusion were analysed.
- Thrombolysis was associated with early neurological improvement.
- The rate of intracerebral haemorrhage was not increased.

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ABSTRACT

Introduction: A significant proportion of ischaemic stroke patients present with unknown symptom onset time. DWI-FLAIR mismatch on MRI can help to identify those eligible for thrombolysis. We set out to analyse the short-term efficacy and safety of thrombolysis in a real-world setting.

Methods: A retrospective single-centre observational study was conducted. We collected data between January 2017 and April 2020. Patients with a large vessel occlusion (LVO) were excluded. Outcomes were compared between thrombolysed patients and those who did not receive alteplase due to lack of DWI-FLAIR mismatch or other contraindications. We analysed baseline and discharge NIHSS scores for efficacy and defined good outcome as any neurological improvement (ANI) on the NIHSS. In terms of safety, the presence and severity of intracerebral haemorrhage on follow-up imaging was analysed, and mortality at 90 days assessed.

Results: Seventy-one patients were included in this study, of whom 29 received thrombolysis. Significantly more patients had ANI in the thrombolysed group (OR, 3.16; 95% CI, 1.178–8.479; $p = 0.020$). In a multivariable logistic regression analysis, only thrombolysis correlated with ANI (OR, 3.051; 95% CI, 1.135–8.206; $p = 0.027$). Two thrombolysed patients suffered intracerebral haemorrhage (6.90%), of whom one was symptomatic and eventually fatal. We did not find a significant difference in 90-day mortality between the two groups (OR, 0.81, 95% CI, 0.134–4.856; $p = 1.000$).

Conclusions: Our real-world data demonstrate that thrombolysis based on DWI-FLAIR mismatch in patients without LVO has an early beneficial effect. The rate of intracerebral haemorrhage was similar to this complication reported in large thrombolysis trials with known onset times.

1. Introduction

Determining the exact symptom onset time in acute ischaemic stroke is often difficult. Approximately 14–27% of stroke patients present to the emergency department with unknown symptom onset time [1]. A commonly encountered scenario is a wake-up stroke). Furthermore, agitated or aphasic patients and those with neurocognitive deficits might not be able to tell when their symptoms started, making treatment decisions challenging.

Current guidelines recommend systemic thrombolysis within 4.5 h after symptom onset [2, 3]. If the onset time is unknown and the time last seen well is beyond 4.5 h, then specific imaging modalities can help to establish eligibility for thrombolytic therapy. One option is the MRI based diffusion-weighted imaging (DWI) – fluid-attenuated inversion recovery

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(FLAIR) mismatch concept. DWI detects ischaemia induced changes in cellular water diffusion within minutes [4, 5]. In comparison, T2 weighted imaging and consequently FLAIR imaging have a sensitivity to only detect net water increase and vasogenic oedema that follows cytotoxic oedema [6, 7]. Therefore, it takes at least 1–4 h for the ischaemic stroke to become visible on FLAIR imaging. The PRE-FLAIR (Identification of Stroke Patients $\leq$3 and $\leq$4.5 Hours of Symptom Onset by Fluid Attenuated Inversion Recovery Imaging and Diffusion-Weighted Imaging) study dem onstrated that the DWI-FLAIR mismatch pattern identifies ischaemic lesions within 4.5 h after symptom onset with 78% specificity and 83% positive predictive value [8]. In a multicentre, randomised, double-blind, placebo-controlled trial (Efficacy and Safety of MRI-based Thrombolysis in Wake-up Stroke, i.e. the WAKE-UP trial), alteplase treatment was administered for patients with unknown stroke onset times who were last seen well more than 4.5 h before symptoms were noticed, and had DWI-FLAIR mismatch [9]. Mismatch was defined as a DWI lesion without corresponding marked FLAIR hyperintensity. Patients treated with alteplase had significantly better functional outcomes at 90 days than those who received placebo. However, severe parenchymal haemorrhages were also significantly more common in the treatment arm. Nevertheless, the WAKE-UP trial provided high-quality evidence on the benefit of alteplase treatment in patients with unknown symptom onset times and a DWI-FLAIR mismatch on MRI.

Our study aimed to share our real-world experience and the challenges of selecting patients for thrombolysis based on the DWI-FLAIR mismatch pattern. We set out to analyse the short-term efficacy and safety of alteplase treatment. We also compared the outcome of treated patients to those not eligible for thrombolysis due to no DWI-FLAIR mismatch or other contraindications. Our analysis included patients with unknown symptom onset times as well as cases where MRI was performed because of diagnostic uncertainty within 4.5 h after symptom onset.

2. Methods

2.1. Patients

A retrospective single-centre observational study was conducted between. January 2017 and April 2020. We identified patients with a suspected clinical diagnosis of acute ischaemic stroke, where an MRI showed DWI hyperintensity. Patients with large vessel occlusions (LVO), who were candidates for thrombectomy, were excluded because our main goal was to analyse the effects of thrombolytic therapy alone. Based on the DWI-FLAIR mismatch pattern, the indication for thrombolysis was established by an experienced attending stroke neurologist. All procedures were carried out in accordance with the Hungarian Acute Ischaemic Stroke Diagnostic and Treatment Recommendations [10]. We used a more permissive protocol than the WAKE-UP trial: patients with partial DWI-FLAIR mismatch were also eligible for treatment, and pre-stroke functional dependence or age were not contraindications for thrombolytic therapy. Patients who received alteplase had repeat imaging (CT or MRI) approximately 24 h after thrombolysis. All patients or their legal representatives gave informed consent before treatment. The Ethics Committee of the University of Szeged, Albert Szent-Györgyi Health Centre approved our study (ID: 6/2017-SZTE), which was conducted according to the revised Declaration of Helsinki.

Parameters of patients treated with alteplase were compared to those who did not receive this treatment due to a matched DWI-FLAIR pattern or other contraindications. For each patient, we recorded detailed demographic characteristics and vascular risk profile. Blood glucose was measured from serum samples taken upon arrival to the Emergency Department (ED). Blood pressure readings were recorded upon arrival to ED.

2.2. Imaging protocol

Each patient underwent multimodal brain MRI acutely, performed with a 1.5 T GE Signa Excite MRI scanner. The acute stroke MRI protocol included DWI, FLAIR, and susceptibility-weighted angiography (SWAN). DWI-FLAIR mismatch was defined as an ischaemic DWI lesion with no corresponding signal change on the FLAIR sequences. Partial mismatch was defined as a corresponding FLAIR signal change smaller than the DWI hyperintensity. Figures 1, 2, and 3 show examples for DWI-FLAIR complete mismatch, partial mismatch and matched pattern. The attending radiologist and neurologist rated the mismatch patterns visually.

2.3. Outcome measures

We analysed baseline and discharge National Institutes of Health Stroke Scale (NIHSS) scores as outcome of efficacy. We defined good short term outcomes as any neurological improvement (ANI), as indicated by a lower NIHSS score at discharge compared to baseline.

In terms of safety outcomes, we analysed the occurrence of intracerebral haemorrhage (ICH) on the 24-hour repeat imaging after thrombolysis. The extent of the haemorrhage was graded according to the European Cooperative Acute Stroke Study II (ECASS II) [11]. Symptomatic ICH (sICH) was also defined according to the ECASS II trial criteria (i.e., any haemorrhage leading to death or neurologic deterioration causing at least 4 point increase in the NIHSS score compared to baseline). Hypersensitivity reaction to alteplase, transfer to intensive care unit (ICU), and mortality within 90 days after stroke were recorded. Comparisons of outcomes between genders were also performed.

2.4. Statistical analysis

The outcome measures were categorical variables. For continuous variables, the distribution of data was tested with the Shapiro-Wilk test. Normally distributed variables were expressed as mean $\pm$ SD and non-normally distributed data as median and IQR. Continuous variables were compared with independent samples t-test for normally distributed data or Mann-Whitney U test in the case of non-normal distribution. Pearson's chi-squared test of independence was applied to compare categorical variables, but we used Fisher's exact test where sample sizes were small (i.e. equal to or less than 5). Statistical significance was met when the p-value was <0.05. To compare ANI and mortality between the groups, 95% confidence intervals (CI) and odds ratios (OR) were calculated by standard approaches. We also performed multivariable logistic regression to analyse the correlation between thrombolysis and ANI. We applied a backward likelihood ratio model selection method. Variables included in the analysis were age, gender, admission blood glucose, admission systolic and diastolic blood pressure, baseline NIHSS and thrombolysis. OR and 95% CI were again calculated. Statistical significance was met when the p-value was <0.05. All statistical analyses were performed with IBM SPSS version 22 statistical software (SPSS Inc., Chicago, USA).

3. Results

We identified 121 patients with a clinical diagnosis of acute ischaemic stroke and DWI hyperintensity on their MRI. Patients with known and unknown stroke onset times were both included. MRI images were not available for review in 17 patients due to technical reasons. Twenty-five patients with LVOs were also excluded. Furthermore, eight patients were excluded because their symptom onset was confirmed as beyond the 4.5-hour time window. Eventually, 71 patients were included in our final analysis. Figure 4 shows the flowchart of patient selection.

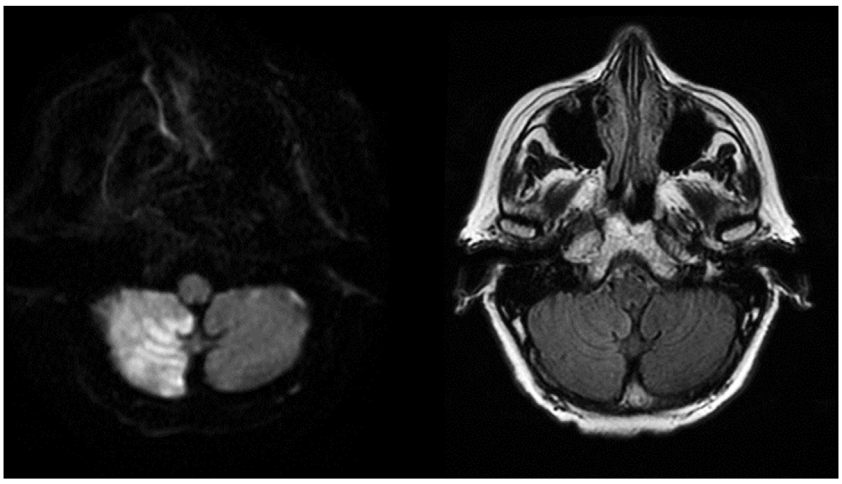

Figure 1. Complete DWI-FLAIR mismatch of a right cerebellar infarct.

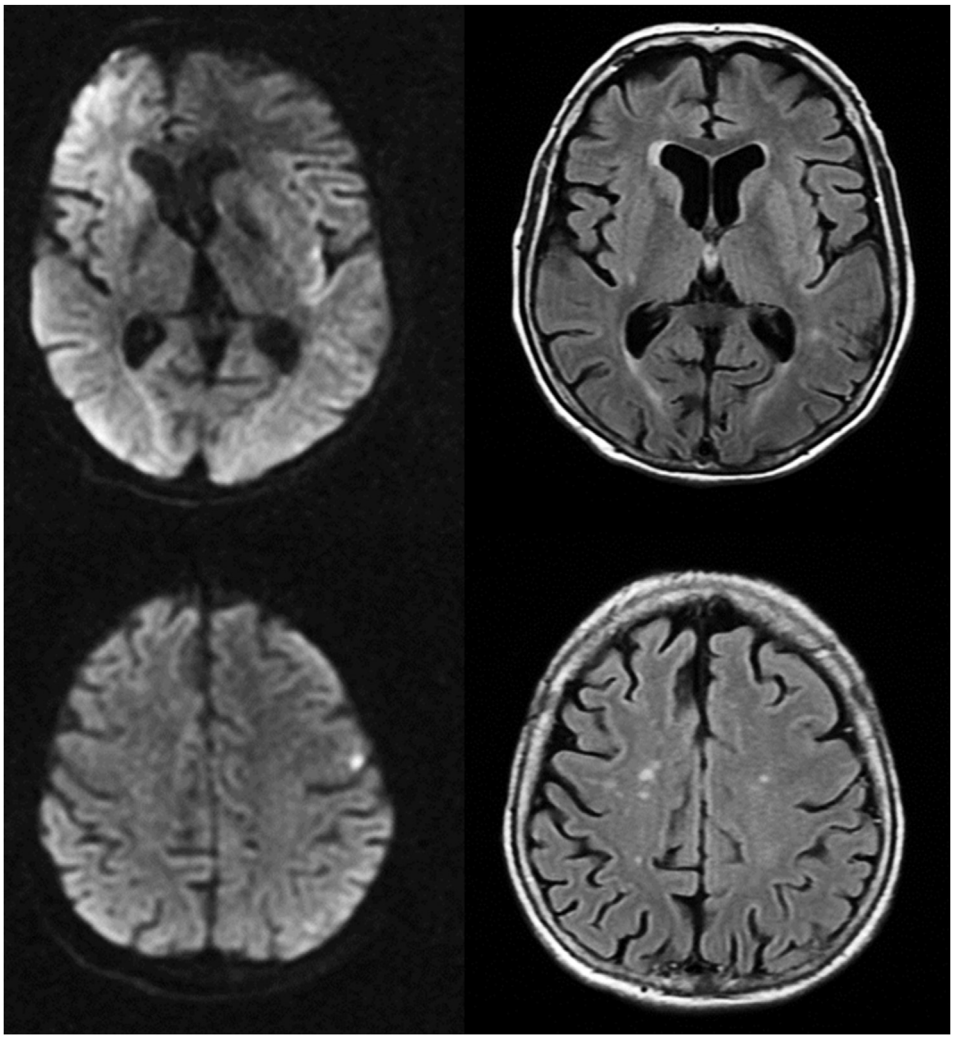

Figure 2. Example for partial mismatch. Superior row: left insular DWI hyperintensity with corresponding FLAIR signal change. Inferior row: the left frontal cortical DWI lesion is not yet visible on FLAIR.

Twenty-nine patients received intravenous thrombolysis. One patient received alteplase despite having a matched DWI-FLAIR pattern because the onset of symptoms was known to be within 4.5 h. In this particular case, MRI was ordered due to diagnostic uncertainty. Six patients had partial DWI-FLAIR mismatch on MRI. Two of these patients were not administered thrombolysis due to sulcal siderosis in one and a previous intracerebral haemorrhage in the other case, as detected on SWAN. Four patients had DWI-FLAIR mismatch but did not receive alteplase due to

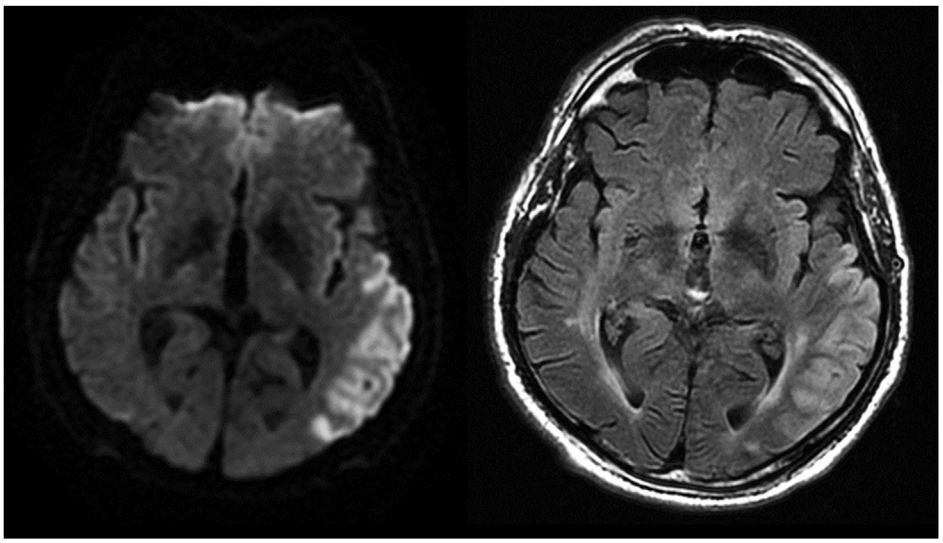

Figure 3. Matched DWI-FLAIR pattern of a left temporal infarct.

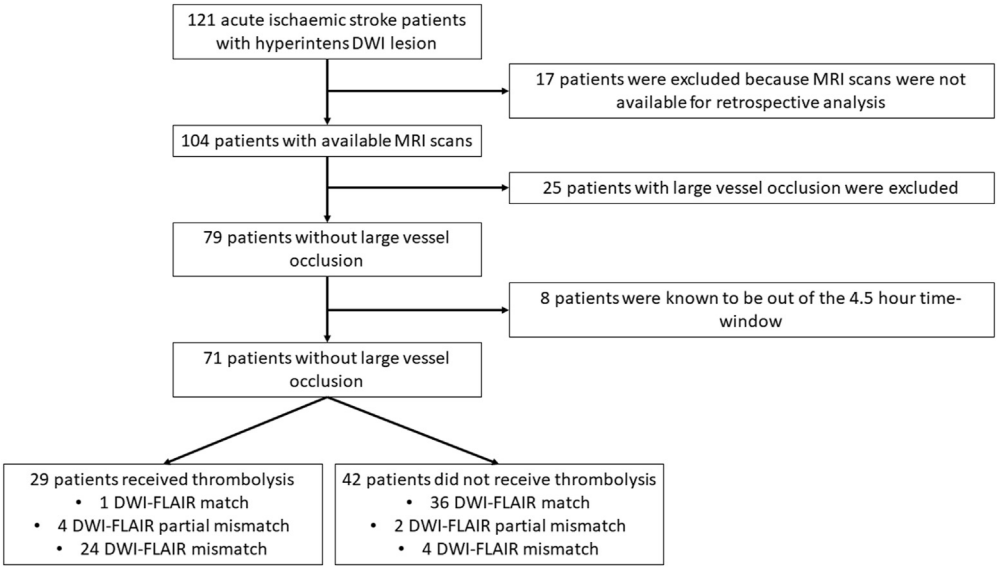

Figure 4. Flowchart demonstrating patient selection in our study.

contraindications: two had signs of previous clinically significant intracerebral haemorrhage on SWAN, one had a haemorrhagic transformation of the culprit infarct, and one had pancreatic cancer and more than ten cortical microbleeds on SWAN. Table 1 presents the demographics and clinical characteristics of our study population. One patient in the non-thrombolysed group suffered a stroke in the emergency department, therefore, a door-to-imaging time is not available for this case. We detected significantly more lacunar infarcts in non-thrombolysed patients ($p = 0.042$). Otherwise, the two groups were well balanced.

3.1. Efficacy and safety outcomes

Significantly more patients had ANI in the thrombolysed group (OR, 3.16; 95% CI, 1.178–8.479; $p = 0.020$). In the multivariable logistic regression analysis, thrombolysis was the only variable that correlated with ANI (OR, 3.051; 95% CI, 1.135–8.206; $p = 0.027$). There were no differences between men and women regarding ANI ($p = 0.451$ among thrombolysed patients and $p = 0.320$ for non-thrombolysed patients).

Only patients in the thrombolysis arm had follow-up imaging. There were no clinical indications for a repeat scan in any of the non-thrombolysed patients. Two thrombolysed patients had intracranial haemorrhage on follow-up imaging (6.90%). One patient had parenchymal haemorrhage type I (PH 1), and another had a remote PH 2. Only the patient with PH 2 had a sICH (3.45%). He died five days after thrombolysis. The initial MRI scan showed a complete DWI-FLAIR mismatch for a right hemispheric lacunar infarct without hypointense signal changes on SWAN. The follow-up CT (Figure 5(A)–(D)) showed a large left parieto-temporo-occipital haemorrhage with perifocal oedema, mass effect, and propagation into the ventricles.

There was no hypersensitivity reaction to alteplase, and there was no need for ICU transfer for ventilation or vasopressor support in either group.

Eighteen patients were lost to long term follow-up. Of the remaining 53 individuals, six died within 90 days after stroke (11.32%): two in the thrombolysed ($n = 20$, 10.00%) and four in the non-thrombolysed group ($n = 33$, 12.12%), with statistically non-significant odds (OR, 0.81, 95%

Table 1. Demographics and clinical characteristics of the study population.

	Thrombolysis (n = 29)	No thrombolysis (n = 42)	p value
Mean age ± SD	73.34 ± 8.66	71.52 ± 9.40	0.404
Male sex – no. (%)	16 (55.17%)	22 (52.38%)	0.817
Medical history – no. (%)			
Hypertension	26 (89.66%)	40 (95.24%)	0.393
Hyperlipidaemia	19 (65.52%)	34 (80.95%)	0.142
Diabetes mellitus	11 (37.93%)	14 (33.33%)	0.690
Smoking	8 (27.59%)	12 (28.57%)	0.928
Excess alcohol consumption	6 (20.69%)	8 (19.05%)	0.864
Atrial fibrillation	8 (27.59%)	12 (28.57%)	0.928
Carotid stenosis > 50%	4 (13.79%)	5 (11.90%)	1.000
Symptomatic carotid stenosis	4 (13.79%)	3 (7.14%)	0.433
Previous carotid endarterectomy or stenting	2 (6.90%)	1 (2.38%)	0.563
Coronary artery disease	3 (10.34%)	5 (11.90%)	1.000
Peripheral artery disease	1 (3.45%)	1 (2.38%)	1.000
Clinical parameters			
Median blood glucose (IQR) – mmol/l	7.70 (6.55–9.75)	6.70 (n = 41, 6.10–9.30)	0.233
Mean systolic blood pressure ± SD – mmHg	167.93 ± 24.09	168.05 ± 30.20	0.986
Mean diastolic blood pressure ± SD – mmHg	90.14 ± 16.33	90.74 ± 18.04	0.885
Median NIHSS score at baseline (IQR)	5.00 (3.00–9.00)	4.50 (3.00–7.00)	0.337
Median NIHSS score at discharge (IQR)	3.00 (2.00–7.50)	4.00 (3.00–6.00)	0.855
Lacunar stroke	5 (17.24%)	17 (40.48%)	**0.042**
Time intervals			
Median door to imaging time (IQR) – min	31.00 (24.50–60.50)	36.00 (n = 41, 22.00–74.50)	0.672
Median imaging to needle time (IQR) – min	34.00 (20.50–42.50)	–	–
Median door to needle time (IQR) – min	70.00 (50.00–109.00)	–	–
Length of hospital stay (IQR) – days	5.00 (4.00–5.50)	5.00 (3.00–6.25)	0.785
Outcome measures			
Any neurological improvement	17 (58.62%)	13 (30.95%)	**0.020**
Haemorrhagic transformation	2 (6.90%)	–	–
sICH	1 (3.45%)	–	–
Transfer to intensive care unit	0 (0.00%)	0 (0.00%)	–
Allergic reaction to alteplase	0 (0.00%)	–	–
Mortality at 90 days	2 (n = 20, 10.00%)	4 (n = 33, 12.12%)	1.000

CI, 0.134–4.856; p = 1.000). Mortality was similar between genders (p = 1.000 for both thrombolysed and non-thrombolysed patients). Autopsies were not performed, and the cause of death was determined on clinical grounds. In the thrombolysed group, one patient died five days after stroke due to the previously mentioned sICH, and one patient died 18 days after stroke due to complications from a sacral pressure sore. In the non-thrombolysed group, one patient died 34 days post-stroke from decompensation of heart failure, and two patients died due to infections at 18 (pneumonia) and 63 (Clostridium difficile) days after stroke, respectively. One patient died at 25 days from complications of a posterior circulation stroke.

4. Discussion

Our real-world data support the use of the DWI-FLAIR mismatch concept for alteplase treatment in acute ischaemic stroke patients without LVO. We analysed ANI to investigate short-term response to alteplase because the actual treatment effect may be better reflected by the NIHSS score rather than the 90-day modified Rankin Scale score (mRS), which is more dependent on a variety of other factors such as comorbidities, polypharmacy, the availability and quality of rehabilitation, support provided by family, and socioeconomic status. It is worth highlighting that the definition of early neurological improvement varies between studies [12]. In the NINDS trial (National Institute of Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study), it was defined as complete resolution of symptoms or at least a 4-point decrease in the NIHSS score 24 h after treatment [13]. Another study analysed the absolute change between baseline and 24-hour NIHSS scores [14].

As the baseline NIHSS scores were relatively low in our study (a median of 5 and 4.5 points in the thrombolysed and non-thrombolysed arms, respectively), we felt that defining early neurological improvement similar to the NINDS trial was not practical. Furthermore, instead of taking the NIHSS score at 24 h, we measured this outcome at discharge, which in our opinion is reasonable practice considering the median length of hospital stay of only five days in both groups. A number of studies support the use of short-term response to thrombolysis, as an outcome, which has a correlation with functional outcome at 90 days [15, 16]. Jantasri et al. demonstrated that a 2-point difference in the NIHSS score at 24 h after thrombolysis predicts functional outcome at 3 months [15]. In another study, early neurological improvement defined as a reduction in NIHSS score by 10 or an absolute score of 4 or less 2 h after thrombolysis, was an independent predictor of favourable outcome at 3 months [16].

One might argue that ANI could be due to spontaneous recovery and not a consequence of thrombolysis. 16–24% of patients with a disabling stroke reportedly achieve good functional outcome at one week or upon discharge, without thrombolysis [17, 18]. Lacunar syndromes were

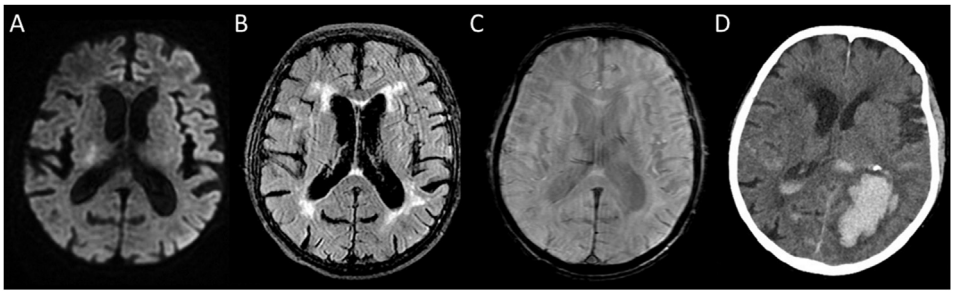

Figure 5. Pre-thrombolysis MRI and 24-hour post-treatment CT of the patient with sICH. (A) DWI demonstrating a right hemispheric lacunar infarct. (B) No corresponding hyperintensity is visible on FLAIR. Periventricular white matter hyperintensities indicate small vessel disease. (C) No microhaemorrhages detected on SWAN. (D) 24-hour repeat CT shows a remote, left parieto-temporo-occipital haemorrhage with perifocal oedema, mass effect, and propagation into the lateral ventricles.

significantly more common in patients with neurological recovery. Lacunar strokes result from lipohyalinosis of small perforating arteries, branch atheromatous disease or microembolisms and they usually have milder clinical presentations compared to large-artery atherosclerosis or cardioembolic strokes [19]. An interesting observation in our study was the significant difference in the proportion of lacunar stroke, which was significantly more common in the non-thrombolysed group. However, ANI was more frequent among thrombolysed patients, which supports the short-term effectiveness of thrombolytic treatment over spontaneous recovery. Our multivariable analysis also demonstrated a significant association between ANI and thrombolysis. Furthermore, the efficacy of thrombolysis in lacunar stroke has been proven in several clinical studies and the post hoc secondary analysis of the WAKE-UP trial [20, 21, 22].

Women differ from men in vascular risk factor profile, stroke subtype and outcome [23, 24, 25]. Women have been found more likely to suffer from stroke-related complications, higher rates of in-hospital death and lesser spontaneous neurological improvement [23]. However, our study did not find differences between genders regarding efficacy and safety outcomes. Kent et al. have also reported no differences in 90-day outcomes [25].

In terms of safety outcomes, we compared our results with those of the WAKE-UP trial. It is worth mentioning that 33.7% of patients in the treated arm of the WAKE-UP trial had occlusion of a large intracranial artery. As discussed earlier, we excluded these patients from our retrospective analysis. Regarding intracerebral haemorrhage, the number of patients with PH1 was not reported in the trial. The percentage of PH2 according to ECASS II was similar: 3.45% in our study compared to 4.0% in the randomised clinical trial. In a pooled analysis of thrombolysis trials, the rate of severe intracranial haemorrhage was 5.2% [26]. In the included studies, symptom onset times were known, and alteplase could be given up to 6 h after symptom onset. The percentage of sICH was also similar: 3.45% in our population vs 2.8% in the WAKE-UP trial. The patient with remote PH2 and consequent sICH had complete mismatch on baseline imaging. No relevant haemorrhagic events occurred in patients with partial FLAIR hyperintensity.

In patients who received alteplase, the 10% mortality at 90 days in our population was higher than the 4.1% in the WAKE-UP study. Potential explanations are that our patients were older with more vascular risk factors present, as well as more disabled at baseline. We did not contraindicate alteplase treatment based on mRS, whereas only patients with mRS 0–1 were included in the WAKE-UP trial.

Our study included only four patients who had thrombolysis with DWI-FLAIR partial mismatch. Therefore, meaningful conclusions about the safety and efficacy of alteplase treatment in patients with such imaging patterns cannot be drawn. Jakubicek et al. reported that 27 thrombolysed patients with partial mismatch did not have higher rates of sICH compared to 37 patients without FLAIR signal change [27]. The beneficial effects of alteplase were similar in the two groups. They implied that the mismatch pattern used in the WAKE-UP trial might be over-selective. Similarly, a report from the Bernese stroke registry found no association between FLAIR hyperintensities and sICH after thrombolysis and thrombectomy [28]. Treatment in that study was indicated based on an "eyeball" assessment of whether the corresponding FLAIR signal change exceeded 50% of the DWI lesion or not. Among 159 patients included, 89 had partial DWI-FLAIR mismatch, in whom the rate of sICH was 6.7%. In our opinion, the concept of alteplase treatment in partial DWI-FLAIR mismatch should be tested in large, prospective, randomised clinical trials to assess whether a broader range of patients could benefit from thrombolysis.

Limitations of our study include the small sample size and the retrospective observational nature of data collection. However, the two groups in our study were similar in terms of vascular risk factor profile and medical management pre-stroke. The non-thrombolysed group comprised of patients with onset times most probably beyond 4.5 h based on their DWI-FLAIR pattern. In addition to comparing the rates of ANI between the two groups, we also performed a logistic regression analysis to investigate the predictors of ANI. This regression analysis identified alteplase treatment as the only variable associated with ANI.

5. Conclusions

In conclusion, our real-world data demonstrate that thrombolysis provides short-term benefit in acute ischaemic stroke patients with a DWI-FLAIR mismatch, in the absence of LVO. The rate of haemorrhagic complications was similar to those published in large clinical thrombolysis trials with known onset times. Randomised studies are warranted to test the efficacy and safety of alteplase treatment in patients with partial DWI-FLAIR mismatch.

Declarations

Author contribution statement

Ádám Annus: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Franciska Zita Gera: Performed the experiments.

László Sztriha: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Péter Klivényi: Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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Kynurenic acid and kynurenine aminotransferase are potential biomarkers of early neurological improvement after thrombolytic therapy: A pilot study

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Conflict of interest
None declared

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Abstract

Background. Biomarkers for predicting treatment response to thrombolysis in acute ischemic stroke are currently lacking. Both, animal models and clinical studies have provided evidence that the kynurenine (KYN) pathway is activated in ischemic stroke.

Objectives. In our pilot study, we aimed to investigate whether KYN pathway enzymes and metabolites could serve as potential biomarkers for treatment response in the hyperacute phase of ischemic stroke.

Materials and methods. We included 48 acute ischemic stroke patients who received thrombolysis. Blood samples were taken both before and 12 h after treatment. Concentrations of 11 KYN metabolites were determined using ultra-high-performance liquid chromatography-mass spectrometry. To assess the treatment response, we used early neurological improvement (ENI), calculated as the difference between the admission and discharge National Institutes of Health Stroke Scale (NIHSS) scores. We performed receiver operating characteristic (ROC) analysis for KYN pathway metabolites and enzymes that showed a correlation with ENI.

Results. In the samples taken before thrombolysis, significantly lower concentrations of kynurenic acid (KYNA) and kynurenine aminotransferase (KAT) activity were found in patients who had ENI ($p = 0.01$ and $p = 0.002$, respectively). According to the ROC analysis, the optimal cut-off value to predict ENI for KYNA was 37.80 nM (sensitivity (SN) 69.2%, specificity (SP) 68.4%) and 0.0127 for KAT activity (SN 92.3%, SP 73.7%).

Conclusions. Our research is the first clinical pilot study to analyze changes in the KYN pathway in ischemic stroke patients who received thrombolytic treatment. Based on our results, baseline KYNA concentration and KAT activity could serve as potential biomarkers to predict early treatment response to thrombolysis.

Key words: kynurenine, biomarker, ischemic stroke, acute stroke, thrombolysis

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Introduction

Biomarkers are objective indicators of physiological or pathological processes and have valuable applications in predicting and monitoring clinical response to therapeutic interventions.[1] At present, biomarkers aiding in prediction of response to intravenous thrombolysis treatment and prognosis in acute ischemic stroke are lacking in routine clinical practice.[2] However, a few blood biomarkers have shown promise: copeptin, a fragment of vasopressin produced in the hypothalamus, was shown to increase the prognostic accuracy of the National Institutes of Health Stroke Scale (NIHSS) in predicting functional outcome and mortality.[3] Matrix metalloproteinase-9 (MMP-9) levels correlated with hemorrhagic transformation after thrombolytic therapy, and S100B was elevated in patients with malignant middle cerebral artery syndrome.[4,5] Higher activated/inactivated thrombin-activatable fibrinolysis inhibitor levels correlated with higher NIHSS scores 2 days after treatment, and with poor outcome on the modified Rankin Scale (mRS) score at day 90.[6] Faille et al. reported that low admission levels of soluble thrombomodulin and soluble endothelial protein C receptor in patients with arterial occlusion were associated with higher recanalization rates after thrombolytic therapy.[7] In another study, low endogenous thrombin potential before thrombolysis was found to be an independent predictor of both short- and long-term mortality following treatment.[8]

The kynurenine (KYN) pathway is the main route of tryptophan (TRP) metabolism. Animal models and clinical studies have unequivocally proven that the KYN pathway is activated in acute ischemic stroke.[9–15] The first step of the pathway is the metabolism of TRP to KYN by indoleamine-2,3-dioxygenase (IDO). Inflammatory cytokines (e.g., interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) and interferon γ (INF-γ)) were shown to increase the expression of IDO.[16,17] Therefore, the activation of the KYN pathway following ischemic brain injury is likely part of a secondary inflammatory reaction.[18,19] The most well-studied metabolites of the pathway are kynurenic acid (KYNA), 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN). Kynurenic acid is metabolized by kynurenine aminotransferase (KAT) from KYN. It is a known endogenous, competitive inhibitor of the N-methyl-D-aspartate receptor (NMDAR) and is therefore thought to have neuroprotective properties.[20] In contrast, 3-HK and QUIN are neurotoxic compounds that produce free radicals and cause oxidative stress. Further metabolites and enzymes of the KYN pathway that were analyzed in our study are highlighted in Fig. 1.

The KYN pathway is linked to a number of traditional cerebrovascular risk factors that could influence serum levels of KYN metabolites.[21] It has been demonstrated that IDO expression regulates blood pressure in mouse models of systemic inflammation.[22] Administration of KYNA into the rostral ventrolateral medulla of spontaneously hypertensive rats, decreased mean arterial blood pressure by approx. 40 mm Hg.[23] Median blood KYN levels of patients with stable angina pectoris were higher in hypertensive patients compared to normotensive individuals.[24]

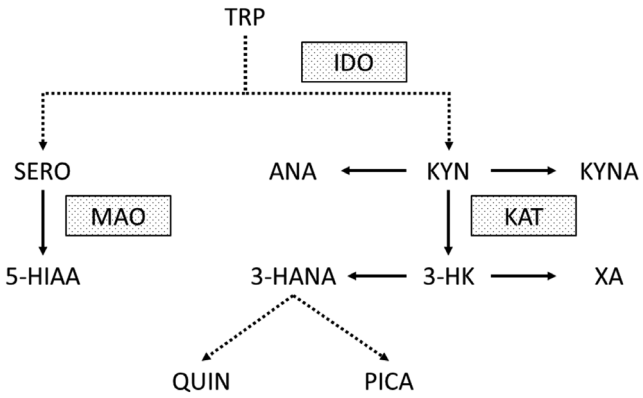

Fig. 1. Kynurenine pathway and other tryptophan metabolites measured in our pilot study. Dotted lines indicate more than 1 enzymatic step. Dotted boxes highlight the most relevant enzymes of the pathway

3-HANA – 3-hydroxyanthranilic acid; 3-HK – 3-hydroxy-kynurenine; 5-HIAA – 5-hydroxy-3-indoleacetic acid; ANA – anthranilic acid; IDO – indoleamine 2,3-dioxygenase; KAT – kynurenine aminotransferase; KYN – kynurenine; KYNA – kynurenic acid; MAO – monoamine oxidase; PICA – picolinic acid; QUIN – quinolinic acid; SERO – serotonin; TRP – tryptophan; XA – xanthurenic acid.

Elevated xanthurenic acid (XA) levels have been found in diabetic patients.[25] Xanthurenic acid forms a complex with insulin that does not activate insulin receptors.[26] Therefore, elevated XA levels contribute to insulin resistance.[27]

Aging, the most relevant non-modifiable risk factor for cerebrovascular diseases, showed a significant association in a multivariate linear regression analysis with serum concentrations of KYN, TRP, and IDO activity.[28] In the Hordaland Health Study, an inverse association was found between heavy smoking and anthranilic acid (AA), TRP, KYN, KYNA, XA, and 3-hydroxyanthranilic acid (3-HANA).[29]

Objectives

Our aim in this single-center pilot study was to investigate whether metabolites of the KYN pathway and activity of relevant enzymes measured before and 12 h after thrombolytic therapy in ischemic stroke could serve as potential biomarkers for predicting treatment response and prognosis.

Patients and methods

Patients and outcomes

Our inclusion criteria were patients with a diagnosis of acute ischemic stroke who underwent intravenous thrombolysis with alteplase between January and December 2018. We excluded patients who received thrombectomy and those who had a baseline mRS score >2. The pilot study

was conducted in accordance with the revised Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the University of Szeged, Albert Szent-Györgyi Clinical Centre (Project GINOP 2.3.2-15-2016-00048). All patients or their relatives gave informed consent for inclusion before the participation in the study.

C-reactive protein (CRP) and white cell count were measured from blood samples taken upon arrival in the emergency department. The time when the alteplase bolus was administered is referred to as "needle time". All patients underwent repeat imaging approx. 24 h after treatment. The NIHSS and mRS scores were established by a certified expert. The NIHSS scores were calculated on admission before thrombolysis, and at discharge from the stroke unit.

Efficacy endpoints were early neurological improvement (ENI) and good functional outcome at 30 and 90 days after the stroke. We defined ENI as a ≥4 point decrease in the NIHSS score from admission to discharge. The criterion for good functional outcome was an mRS score ≤2.

Sampling

Peripheral venous blood samples were taken just before thrombolysis and 12 h after the initiation of treatment (samples A and B, respectively). Blood samples were centrifuged at 3000/min for 13 min, and sera were then stored at –80°C until further analysis. Due to restricted opening times of our biobank facility, samples were only processed on weekdays.

Measurement of kynurenines by ultra-high-performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS)

Reagents and chemicals

All reagents and chemicals were of analytical or liquid chromatography-mass spectrometry (LC-MS) grade. Tryptophan and its metabolites and d4-picolinic acid (PICA) were purchased from Sigma-Aldrich (St. Louis, USA). The d3-3-HK was obtained from Buchem BV (Apeldoorn, the Netherlands). The other deuterated internal standards (ISs; d4-serotonin (SERO), d4-KYN, d3-3-3-HANA, d5-TRP, d5-5-hydroxy-3-indoleacetic acid (5-HIAA), d5-KYNA, d4-XA and d3-QUIN) were purchased from Toronto Research Chemicals (Toronto, Canada). Acetone, methanol (MeOH) and water were obtained from VWR Chemicals (Monroeville, USA). Formic acid (FA) was purchased from Thermo Fisher Scientific (Portsmouth, USA).

Preparation of standard, IS and quality control solutions

Stock solutions, calibration standards and quality control (QC) samples were prepared as described previously.[30] Calibration standards consisted of 100 µL of "blank" serum, 10 µL of standard solution mix (156.25–5000 nM SERO, 312.5–10,000 nM KYN, 7.8–250 nM 3-HANA, 6.25–200 µM TRP, 7.8–250 nM 5-HIAA, 6.25–200 nM ANA, 4.7–150 nM KYNA, 6.25–200 nM 3-HK, 1.5–50 nM XA, 3.125–100 nM PICA, and 62.5–2000 nM QUIN in 0.1% (v/v) aqueous FA), were treated with 370 µL of ice-cold acetone: MeOH (1:1, (v/v)) containing 10 µL of the SIL-IS mix (1500 nM d4-SERO, 1000 nM d4-KYN, 65 nM d3-3-HANA, 5250 nM d5-TRP, 200 nM d5-5-HIAA, 50 nM d5-KYNA, 90 nM d3-3-HK, 25 nM d4-XA, 80 nM d4-PICA, and 300 nM d3-QUIN) to precipitate proteins. After centrifugation, 400 µL of supernatant were transferred to a new tube, spun for 15 s and split into 2 equal parts. After concentration under a vacuum (Savant SC 110 A Speed Vac Plus; Savant, Holbrook, USA), half of the sample was treated with 70 µL of derivatizing reagent (n-butanol-acetyl chloride, 9:1, (v/v)) and was incubated for 1 h at 60°C. The mixture was dried under nitrogen before reconstitution. Both parts of the sample were dissolved in 100–100 µL of the starting eluent, vortexed, centrifuged, and combined.

Preparation of human serum samples for analysis

The human serum samples were prepared as described previously.[30] Briefly, to 100 µL of each serum sample, 10 µL 0.1% (v/v) of aqueous FA and 370 µL of ice-cold acetone–MeOH (1:1, (v/v)) containing 10 µL of the SIL-IS mix (the same as used in the preparation of the calibration standards) were added, and 400 µL of supernatant was treated as above.

Instrumentation and UHPLC-MS/MS analysis

The UHPLC separation of TRP and its metabolites was performed on a pentafluorophenyl (PFP) column (100 Å, 100 mm × 2.1 mm, particle size 2.6 µm; Phenomenex, Torrance, USA) connected to an ACQUITY I-Class UPLC™ liquid chromatography system (Waters, Manchester, UK) using 0.1% (v/v) aqueous FA as solvent A and MeOH containing 0.1% (v/v) FA as solvent B. All mass spectrometric measurements were carried out on an online connected Q Exactive™ Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, USA), operating in the positive electrospray ionization mode. For quantitative mass spectrometric analysis through MS/MS, the parallel reaction monitoring (PRM) data acquisition mode was chosen. The optimization of parameters and the validation of the UHPLC-MS/MS analysis for human serum were carried out previously.[30]

Statistical analyses

Our outcome measures were categorical variables (ENI, good outcome at 30 and 90 days). Based on the Shapiro–Wilk test, some KYN metabolites and enzymatic activities

did not show normal distribution (namely, 3-HANA, TRP, ANA, 3-HK, PICA, XA, monoamine oxidase (MAO), and KAT). Therefore, based on the distribution, continuous variables were either expressed as mean ± standard deviation (SD) or median and interquartile range (IQR). Kynurenine metabolite concentrations and enzymatic activities measured at the 2 timepoints were compared with either paired sample t-test (if the distribution was normal), or Wilcoxon matched-pairs signed-ranks test (when data was nonparametric) for cases where both samples were taken. To compare means of concentrations and enzymatic activities between groups with and without ENI or good functional outcome, we used the independent sample t-test or Mann–Whitney U test (depending on the distribution of the data). Boxplots were drawn to allow for better visualization of statistically significant findings. Furthermore, if statistical significance was met, we performed receiver operating characteristic (ROC) analysis. We calculated area under the curve (AUC), as well as sensitivity (SN) and specificity (SP) for different cut-off values. Due to the small sample size of our pilot study, logistic regression was not performed. A p-value of <0.05 was regarded statistically significant. Confidence intervals (CI) of 95% were presented where appropriate. Analyses were carried out with IBM SPSS v. 24 (IBM Corp., Armonk, USA) statistical software.

Results

Our pilot study included 48 patients. Thirty-nine were known to be within the 4.5 h thrombolysis time window. In the remaining 9 patients with unknown stroke onset time, intravenous alteplase was administered on the basis of a diffusion-weighted imaging (DWI)-fluid-attenuated inversion recovery (FLAIR) mismatch demonstrated on an acute brain magnetic resonance imaging (MRI), as per the WAKE-UP trial.[31] The flowchart of patient selection is shown in Fig. 2. The clinical characteristics of our study population are highlighted in Table 1. Seventeen patients had large vessel occlusion (LVO), but mechanical thrombectomy was not performed due to limited availability of this service in our center at the time. We collected 32 blood samples before thrombolysis and 36 samples 12 h after treatment. Twenty-three patients had samples taken at both timepoints. The UHPLC-MS/MS method provided simultaneous quantification of TRP and its 10 most important metabolites (SERO, KYN, 3-HANA, 5-HIAA, ANA, KYNA, 3-HK, XA, PICA, and QUIN).[30] Concentrations of the measured KYN metabolites of the 23 patients who had sampling at both timepoints are shown in Table 2. Significant changes in paired serum levels were observed for KYN, ANA, KYNA, XA, PICA, and QUIN. Enzymatic activity of IDO, MAO and KAT were calculated by the following ratios: KYN/TRP, 5-HIAA/SERO and KYNA/KYN, respectively. Enzymatic activities are also demonstrated in Table 2. The activity of IDO and MAO decreased significantly after 12 h.

Table 1. Clinical data of our study population (n = 48). Some data were not available for all patients. These are highlighted after each criteria accordingly

Patient characteristics	Value
Mean age ±SD [years]	67.33 ±12.04
Female	24 (50%)
Male	24 (50%)
Hypertension	41 (85.42%)
Diabetes mellitus	13 (27.08%)
Hyperlipidemia	39 (81.25%)
Smoking	14 (29.17%)
Atrial fibrillation	7 (14.58%)
Coronary artery disease	10 (20.83%)
Mean baseline NIHSS score ±SD	8.81 ±4.29
Mean baseline mRS score ±SD	0.79 ±0.77
Large vessel occlusion	17 (35.42%)
Mean SOtN time ±SD (min, n = 39)	136.59 ±53.9
Mean DtN time ±SD (min, n = 47)	57.45 ±35.72
Length of stay in stroke unit [days]	4.91 ±2.05
Intracerebral hemorrhage after treatment	4 (8.33%)
Mean C-reactive protein ±SD [mg/L], n = 47)	10.44 ±18.78
Mean white cell count ±SD [g/L]	8.07 ±2.31
Mean discharge NIHSS score ±SD	6.71 ±7.89
Early neurological improvement	19 (39.58%)
Mean mRS score at 30 days ±SD (n = 45)	2.47 ±1.84
Mean mRS score at 90 days ±SD (n = 40)	2.38 ±1.9
Good functional outcome at day 30 (n = 45)	27 (60%)
Good functional outcome at day 90 (n = 40)	24 (60%)

DtN – door to needle; mRS – modified Rankin Scale; NIHSS – National Institutes of Health Stroke Scale; SD – standard deviation; SOtN – stroke onset to needle.

Patients with ENI had significantly lower concentrations of KYNA and lower KAT activity in sample A (independent sample t-test, p = 0.01, df = 30, t = –2.722; and Mann–Whitney U test, p = 0.002, z = –3.050, respectively, Fig. 3). There was no statistically significant difference in sample B. Regarding the presence or absence of good outcome at 30 and 90 days, concentrations and enzymatic activities did not statistically significantly differ in samples A or B. Receiver operating characteristic analysis for ENI was performed using KYNA levels and KAT activity measured before treatment (Fig. 4). The AUC for KYNA concentrations was 0.74, 95% CI = 0.57–0.91, p = 0.02. The optimal cut-off value to predict ENI was 37.8 nM (SN 69.2%, SP 68.4%). Similarly, the AUC for KAT activity was 0.82, 95% CI = 0.67–0.98, p = 0.002. The optimal cut-off activity was 0.0127 (SN 92.3%, SP 73.7%).

Discussion

To our knowledge, this is the first study to analyze the changes in KYN metabolite serum levels and enzymatic

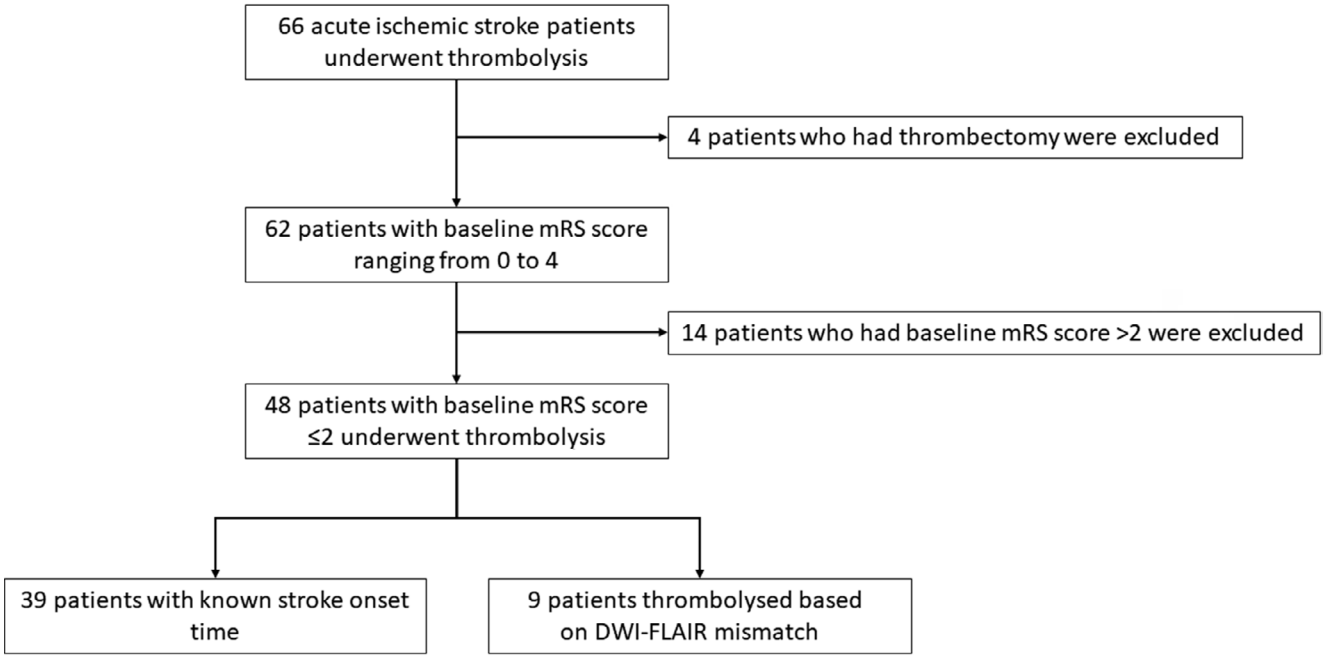

Fig. 2. Flowchart showing patient enrollment in our pilot study

DWI – diffusion weighted imaging; FLAIR – fluid attenuated inversion recovery; mRS – modified Rankin Scale.

Table 2. Mean and median concentrations of KYN metabolites taken before thrombolysis (sample A) and 12 hours after (sample B) alteplase treatment. Only the results of 23 patients who had sampling at both timepoints are presented. The unit of measurement for all metabolites is nanomoles (nM). Enzymatic activities are also shown (these ratios do not have units of measurement). Concentrations are expressed as mean ±SD if the distribution was normal or median and interquartile range when data was nonparametric. Concentrations and enzymatic activities were compared with either paired sample t-test (if the distribution was normal) or Wilcoxon matched-pairs signed-ranks test (when data was nonparametric)

Metabolites and enzymes	Sample A (nM) (n = 23)	Sample B (nM) (n = 23)	p-value
SERO	655.34 ±301.3	680.97 ±263.54	0.44
KYN	3669.52 ±1044.79	3413.1 ±1114.47	0.03
3-HANA	53.07 (40.9–79.74)	43.33 (35.58–62.01)	0.05
TRP	46,371.75 (41,588.69–54,644.26)	50,599.91 (41,798.89–53,961.54)	0.56
5-HIAA	96.58 ±35.47	88.15 ±36.29	0.18
ANA	53.75 (38.73–68.44)	43.8 (27.07–60.2)	0.01
KYNA	45.44 ±20.02	37.69 ±14.55	0.004
XA	11.27 (5.8–16.51)	4.58 (2.56–7.93)	0.001
3-HK	125.68 (79.33–175.98)	115.73 (87.04–161.72)	0.26
PICA	43.33 (34.09–52.22)	29.47 (24.12–39.6)	<0.001
QUIN	673.69 ±230.82	620.26 ±236.66	0.001
IDO	0.08 ±0.02	0.07 ±0.02	0.02
MAO	0.16 (0.11–0.21)	0.12 (0.08–0.2)	0.02
KAT	0.01 (0.01–0.02)	0.01 (0.01–0.01)	0.14

3-HANA – 3-hydroxyanthranilic acid; 3-HK – 3-hydroxykynurenine; 5-HIAA – 5-hydroxy-3-indoleacetic acid; ANA – anthranilic acid; IDO – indoleamine 2,3-dioxygenase; KAT – kynurenine aminotransferase; KYN – kynurenine; KYNA – kynurenic acid; MAO – monoamine oxidase; PICA – picolinic acid; QUIN – quinolinic acid; SD – standard deviation; SERO – serotonin; TRP – tryptophan; XA – xanthurenic acid.

activity in acute ischemic stroke patients who received thrombolytic treatment.

The main finding of our pilot study is that ischemic stroke patients with ENI after thrombolysis have significantly lower concentrations of KYNA and lower KAT activity at baseline. Therefore, we propose that pre-thrombolysis KYNA levels and KAT activity are potential biomarkers of ENI. It should be highlighted that ENI was defined as the difference between the admission and discharge NIHSS scores, although we are aware that ENI is usually calculated with the NIHSS score taken 24 h after symptom onset or treatment.[32] The mean time difference between

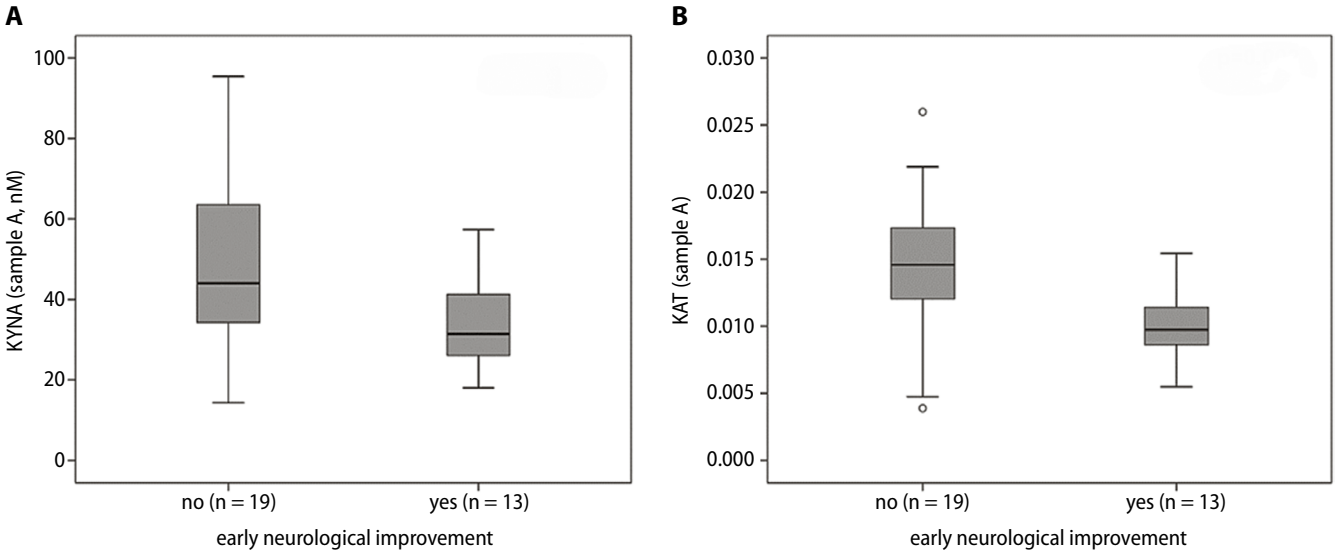

Fig. 3. Boxplots highlighting the significant difference of KYNA levels (A) and KAT activity (B) between patients with and without early neurological improvement. Measurements were made from samples taken before alteplase treatment. For each box, the horizontal line inside the box shows the median. The ends of the boxes represent the 1st and 3rd quartiles. The whiskers extend to the highest and lowest values not considered outliers (defined as 1.5 times the interquartile range (IQR)). Outliers are shown as circles

KAT – kynurenine aminotransferase; KYNA – kynurenic acid.

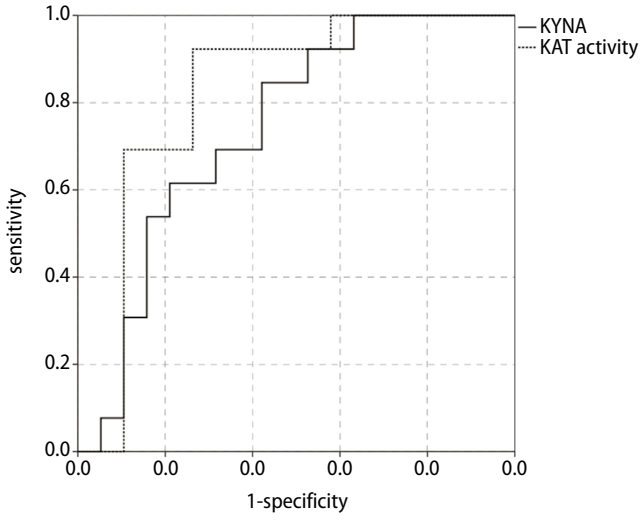

Fig. 4. Receiver operating characteristic (ROC) curve showing the accuracy of KYNA concentration and KAT activity (measured before thrombolysis) in predicting early neurological improvement

KAT – kynurenine aminotransferase; KYNA – kynurenic acid.

the admission and discharge date in our population was 4.91 days. We believe that this five-day difference does not confound the treatment effect size of intravenous alteplase. Our view is that the true effect of thrombolysis is better reflected in the short-term NIHSS change than in the 30 and 90 day mRS scores, which are more dependent on a number of additional factors, such as the pre-stroke condition of patients, comorbidities, polypharmacy, the availability and quality of rehabilitation, and support provided by family.

Based on our previous research in animal stroke models, we had hypothesized that levels of neuroprotective KYNA would be higher in patients with better outcomes.[13,33] However, lower concentrations of KYNA and lower KAT activity were found to predict good treatment response, with good sensitivity and specificity. Our findings support previous observations by Darlington et al. of higher KYNA levels being detected in patients who died within 21 days after ischemic stroke compared to those who survived.[11] It is unclear why increased levels of KYNA are associated with worse clinical outcome in ischemic stroke. One possible explanation is that KYNA is an endogenous NMDAR antagonist; therefore, it could further decrease synaptic activity in brain ischemia and, consequently, worsen brain function.[11,34] It has also been reported that KYNA can interfere with mitochondrial respiration, resulting in reduced ATP synthesis and increased levels of oxidative stress.[35]

We did not find any correlation between KYN metabolites or enzymes and good functional outcome measured 30 and 90 days after the stroke. This finding was somewhat surprising, given the results of the ROC analysis. Brouns et al. reported that the KYN/TRP ratio correlated with the mRS score 3 months after the ischemic stroke.[10] Possible explanations behind the lack of correlation in our study are the small sample size and the effect of thrombolytic treatment.

Limitations

The main limitations of our pilot study are the small sample size and the absence of a control group. An ideal control group would have been acute ischemic stroke patients who did not receive alteplase treatment. However, this would have been unethical, given the evidence supporting thrombolytic treatment.[36]

We could not obtain blood samples from every patient at both timepoints due to occasional limited availability of our biobank facility, and the loss of several patients before follow-up. Due to the small number of individuals included in our study, logistic regression analysis was not applicable.

Also, it is important to note that KYN metabolites were measured from the serum of patients. Therefore, our findings do not necessarily reflect the intracerebral changes in KYN metabolites and enzymes in the setting of acute ischemic stroke. It has been previously reported that approx. 40% of KYN is produced locally in the central nervous system and the remaining 60% is taken up from circulation.[37] Metabolites that can cross the blood-brain barrier (BBB) via large neutral amino acid transporters are TRP, KYN and 3-HK.[20,38] Kynurenic acid only has a limited ability to traverse across the BBB.

Conclusions

In conclusion, our pilot study provides further evidence that the KYN pathway is already activated within the first few hours after symptom onset in acute ischemic stroke. We propose that baseline serum KYNA concentration and KAT activity are potential biomarkers predicting early treatment response to thrombolytic therapy in stroke. Consequently, genetic polymorphisms of KAT could also hold promise as a further research target. Future studies with larger samples, well-chosen control groups and rigorous methodology are needed.

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24-Hour Near-Infrared Spectroscopy Monitoring of Acute Ischaemic Stroke Patients Undergoing Thrombolysis or Thrombectomy: A Pilot Study

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Introduction: Monitoring of acute ischaemic stroke patients during thrombolysis or thrombectomy is based mostly on frequent physical examinations, since no objective measurement of cerebrovascular haemodynamics is available in routine clinical practice. Near-infrared spectroscopy (NIRS) is a bed-side, noninvasive assessment tool that could help monitor these patients and potentially guide therapeutic interventions. Our goal in this pilot study was to investigate whether NIRS is a suitable method to monitor leptomeningeal collateral circulation via changes in cortical oxygen saturation in the first 24 hours of acute ischaemic stroke. *Patients and methods:* Our study included 5 patients with acute anterior circulation infarcts. All patients received thrombolytic therapy and 1 had thrombectomy. 24-hour continuous NIRS monitoring was performed on all participants. *Results:* We aimed to give a detailed description of each NIRS recording and explain how the observed findings could correlate with changes in anterior watershed territory collateral circulation and clinical outcome. *Conclusion:* Our pilot study supports the use of NIRS monitoring in acute ischaemic stroke. We believe that this technique could provide real-time information on the dynamic changes of leptomeningeal collateral circulation and help monitor the effects of thrombolysis and thrombectomy.

Key Words: Near-infrared spectroscopy (NIRS)—ischaemic stroke—thrombolysis—collateral circulation

Introduction

Intravenous administration of recombinant tissue plasminogen activator is the treatment of choice in eligible acute ischaemic stroke patients who arrive to the hospital within the therapeutic time window.[1] If large vessel occlusion is present, mechanical thrombectomy should be performed as well. Monitoring of patients during these procedures is based mostly on frequent physical examinations. For the time being, no objective measurement of the patients' cerebrovascular haemodynamics is used in routine clinical practice. Near-infrared spectroscopy (NIRS) is a bed-side, noninvasive, continuous, real-time assessment tool which could help monitor patients with acute ischaemic stroke. It is most commonly used during cardiac surgery and carotid endarterectomy to detect and prevent cortical desaturations which might lead to permanent neurological sequelae.[2] To our knowledge, only a few observational and pilot studies have been published that investigated the potential of NIRS monitoring during acute ischaemic stroke.[3-6]

NIRS utilizes a light source which emits photons in the near-infrared range (700 nm-1100 nm). These photons can

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penetrate thought the skull and a few centimetres deep into the brain tissue. The emitted light is partly redirected, scattered, and absorbed. The absorption spectrum of oxyhaemoglobin (Hb_{oxy}) and deoxyhemoglobin (Hb_{deoxy}) is different at various wavelengths.[7] This difference allows for calculation of Hb_{oxy} and Hb_{deoxy} concentrations based on the difference in intensity of emitted and received light, using the Beer-Lambert equation:

$$A = lg\frac{I_0}{I} = \varepsilon \times c \times l$$

(A: absorption, I_0: intensity of emitted light, I: intensity of received light, ε: absorption coefficient, c: concentration, l: photon pathlenght).

Total haemoglobin (Hb_T) concentration equals the sum of Hb_{oxy} and Hb_{deoxy} concentrations and is proportional to cerebral blood volume (CBV).[8] Therefore NIRS can be used to measure cortical blood oxygenation/saturation (the fraction of Hb_{oxy} relative to Hb_T) and serve as an indicator for the balance between cerebral oxygen delivery and consumption.[2,9] Mean cortical saturation measured with NIRS comprises of approximately 70% venous and 30% arterial blood.[7] It has been established that the relative change in regional O_2 saturation (rSO_2) and not the absolute rSO_2 is considered as a marker of cerebral haemodynamics, since absolute values show great interindividual variability.[10,11] Other limitations of NIRS are environmental and individual features that influence absolute rSO_2 values. These features are summarized in Table 1.[3,12] Combined effect of the listed factors can sometimes make the interpretation of NIRS measurements uncertain.

It has been established that collateral circulation plays a pivotal role in reducing progression of ischaemic brain damage.[13] Patients with good collaterals develop smaller infarcts, respond better to mechanical thrombectomy, show better clinical outcome, and have a lesser chance for haemorrhagic transformation after thrombolysis.[14-16] However, real-time assessment of collateral circulation in the setting of acute ischaemic stroke is lacking. We know from multimodal MRI studies that augmented CBV, preserved cerebral blood flow (CBF) and delayed mean transit time imply the presence of collateral flow.[17] Taussky et al showed a linear correlation between rSO_2 and CBF measured with CT perfusion.[18] Therefore, since CBV and CBF correlates with rSO_2 values, our goal in this pilot study was to investigate whether NIRS is a suitable method to monitor anterior watershed territory leptomeningeal collateral circulation via changes in cortical oxygenation during thrombolysis and thrombectomy. Due to the small sample size of our pilot study, we could not draw statistical conclusions. Instead, we aimed to give detailed analysis of the 5 NIRS recordings and explain how these findings could correlate with cerebrovascular haemodynamics and clinical picture.

Table 1. *Factors influencing rSO_2 values*

Contamination form hair and skin
Sweating
Skull thickness
Extracranial circulation
O_2 extraction of brain tissue (e.g.: reduced O_2 extraction of infarcted or oedematous territory)
Blood pressure
Peripheral oxygen saturation
Haemoglobin concentration in blood
Level of consciousness

Patients and Methods

The study was approved by an independent ethics committee (University of Szeged, Faculty of Medicine, Ethics Committee, ID: 211/2016-SZTE). All patients or first degree relatives gave written informed consent prior to NIRS monitoring. Our study population included 5 acute stroke patients who had left sided anterior circulation infarcts. Detailed patient characteristics are highlighted in Table 2. All participants received alteplase as recommended by the 2018 american heart association (AHA)/american stroke association (ASA) acute ischaemic stroke guideline.[1] One patient also had mechanical thrombectomy due to left M1 occlusion (Patient 3). INVOS™ 5100C Cerebral/Somatic Oximeter (Medtronic, Minneapolis, MN) was used for 24-hour continuous monitoring. Application of the NIRS sensors did not delay the start of thrombolysis. The sensors were placed over bilateral frontal areas, as recommended by the manufacturer. The studied brain areas correspond to the anterior watershed territories. Baseline rSO_2 was measured before the initiation of intravenous recombinant tissue plasminogen activator. rSO_2 measurements were made approximately every 30 seconds. We analyzed the 5 minute average rSO_2 values registered at the start of thrombolysis and also 1 hour, 6 hours, 12 hours, 18 hours, and 24 hours after the initiation of alteplase treatment. Interhemispheric rSO_2 ($IH\Delta rSO_2$) difference was calculated as rSO_2 on the affected side minus rSO_2 measured above the contralateral side. Based on previous articles, 4% change in rSO_2 value, and 2% change in $IH\Delta rSO_2$ was considered significant.[5,19] Simultaneously, blood pressure, peripheral O_2 saturation (SpO_2), heart rate, and electrocardiography were also monitored. The patients' SpO_2 was above 92% while breathing ambient air, therefore they did not receive O_2 supplementation during the study period. The only exception was Patient 3 who underwent thrombectomy. He was intubated because he could not cooperate to the procedure due to severe aphasia. The patients' clinical outcome was assessed with the National Institutes of Health Stroke Scale (NIHSS) and modified Rankin scale (mRS). A mRS score of 0-2 at 90 days was considered as good functional outcome. If large vessel occlusion (LVO) was present, collateral circulation on imaging was assessed by a neuroradiologist using a 3 grade scale (good-intermediate-poor). Initially, Patients 1-3 had CT angiography (CTA) and Patients 4-5 had MR angiography –

Table 2. *Patient characteristics*

	Patient 1	*Patient 2*	*Patient 3*	*Patient 4*	*Patient 5*
Sex	Female	Male	Male	Female	Female
Age	80	67	63	66	78
Vessel territory	Left MCA	Left ICA	Left MCA	Left MCA	Left MCA
LKW to treatment time (min)	128	97	200	245	228
Hypertension	yes	yes	yes	yes	yes
Hyperlipidaemia	no	yes	yes	yes	yes
Diabetes mellitus	no	no	no	no	no
Atrial fibrillation	no	no	no	no	no
Ischaemic heart disease	yes	no	yes	no	yes
Smoking	no	no	yes	yes	yes
Haemoglobin (g/l)	142	140	139	153	121
Large vessel occlusion	Left M2	Left ICA	Left M1	0	Right ICA (chronic)
Collateral score	Good	Good	Intermediate	Not applicable	Not applicable
Stroke subtype	CE	LAA	CE	SVD (striatocapsular infarct)	CE
NIHSS baseline	14	15	17	7	9
NIHSS 24-hour	9	12	12	10	4
NIHSS discharge	4	9	8	10	1
mRS 3 months	2	2	1	3	1

Abbreviations: CE: cardioembolic, ICA: internal carotid artery, LAA: large-artery atherosclerosis, LKW: last known well, MCA: middle cerebral artery, mRS: modified Rankin score, NIHSS: National Institutes of Health Stroke Scale, SVD: small vessel disease, VA: vertebral artery.

time of flight imaging. Figure 1 shows CT and MRI scans approximately 24 hours after thrombolysis for each patient.

Results

Descriptive Analysis of Each Patients' NIRS Recordings

Patient 1 suffered a left middle cerebral artery (MCA) territory stroke due to M2 occlusion. Collateral circulation was good based on CTA. During NIRS monitoring, no relevant rSO_2 difference was observed between the 2 hemispheres ($IH\Delta rSO_2$ was between −2% and 0%). rSO_2 values were quite stable on both sides. The patient had a good clinical outcome at 3 months.

Patient 2 had clinical signs of left hemispheric stroke. CTA revealed a left internal carotid artery (ICA) occlusion. Good collaterals were detected on CTA and rSO_2 absolute values were higher above the ipsilateral side (average $IH\Delta rSO_2$ was 3%). rSO2 levels gradually rose in the first 12 hours on both sides. This might indicate subtle increase in CBV and CBF in the leptomeningeal collaterals. The patient's NIHSS score decreased in the first few days and eventually showed good clinical outcome at 3 months.

Patient 3 had a left M1 occlusion and underwent endovascular thrombectomy after thrombolysis. Collaterals were graded as intermediate on CTA. Initially, a significant $IH\Delta rSO_2$ was observed. The affected side had a lower absolute rSO_2 value (55% versus63%). This difference did not change after thrombolysis (1 hour post thrombolysis $IH\Delta rSO_2$ was −7%). However, after thrombectomy there was a significant increase in rSO_2 on the ipsilateral side and consequently $IH\Delta rSO_2$ substantially decreased. $IH\Delta rSO_2$ absolute values even became positive after 12 hours. These findings possibly indicate that NIRS sensors were either placed above ischaemic territory or the leptomeningeal collateral circulation was insufficient. As expected from the NIRS recording of the first 24 hours, the patient's recovery went well (mRS 1 at 90 days).

Patient 4 was the only participant who did not achieve good functional outcome at 90 days (mRS was 3). She suffered a left MCA territory infarction, MR angiography – time of flight imaging did not show LVO. Before thrombolysis, absolute rSO_2 was significantly higher on the affected side (69% versus 61%). After 1 hour, a marked increase of rSO_2 was observed above both hemispheres (+9%, $IH\Delta rSO_2$ remained 8%). $IH\Delta rSO_2$ then steeply decreased to −2% at 12 hours. The patient's NIHSS score worsened. Control CT scan revealed a left striatocapsular infarct. The striatocapsular territory is supplied by perforator arteries stemming from the proximal part of M1 and does not have collateral circulation.[20] Since rSO_2 increased similarly above both hemispheres in the first hour, it is possible that the ischaemic insult provoked an increase in global cerebral perfusion.

Patient 5 had markedly elevated rSO_2 values above the ipsilateral hemisphere (82% versus 69%). The significantly high $IH\Delta rSO_2$ was possibly a consequence of chronic right ICA occlusion which led to long-term, effective Willisian collateralization and consequent enlargement of left ICA, MCA, and anterior cerebral artery (ACA) (Fig. 1**,F**). Increased blood flow in the left MCA and ACA could explain the high rSO_2 values above the ipsilateral watershed area, implying well-developed leptomeningeal collaterals. The $IH\Delta rSO_2$ value remained high throughout the 24-hour monitoring. The patient had a good functional outcome at 3 months.

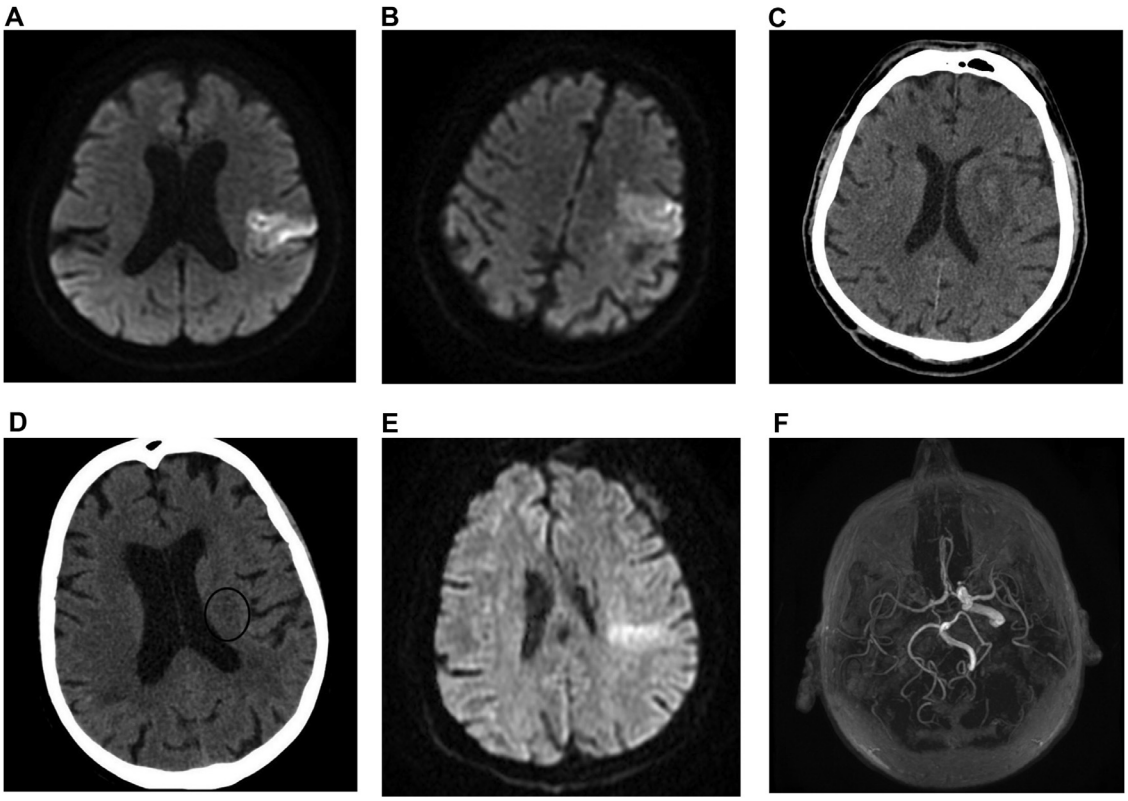

Figure 1. ***CT and MRI scans of patients approximately 24 hours after thrombolysis.*** *(A) Diffusion-weighted imaging (DWI) scan of Patient 1, showing left MCA territory cortical ischaemia. (B) DWI scan of Patient 2 shows a similar brain infarct. (C) Noncontrast CT (NCCT) scan of Patient 3 after thrombectomy. The infarct mainly involves the left basal ganglia and internal capsule. (D) NCCT of Patient 4 showing slight hypodensity in the left corona radiata (striatocapsular infarct). (E) DWI scan of patient 5 shows a left MCA territory infarct. (F) MRA-TOF reconstruction of Patient 5 demonstrating enlarged left ICA and MCA. Abbreviations: MRA-TOF, MR angiography – time of flight; MCA, middle cerebral artery.*

Data of NIRS recordings and diagrams are found in the **Supplementary material.**

Analysis of Combined Results

The initial rSO_2 above the affected hemispheres showed greater variability compared to the contralateral sides. Patient 5's results might have led to bias, therefore we only examined the first 4 patients' results. Still, the difference in variability remained significant, which was most prominent in the first 12 hours. This finding probably reflects impaired autoregulation on the affected side. Graphs demonstrating rSO_2 variability are found in the **Supplementary material.**

Discussion

Most of the previous studies that used NIRS in the setting of acute ischaemic stroke aimed to study the oxygenation of ischaemic brain area. Instead, we tried to investigate whether NIRS is feasible in evaluating leptomeningeal collaterals located at the anterior watershed areas. We believe that a good example for our hypothesis is the case of Patient 5. Due to a chronic right ICA occlusion, we measured significantly higher rSO_2 values above the left hemisphere. The explanation of this finding is possibly the increased blood flow in the left ICA, MCA, and ACA which provides adequate blood perfusion to both hemispheres through the circle of Willis. Long-term increased flow led to enlargement of these vessels and subsequently well-developed leptomeningeal collateral circulation in the monitored hemisphere.

Ritzenthaler et al performed 24-hour NIRS monitoring in 17 acute stroke patients who underwent mechanical thrombectomy.[4] All their patients had lower absolute rSO_2 values above the affected hemisphere. They did not find a significant relationship between initial ipsilateral rSO_2 and collateral circulation (assessed with American Society of Interventional and Therapeutic Neuroradiology Collateral Flow Grading scale, ASITN). Explanations behind their finding might be that NIRS sensors were above ischaemic territory or the leptomeningeal collateral circulation was insufficient in all participants. Since the authors reported patients with ASITN score of more than 3 (indicating good collateral flow), the latter explanation seems unlikely. In our study, Patient 3 demonstrated a similar NIRS trend to those cases published in Ritzenthaler's article. After successful recanalization, $IH\Delta rSO_2$ significantly decreased. Patient 2 had left ICA occlusion, but still had higher rSO_2 on the ipsilateral side possibly due to well-developed leptomeningeal collaterals.

In another study, NIRS monitoring was also used during thrombectomy in 43 acute ischaemic stroke patients.[3] Hametner et al. reported that absolute values of median IHΔrSO_2, measured at the end of thrombectomy, were significantly lower in patients who died by 90 days. In addition, patients whose variability in rSO2 values were lower, showed significantly worse 90-day outcomes (mRS score 3-6). Due to the small sample size of our study, we could not draw significant statistical correlations related to NIRS parameters and clinical outcome. Instead, we aimed to give individual descriptions of each patients' monitoring.

Damian and Schlosser investigated patients with MCA occlusions who had consequent brain oedema.[19] NIRS monitoring was performed in the subacute phase of stroke (at least 12 hours, but within 4 days after the ictus). Interestingly, 22 out of 24 patients had higher absolute rSO_2 values above the ipsilateral frontal area. These data are quite the opposite of that published by Ritzenthaler et al. We hypothesize that this difference is because the measurements were made at different time points (subacute versus acute phase of stroke). It is possible, that the observed positive absolute IHΔrSO_2 values in Damien and Schlosser's study reflects increased compensatory leptomeningeal collateral circulation, which developed on the affected side a few days after the cerebrovascular insult. The article reported good clinical outcome (Glasgow Outcome Scale 3-4) in cases where average IHΔrSO_2 values increased over time. Outcomes were assessed between 6-24 weeks, after rehabilitation. In all 5 cases, where the initial IHΔrSO_2 decreased, the patients died. Another important finding of the study was that clinical signs of progressing brain oedema and unfavourable rSO_2 changes were reversible in some cases by hemicraniectomy, hyperventilation, hypothermia, or improved systemic perfusion.[19] Therefore, correct interpretation of NIRS monitoring could guide therapeutic interventions. Previous studies showed, that decrease in systemic blood pressure and/or SpO_2 correlates well with a drop in rSO_2.[3,5] We believe, that NIRS parameters can guide clinicians in finding the target blood pressure and SpO_2 values of each individual patient. For example, some patients with acute ICA occlusion could benefit from increasing blood pressure to maintain adequate collateral circulation until thrombectomy can be performed to achieve recanalization. A preclinical study investigated this concept and found that mild induced hypertension increased cortical collateral blood flow and significantly reduced infarct volume in mice with transient distal MCA occlusion.[21]

NIRS monitoring would provide additional information if more sensors were placed over the cerebral hemispheres. This way, rSO_2 could be simultaneously measured over the ischaemic territory and watershed areas. Rummel et al used multichannel NIRS monitoring during transient balloon occlusion of cerebral arteries.[8] They demonstrated that different rSO_2 changes are observed over the ischaemic core and watershed areas during transient LVOs due to

Table 3. *Relevant findings of previous studies with NIRS*

Study	Timing of monitoring	Findings
Ritzenthaler et al[4]	First 24 h, including thrombectomy	Lower absolute rSO_2 values above the affected hemisphere in all patients (n = 17) No significant relationship between initial Ipsilateral rSO_2 and collateral circulation
Hametner et al[3]	During thrombectomy + 6 h or time to extubation	Correlation was found between $rSO_{2\ and}$ MRI parameters (MTT and T_{max}) Median IHΔrSO_2 at the end of thrombectomy was significantly lower in patients who died by 90 d Variability in rSO_2 were lower in patients with mRS score 3-6 at 90-d
Damian and Schlosser[19]	Subacute phase (12 h to 4 d after stroke)	Significant association between changes in MAP and rSO_2 22 out of 24 patients had higher absolute rSO_2 above the ipsilateral frontal area Good clinical outcome when average IHΔrSO_2 values increased Progression of brain oedema and unfavourable rSO_2 changes were reversible by therapeutic interventions (e.g.: hemicraniectomy)
Moreau et al (multichannel monitoring)[12]	Acute phase (9 h ≥ after symptom onset)	At least 1 ipsilateral region showed reduced rSO_2 compared to unaffected side rSO_2 values were significantly higher after haemorrhagic transformation
Rummel et al (multichannel monitoring)[8]	Transient balloon occlusion of cerebral arteries	Different rSO_2 changes over ischaemic core and watershed areas

Abbreviations: IHΔrSO2, interhemispheric rSO_2 difference; MAP, mean arterial pressure; MRI, magnetic resonance imaging; mRS, modified Rankin scale; MTT, mean transit time; T_{max}, time-to-maximum; rSO_2, regional oxygen saturation.

haemodynamic changes in collateral flow. Moreau et al also applied multichannel NIRS monitoring in 5 acute ischaemic stroke patients who had LVO. The sensors were placed over the frontal, parasagittal frontal, Rolandic sulcus, Broca, and Wernicke areas of the brain.[12] The symptom onset to monitoring time was within 9 hours. They found that, at least 1 region of the infarcted hemisphere showed reduced rSO_2 values compared to the unaffected, contralateral side. One of their patient's suffered a haemorrhagic transformation a few days after the ischaemic event. Not surprisingly, rSO_2 values were significantly higher above the affected hemisphere compared to the contralateral side. This finding is probably explained by the presence of still highly oxygenated blood within the brain tissue.[12]

Table 3 highlights the most important findings of previous NIRS studies.

In summary, the results of our pilot study support the use of NIRS monitoring in the setting of acute ischaemic stroke. We believe that this technique could provide valuable information on the state of leptomeningeal collaterals and help monitor the effects of thrombolysis and thrombectomy. In addition, rSO_2 values could guide individual management of patients' blood pressure and oxygen supplementation to widen the therapeutic time window for recanalization.[17] However, future studies, preferably with multichannel NIRS monitoring are warranted to gain further information on the relation between leptomeningeal collaterals, ischaemic territory, and rSO_2 absolute values and trends.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jstrokecerebrovasdis.2019.05.026.

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