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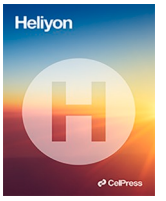

Research article

N-acetyl aspartate levels early after ischemic stroke accurately reflect long-term brain damage

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ABSTRACT

Background: Estimation of brain damage following an ischemic stroke is most often performed within the first few days after the insult, where large amounts of oedematous fluid have accumulated. This can potentially hamper correct measurement of infarcted area, since oedema formation poorly reflects infarct size. This study presents a non-invasive, easily applicable and reliable method to accurately predict long-term evolution and late-stage infarction.

Objective: We performed a longitudinal analysis of brain infarct evolution after MCAO in mice, in order to determine whether water-compensated N-Acetylaspartate (NAA) levels in the infarct area, measured 24 h after the insult, is a suitable marker for late-stage infarction and thereby prognosis.

Methods: Twenty mice were divided into 4 groups and scanned longitudinally at different timepoints after MCAO, followed by euthanisation for histology: Group 1) MRI/MRS at day 1 after MCAO (n = 4), Group 2) MRI/MRS at days 1 and 7 after MCAO (n = 5), Group 3) MRI/MRS at days 1, 7, and 14 after MCAO (n = 3), and Group 4) MRI/MRS at days 1, 7, 14, and 28 after MCAO (n = 4). At days 1, 7, 14, and 28, NAA levels were correlated with histological determination of neuronal death based on Nissl and H&E stainings.

Results: Twenty-four hours after the insult, NAA levels in the infarcted area decreased by 35 %, but steadily returned to normal after 28 days. In the acute phases, NAA levels strongly correlated with loss of Nissl substance ($r^2 = -0.874$, $p = 0.002$), whereas NAA levels in later stages reflect glial metabolism and tissue reorganisation. Most importantly, NAA levels 24 h after MCAO was highly correlated with late stage infarction at days 14 and 28 ($r^2 = 0.73$, $p = 0.01$), in contrast to T2 ($r^2 = 0.06$, $p = 0.59$).

Conclusions: By using a fixed voxel, which is easily positioned in the affected area, it is possible to obtain reliable measures of the extent of neuronal loss at early time points independent of oedema and brain deformation. Importantly, NAA levels 24 h after MCAO accurately reflects late-stage infarction, suggesting that NAA is a useful prognostic biomarker early after an ischemic stroke.

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1. Introduction

Ischemic brain damage involves a sequential cascade of multiple pathomechanisms, and in stroke patients it may take weeks before the damaged tissue matures into a state of terminal liquefactive necrosis with gliosis [1]. This multifactorial pathogenesis challenges drug testing studies with respect to both time and methodology, and may partly explain why the transitional aspects of animal stroke models are so poor [2,3].

We have previously shown that in most drug testing studies, infarct size estimation as a measure of neuronal survival is performed 24–48 h after stroke, when oedema formation peaks [4][1]. This greatly impacts the reliability of the measurements, since the methodologies most often applied either rely on oedema formation as a measure of total brain damage (e.g. T2, DWI) or perform the analyses on processed tissue, which allows for severe tissue derangements, and consequently loss of validity.

Therefore, suitable methods and markers that correctly predict the amount of brain damage, and thus long-term outcome, are needed. On these grounds we have revisited N-acetylaspartate (NAA), the most abundant CNS metabolite that can be detected by magnetic resonance microscopy (MRS). NAA is highly concentrated in neurons, and has been used as a marker for brain damage, in a range of rodent stroke studies [5–9]. The general consensus from these studies and others seem to be that NAA is a good marker in acute ischemic tissue, whereas redistribution in glial cells and trapping in necrotic debris restricts its use a stable long-term marker of neuronal loss [5]. Thus, if determined early after the ischemic episode, NAA correctly reflects the amount of brain damage in the infarcted area. Whether this reflects long-term outcome and prognosis is currently unknown.

We here report that NAA levels - normalized to amount of accumulated water, as this severely impacts the readings [10] - in the infarcted area 24 h after ischemia, accurately predicts long-term evolution and outcome in mice subjected to permanent MCAO.

2. Materials and methods

2.1. Middle cerebral artery occlusion

A total of 23 male C57BL/6J mice (7–10 weeks old, Taconic, Ejby, Denmark) were housed at the Experimental Stroke Unit, University of Copenhagen and were allowed to acclimatize for at least seven days prior to surgery. The mice were individually housed and given free access to water and food.

Mice were subjected to focal cerebral ischemia by permanent occlusion of the distal part of the left middle cerebral artery (MCA) as previously described [11]. The same person anesthetized and performed MCA surgery on all mice. The surgeon was well experienced with the procedures and blinded to the different groups. Before surgery, mice were placed in an induction chamber, and anesthesia was induced with 5 % isoflurane (Forene, Abbot Scandinavia, Stockholm, Sweden) delivered in pure oxygen. After the paw withdrawal reflex was absent, mice were shaved at the incision site, the skin was disinfected with 80 % ethanol, and then the mice were attached to an anesthetic face mask for spontaneous respiration. During surgery isoflurane was maintained at approximately 2.5 % to ensure sufficient anesthesia. To maintain normal body temperature, mice were placed on a heating pad (HB101, Panlab, Cornella, Spain). The rectal body temperature was maintained at 36–38 °C.

Before the incision was made through the skin, at the incision site the mice received subcutaneous lidocaine (10 mg/mL, Glostrup Apotek, Glostrup, Denmark). All skin incisions were performed through the temporal muscle between the lateral part of the orbit and the external auditory meatus. To access the MCA, a 1.5 mm diameter hole was drilled directly over the distal part of the left MCA, the

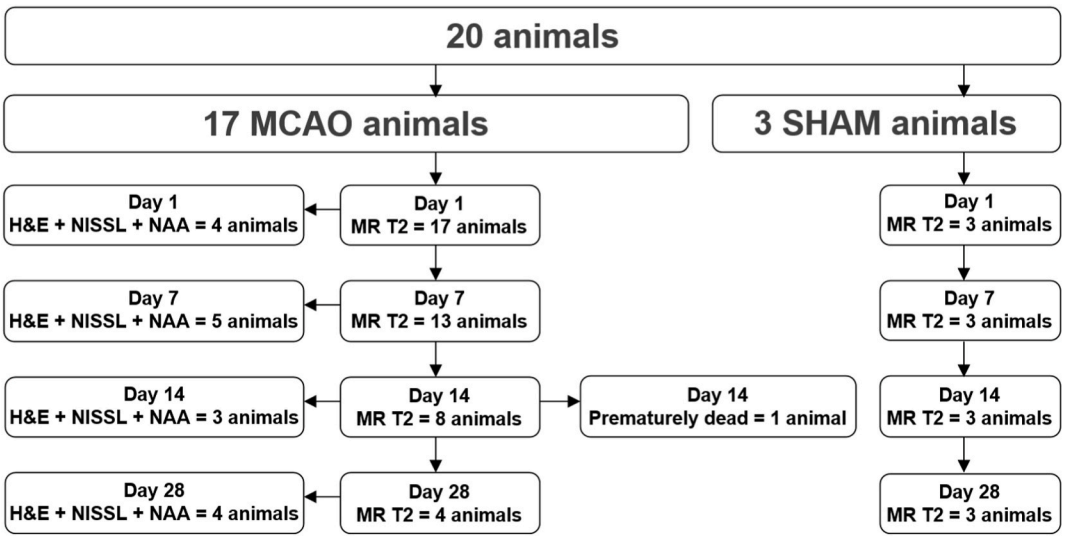

Fig. 1. Flowchart.

dura mater was removed, and the MCA was occluded by applying bipolar forceps coupled to an electrosurgical unit (ERBE VIO 100 C, Mediplast NC Nielsen, Balling, Denmark). The skin was then closed with interrupted sutures of 6-0 polyglactin 910 (Vicryl, Ethicon, St Stevens Woluwe, Belgium). From induction of anesthesia to regaining righting reflex, the time of the procedure was 10–15 min.

Sham animals underwent the same procedure except from vessel coagulation. After surgery, the mice received subcutaneous administration of lidocaine (10 mg/mL, Glostrup Apotek, Glostrup, Denmark) at the incision site, and were housed individually for a maximum of 28 days. Three mice died shortly after surgery.

The Experiment was approved by the Danish Animal Ethics Committee (#2012-DY-2934-00001) and Department of Experimental Medicine, University of Copenhagen.

2.2. MRS and MRI scanning protocols

MR scannings were performed using a 9.4 T Bruker Biospec scanner for small animals (Bruker BioSpin MRI, Ettlingen, Germany). A 20 mm dedicated transmitter/receiver 1H mouse brain volume coil was selected for the MR animal imaging, due to its good signal sensitivity and easy animal setup.

We sampled data at 4 different time points from day 1 to day 28 following MCAO in mice.

The mice were divided into 4 groups and scanned longitudinally at different time-points after MCAO, followed by euthanisation for histology: Group 1) MRI/MRS at day 1 after MCAO (n = 4), Group 2) MRI/MRS at days 1 and 7 after MCAO (n = 5), Group 3) MRI/MRS at days 1, 7, and 14 after MCAO (n = 3), and Group 4) MRI/MRS at days 1, 7, 14, and 28 after MCAO (n = 4). A flow chart for these main experimental groups is shown in Fig. 1. Besides these 4 main experimental groups, 3 animals had sham surgery and MRI/MRS at days 1, 7, 14 and 28. In total 17 animal completed the experiment and MCAO according to the plan, 3 animals had sham surgery and 3 animals died prematurely (2 animals after MCAO at day 1, and 1 animal at day 14).

The mice were anesthetized in an induction chamber using 2.5 % isoflurane, followed by maintenance at 1.5 % in pure air delivered through a nose cone. Body temperature was maintained during the entire scan to be at 37 ± 1 °C by circulating warm water around the animals. Respiration was monitored using a small pneumatic pillow placed under the abdomen of the animal. The body temperature was measured during the entered scan using a rectal thermometer. The respiration rate and temperature were displayed on an MR compatible small animal-monitoring device.

The 2D Standard Bruker TurboRARE T2 weighted images were acquired for infarct size determination and voxel positioning in local spectroscopy scans. The T2 weighted images were acquired with the following imaging parameters: (repetition time/Echo Time: TR/TE = 3272/48 ms; RareFactor = 8; number of acquisitions = 3; no. of slice/thickness = 20/0.7 mm; matrix, 256x256). Contours of

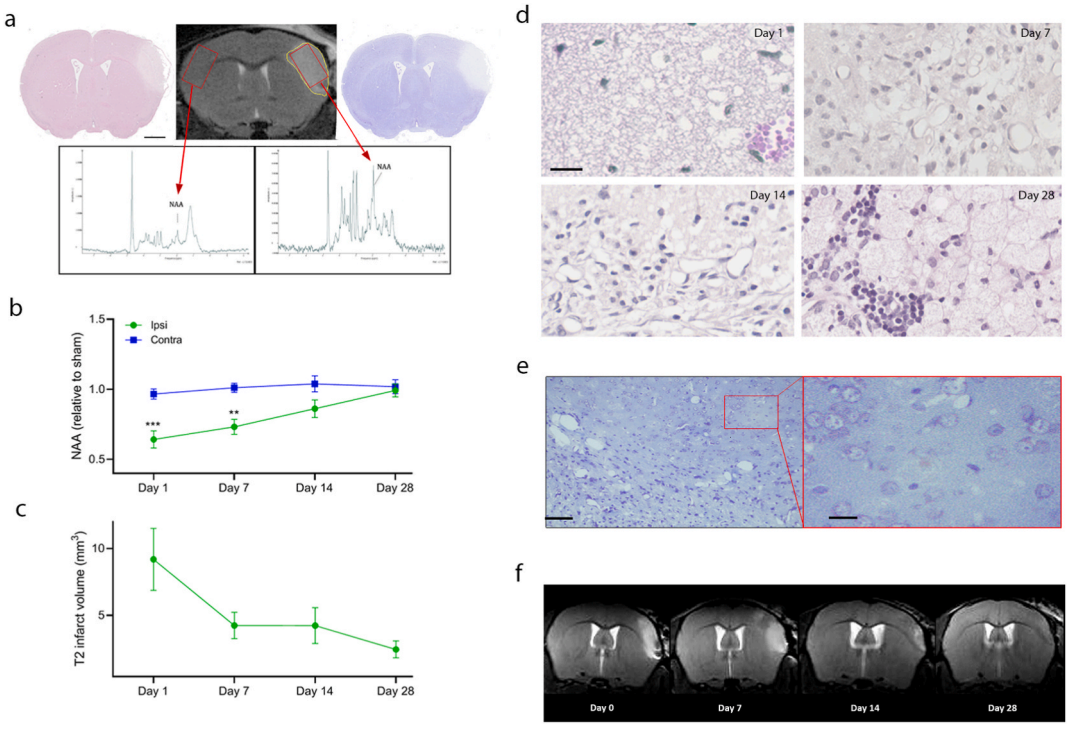

Fig. 2. A: H&E, T2-weighted MRI and cresyl violet images as well as the corresponding MR spectrum, 24 h after infarction. B: Water-normalized NAA levels from day 1–28. C: Infarct size evolution based on T2-weigthed imaging. D: Representative images of the infarcted area from day 1 to day 28. E: Representative image of dorsolateral striatum 7 days after infarction. F: Representative MR images of an infarcted brain from day 1–28 post-stroke. Scale bars: 10 mm (A), 50 μm (D), 100 μm (E, insert: 25 μm). ***:$p < 0.001$, **: $p < 0.01$.

the infarct regions were manually drawn using the IDL-software to obtain infarct volumes.

For in vivo 1H MR spectroscopy the voxel (2x2x2 mm) was positioned in the left MCA-territory and contra-laterally as control, as shown in Fig. 2. Localized shimming in the voxel was performed automatically based on Fastmap, leading to a water line width ranging from 12 to 20 Hz. Signals were obtained using the STEAM pulse sequence with implemented pre-delay outer volume suppression as well as the water suppression method, VAPOR [12].

Parameters for acquisition of the proton spectra in the volume of interest (VOI) are the following: (TR/mixing time/TE, 3000/10/2.35 ms; spectral width, 5000 Hz; number of excitations = 256; scan time, 13 min). Two spectra were acquired: a non-suppressed water spectrum to serve as an internal reference for signal normalization, and a water suppressed in order to quantify the metabolites. The two spectra were acquired with the same scanner calibration parameters i.e. gain and loading factors which were determined automatically from the first run. A baseline correction for macromolecules was also applied. The spectra were corrected for B0 instability due to eddy currents as well as B0 drift using the Bruker built-in routines (B0 map).

2.3. Spectral post processing and analysis

All the acquired MRS spectra were processed and analysed using the jMRUI software package (Version 5.2) [13]. The line-shape using Lorentzian line broadening of 15 Hz and the macromolecular contributions on the baseline were compensated. We used the nonlinear procedures AMARES [14] in jMRUI to compute individual spectral components where 10 metabolite tops were identified, but only total N-acetyl aspartate, total choline and total creatine were included in this study.

2.4. Tissue processing

Mice were overdosed with pentobarbital (200 mg/mL+1 % lidocaine Glostrup Apotek, Denmark) on day 1 (n = 4), 7 (n = 5), 14 (n = 3) and 28 (n = 4), after pMCAO according to Fig. 1. This was followed by perfusion through the left ventricle with 4 % isotonic saline until blood was cleared from the circulation, followed by 100 mL 4 % paraformaldehyde (VWR International, Leuven, Belgium). The brains were post-fixed in paraformaldehyde for at least 24 h before they were embedded in paraffin. Brains were cut into coronal sections (5 μm) at 15 consecutive levels with a distance of 400 μm in between, including the entire infarcted area. Sections were mounted on superfrost plus slides (Thermo Fischer Scientific, Waltham, MA, USA) and kept at 40 °C overnight to dry.

2.5. Histology

Two sections per level were chosen for either H&E or cresyl violet staining. The sections were rehydrated in xylene and decreasing concentrations of ethanol, and then washed in PBS containing 0.05 % Tween-20 (Gibco, Grand Island, NY, USA).

For H&E, the sections were incubated 3 min in 0.1 % Mayers hematoxylin (VWR, Søborg, Denmark) followed by washing, incubation for 2 min in 1 % eosin Y (VWR) and washing again. After staining the sections were dehydrated through increasing concentrations of ethanol and xylene, followed by mounting with DPX mounting media (Cellpath, Concord, NC, USA). For cresyl violet, the sections were incubated 10 min in 0.1 % cresyl violet acetate followed by a brief differentiation step in acetic acid and 95 % ethanol. After staining the sections were quickly dehydrated, followed by mounting with DPX mounting media (Cellpath, Concord, NC, USA).

2.6. Infarct area quantification

Micrographs were obtained by scanning whole sections using a NanoZoomer-2.0HT (Hamamatsu Photonics, Shizuoka, Japan) at 40 × magnification. Infarct area measurements were performed by manual use of the 'Freehand region' function in the NDP.view2 software (Hamamatsu Photonics). On H&E, infarct quantifications were performed by delineating the border between pyknotic and/or eosinophilic neurons, and normal looking neurons. With regards to cresyl violet, the infarcted areas were defined by the absence of Nissl body staining in neuronal cytoplasm. Infarct areas were then calculated by subtracting the area of the non-infarcted ipsilateral hemisphere from the area of the contra-lateral hemisphere. Infarct volumes were calculated by multiplying the infarct areas with the distance between each section (400 μm).

2.7. Statistical analysis

Because of small group sizes, data were not assumed to be normally distributed. NAA concentrations in ipsi-, contra-lateral and sham animals were compared using Kruskal-Wallis one-way ANOVA followed by Dunn's post hoc test. All correlation analyses between NAA and infarct volumes were performed using Spearman's two-tailed method.

Data are presented as Mean ± SEM unless otherwise stated. All analyses were performed using Prism 9 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

Estimation of sample sizes in the MCAO model experiments was predicted from our previous results [15,16] and corresponding to a 50 % reduction in mean infarct volumes. If the α (confidence level) and β error levels (statistical power) were 5 and 50 %, respectively, groups required a sample size of 4.

3. Results

In order to evaluate whether water-compensated NAA levels early after infarction accurately reflect tissue damage and thereby long-term prognosis, infarct evolution was monitored using T2-weighted MRI, cresyl violet and H&E stainings at days 1, 7, 14, and 28 after MCAO. Oedema formation was clearly seen on MRI (depicted in yellow), but no information about cellular fitness can be obtained from such images (Fig. 2a), even though drastic changes in metabolite concentrations were taking place as seen in the MR spectra (Fig. 2a).

NAA levels were significantly reduced in the infarcted area (Fig. 2b). Compared to the contralateral values, water-normalized concentrations were reduced 36 %, 26 % and 17 % at days 1, 7 and 14, respectively. At day 28 NAA levels virtually returned to normal due to collapse of necrotic tissue and glial proliferation. T2-weighted MRI showed huge oedema formation in the acute phases with high variation in between animals (Fig. 2c). The amount of accumulated fluid was profoundly reduced at day 7, and virtually absent at day 28 post-stroke (Fig. 2f).

In contrast to MRI, cresyl violet and H&E provide valuable information about cellular degeneration, which, besides cellular atrophy and pyknosis, is represented by loss of Nissl bodies and occurrence of eosinophilc neurons, respectively. Fig. 2d exemplifies infarct evolution, ranging from oedema and presence of red and pyknotic neurons at day 1, occurrence of new blood vessels and occasional immune cells at day 7, healing with gliosis and increased influx of immune cells at day 14, to liquefaction and dominance of lymphocytes at day 28. Besides the confluent necrosis in cortical areas, small clusters of subtle neuronal degeneration – as evidenced by loss of Nissl substance - were observed in the dorsolateral striatum. This seemed to peak at day 7, and was virtually absent at day 28 (Fig. 2e).

In order to get an idea of how well NAA levels reflect tissue destruction and cellular demise, we determined correlation coefficients between NAA and MRI, cresyl violet and H&E throughout the study period (Fig. 3). At early time points, NAA levels were strongly

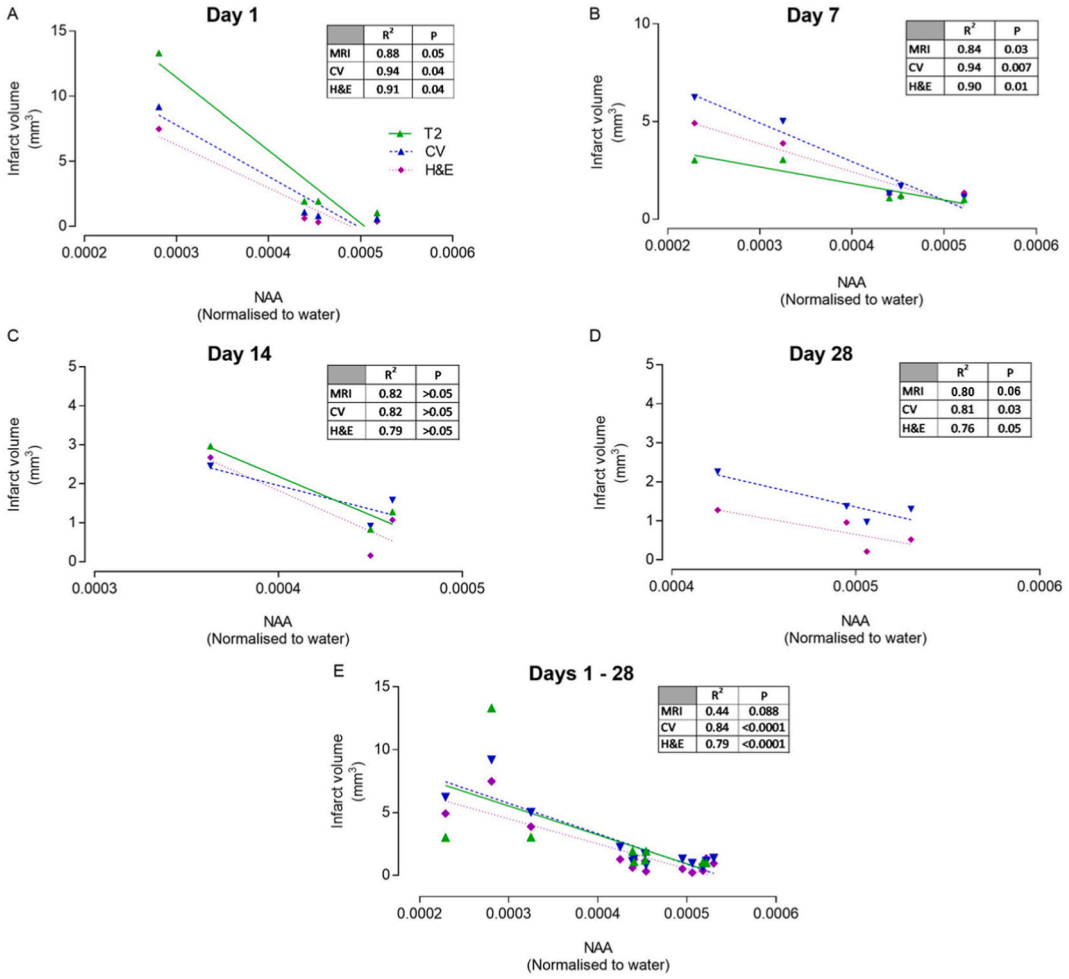

Fig. 3. Correlation between NAA levels and infarct volumes. Water-normalized NAA levels and infarct volumes based on T2 MRI, Nissl and H&E staining are shown at days 1 (A), 7 (B), 14 (C) and 28 (D) after MCAO (E includes all groups). The inserted tables show correlation coefficients (R^2) and p-values between NAA and infarct volumes estimated using T2-weithged MRI, cresyl violet and H&E, respectively.

associated with both cresyl violet and H&E-based infarct estimations (Fig. 3a + b), whereas the correlative value was less clear at later time points (Fig. 3c + d), due to tissue reorganisation, questioning the use of NAA as marker for tissue loss at later stages. The strongest correlations were seen between NAA and loss of Nissl substance in the early phases after MCAO ($r^2 = 0.94$ for days 1 and 7, Fig. 3a + b), as well as across all groups and days ($R^2 = 0.84$, $p < 0.0001$, Fig. 3e), whereas a weaker, but significant, correlation was found between NAA and pyknotic/eosinophilic neurons ($R^2 = 0.79$, $p = 0.0012$).

The overall purpose of this study was to determine if NAA can be used as a predictive marker for tissue loss and chronic disability. Therefore, we compared NAA levels and T2-weighted MRI volume estimates at day 1 with mature infarct sizes, based on cresyl violet, at days 14 and 28 (Fig. 4). The predictive power of NAA compared to MRI is quite evident, as a significant correlation with $r^2 = 0.73$, was seen. T2-weighted MRI, on the other hand, showed high variation and no clear association with late stage infarct size and tissue death, which signifies that caution is imperative when extrapolating and concluding on such data.

4. Discussion

We have systematically investigated infarct evolution in a mouse stroke model based on T2-weighted MRI, NAA levels, cresyl violet and H&E stainings. The study design aimed to answer if NAA normalized to water, is a reliable biomarker for loss of viable tissue and chronic infarction, unbiased by oedema, because oedema is an important confounder in this model.

Results showed that non-invasive spectroscopy-based determination of NAA levels normalized to water correlated significantly with loss of Nissl substance, a reliable marker of neuronal death [17]. Furthermore, NAA exhibited low variation and is easy to quantify with the right equipment, which is of high value in stroke prognosis.

Size and voxel positionings in the infarct core were fixed throughout the study period, which simplifies the procedure. Due to infarct maturation over time, the fixed voxel also included non-infarcted tissue at later time points, which accounts for much of the observed increase in NAA over time. Although neurons are the major source of NAA, the recovery of NAA is most likely also a consequence of increased glial activity [17]. Especially, the latter contribution to NAA levels in the acute phases is minor, and our results signify that NAA is useful as an early predictive marker for late stage tissue death, and thus disability.

Strong correlations between NAA levels and the histology-based infarct volume estimations were observed. The strongest correlations were seen between NAA and Nissl based infarct volume estimations, signifying that loss of NAA in neurons occurs subsequently with detachment of ribosomes and breakdown of the endoplasmic reticulum (ER) in dying neurons. Twenty-four hours after infarction, a complete loss of Nissl substance and a high numbers of pyknotic neurons were seen in the infarct core. H&E-based identification of dying neurons (based on pyknotic and/or eosinophilic neurons) resulted in smaller infarct volumes, compared to cresyl violet stained sections, which coincides with the fact that ER breakdown is an early event in ischemic neurons [17].

In the neighbouring dorsolateral striatum, occasional delayed neuronal death was observed based on loss of Nissl substance and pyknosis. Small degenerating clusters with subtle evidence of healing, in the form of immature vessels, were detected especially 7 days after infarction, most likely due to lack of cortical input [18,19].

To further validate NAA, functional analyses such as TTC (2,3,5-triphenyltetrazolium chloride) staining could have been considered. Our focus was on preservation of tissue morphology though, meaning that the mice were perfused with fixative, making such analyses impossible. Additionally, it was previously found that TTC staining doesn't provide much additional information, compared to Nissl staining [20,21].

A range of studies have consistently demonstrated significant decreases in NAA levels in ischemic brain tissue. Studies on animal stroke models have shown that, like in our study, NAA is significantly reduced in the acute phases, only to recover over a time course of 14–28 days [22–24]. The reason behind this is mostly due to glial cell proliferation and tissue reorganisation, but also other acetylated compounds may contribute to the signal [17]. This makes the use of NAA measurements at late stages during infarct maturation, as a marker for treatment responses and infarct development, highly questionable. In the acute and early stages after infarction, NAA seems to reflect the amount of brain damage and neuronal death quite well, as also shown by Lee et al., who found that reduced NAA levels in

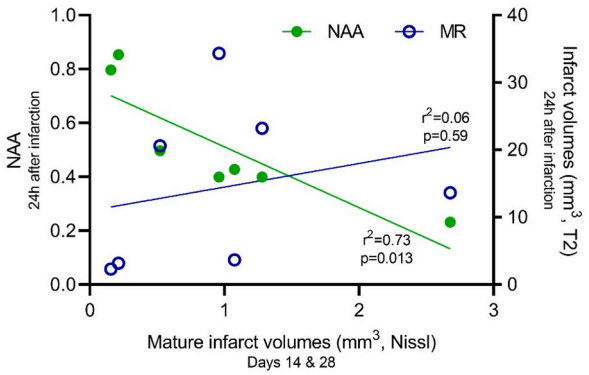

Fig. 4. Correlations between NAA (left y-axis) and T2 volumes (right y-axis) at day 1, and infarct volumes (based on loss of Nissl substance) at days 14 and 28.

the infarct correlate with neuronal eosinophilia and pyknosis [24]. This is in agreement with our data and suggests that NAA levels in the early infarct are a useful surrogate marker for neuronal death. We expand this perspective and suggest that water-compensated NAA levels in the infarct core 24 h after infarction is a reliable marker for long-term brain damage.

Human studies have likewise demonstrated reductions in NAA several hours after the insult, which seem to stay low for weeks thereafter, although with some variation [10,25–27]. This differs from rodent models where tissue reorganisation and NAA recovery seem to occur at a faster rate [22–24]. This is reproduced in our data, but since the purpose of this study is to evaluate whether NAA levels early after infarction (24 h) can be used as a surrogate marker for end-stage tissue damage, this should not affect the translational value of our data.

The predictive value of early NAA measurements has only been investigated in one human stroke study [28]. Two decades ago, Parsons and co-workers found that the NAA/creatine ratio was predictive of long term outcome in parallel to our study, where creatine values remained unchanged (data not shown), which typically is seen in ischemic tissue [29]. Overall, the use of NAA as biomarker for long-term prognosis seems highly promising, e.g. in combination with serological markers [30], and warrants further larger scale clinical studies. Additionally, NAA measurements could also prove useful when testing new and experimental drugs such as herniarin and others [31].

In conclusion, oedema-corrected MR-based estimation of NAA concentrations early after an ischemic insult reflects neuronal death in both early and late phase infarction, suggesting that the metabolite is of prognostic value in stoke patients.

Data availability statement

Data associated with the study has not been deposited into a publicly available repository, but data will be made available upon request.

CRediT authorship contribution statement

Henrik Hasseldam: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Rune Skovgaard Rasmussen:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Henrik Hussein El Ali:** Conceptualization, Investigation, Methodology. **Flemming Fryd Johansen:** Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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