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Metabolic disturbances in brain ischemic regions in an experimental model and in humans.

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Zaburzenia metaboliczne w niedokrwionych regionach mózgu na modelu zwierzęcym i u ludzi.

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C O N T E N T S

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Abstract

Ischemic stroke patients with similar history, symptoms, conventional CT or MR imaging features and general background may experience very different disease courses and outcomes. It is difficult to predict whether and how much the visible ischemic lesion on DWI will enlarge within the days following stroke. The mismatch between the lesion extent as suggested by the clinical stroke severity and the visible lesion extent on DWI has become known as the "clinical-DWI mismatch" but its background remains unknown. We hypothesized that progression of damage in ischemic tissue is associated with baseline metabolic events in tissue that is anatomically and "radiologically" healthy-looking. To explore this, using various MR techniques, we examined brain subregions metabolic factors: lactate concentrations and tissue temperatures, both measured within 24 hours from stroke in 40 patients. These parameters were in turn examined for the association with clinical course of the disease from its first day up to 3 months from onset and with other patient and lesion characteristics. Additionally, in an animal model of acute ischemic stroke, we explored whether induced deep systemic hypothermia (stroke treatment measure) or hyperthermia (pyrexia is a frequent stroke complication) may modulate post-ischemic brain lactate formation. We concluded that baseline "energy metabolism" changes within the healthy looking ipsilateral brain tissue (and not "just" in potential "penumbra") account for the recently described ischemic stroke DWI-clinical

mismatch. These changes may precede progression of symptoms, tissue infarction and thus lesion enlargement as well as the final clinical outcome. Hypothermia or pyrexia might influence stroke severity by modulation of ipsilateral brain tissue energy metabolism. MR spectroscopy techniques could be effectively used for predicting ischemic stroke deterioration, perhaps performed routinely with DWI, and therefore be important information for management decisions.

Abstract in Polish

Streszczenie w języku polskim

Ostry udar niedokrwienny mózgu jest statystycznie trzecią z najczęstszych przyczyn zgonów (po nowotworach i chorobach mięśnia serca) i najczęstszą przyczyną trwałej niepełnosprawności u ludzi dorosłych. Alarmujące statystyki i negatywny wpływ choroby na podstawowe stosunki społeczne, socjalne, psychologiczne i wreszcie ekonomiczne w krajach cywilizowanych skłoniły do planowania i realizacji długoletnich i wieloośrodkowych, zarówno eksperymentalnych jak i klinicznych, badań, których celem jest poznanie patofizjologii a poprzez nią skutecznych metod leczenia choroby. Do tej pory tylko rekombinowany tkankowy aktywator plazminogenu (tromboliza), aspiryna i podstawowa intensywna terapia w oddziale udarowym okazały się postępowaniem skutecznym, a spośród nich wysoce skutecznym jedynie leczenie trombolityczne. Niestety, ze względu na liczne ograniczenia tylko niewielka część chorych ostatecznie może skorzystać z takiej terapii.

Kluczowym elementem w planowaniu postępowania z chorymi z ostrym udarem niedokrwiennym mózgu jest możliwość przewidywania kierunku progresji stanu klinicznego pacjenta w ciągu kolejnych dni od zachorowania. Ostatnie doniesienia na temat dużych rozbieżności pomiędzy obrazem radiologicznym a stanem klinicznym chorego i progresją choroby

potwierdzają, że istnieje szereg innych niż anatomia ogniska niedokrwiennego czynników patofizjologiczno – klinicznych modulujących obraz kliniczny, dalszy przebieg choroby i rokowanie. Podobnie, w ostrej fazie choroby nie jest w praktyce możliwe określenie czy i jak bardzo może powiększyć się ognisko niedokrwienne w dalszym jej przebiegu, co miałoby niebagatelne znaczenie dla szacowania prognozy klinicznej. Taka rozbieżność pomiędzy anatomią radiologiczną ogniska niedokrwiennego a stanem neurologicznym pacjenta z udarem niedokrwiennym mózgu (innym niż wynikałoby to z obrazu radiologicznego mózgowia) funkcjonuje w literaturze jako „rozbieżność kliniczno-radiologiczna" („DWI-clinical mismatch", „radiologial-clinical mismatch").

Wyjaśnienie podstaw patofizjologicznych i klinicznych tego problemu stało się istotą badań szeregu zespołów naukowych z powodu ważnych implikacji praktyczno-klinicznych: przybliżona informacja o dalszym naturalnym przebiegu choroby może być kluczowa przy podejmowaniu decyzji terapeutycznych.

Mechanizmy patofizjologiczne w niedokrwionej tkance mózgu i ich znaczenie praktyczno-kliniczne zostały relatywnie dobrze zbadane w zakresie makroskopowego i mikroskopowego obrazu struktur anatomicznych czy komórkowych. Nieporównywalnie mniej poznane są natomiast zaburzenia metaboliczne w niedokrwionej tkance, a tym samym ich związek

z obrazem klinicznym choroby i implikacje terapeutyczne. Wynika to głównie z szeregu trudności technicznych: z jednej strony niemożności pozyskania materiału bezpośredniego – tkanki mózgu – do takich badań u ludzi, z drugiej zaś trudności w opracowaniu takich modeli eksperymentalnych udarów niedokrwiennych, które byłyby zbliżone do choroby ludzi i nie były jednocześnie obarczone artefaktami biochemicznymi (stosowanie farmaceutyków, np. do znieczulania zwierząt) czy mechanicznymi (procedury chirurgiczne).

Celem niniejszej rozprawy doktorskiej jest:

- wyjaśnienie czy zjawisko tzw. kliniczno - radiologicznej rozbieżności („clinical-DWI mismatch”), progresja objawów klinicznych i powiększanie się obszaru zawału tkanki mózgu u chorych z udarem niedokrwiennym są spowodowane lub poprzedzane przez zaburzenia metaboliczne w tkance mózgu, w której nie stwierdzono cech patologii w badaniach neuroobrazowych („zdrowo wyglądającej”), np. położonej w sąsiedztwie ogniska niedokrwiennego
- opisanie dystrybucji temperatury mózgu u pacjentów w pierwszej dobie udaru niedokrwiennego oraz zbadanie korelacji temperatur obszaru niedokrwienia z cechami neuroobrazowymi i klinicznymi
- zbadanie zależności pomiędzy mózgowym stężeniem mleczanu i temperaturą ciała oraz wyjaśnienie czy udowodniony wpływ temperatury

ciała na stan neurologiczny chorych po udarze niedokrwiennym mózgu (hipotermia jest postulowaną metodą leczenia udarów niedokrwiennych zaś gorączka jest powikłaniem udarów istotnie pogarszającym rokowanie) może być realizowany poprzez modulację metabolizmu mózgu, na modelu zwierzęcym choroby.

Zatem robocza hipoteza naukowa niniejszej rozprawy doktorskiej zakładała, że poudarowe upośledzenie funkcji neurologicznych, stan kliniczny chorego i rokowanie są wynikiem nie tylko wielkości i lokalizacji samego ogniska niedokrwiennego, ale także zaburzeń biochemicznych w regionach mózgu, które nie są zmienione w konwencjonalnych badaniach neuroradiologicznych, w szczególności w sąsiedztwie ogniska niedokrwiennego, tzw. strefie półcienia – „penumbrze".

W poszukiwaniu przyczyn udarowej kliniczno – neuroobrazowej rozbieżności wybrane zostały dwa parametry: stężenie mleczanu w tkance oraz temperatura tkanki mózgu. Obydwa parametry są etycznie i technicznie możliwe do oceny u pacjentów w ostrej fazie udaru niedokrwiennego mózgu. Nieinwazyjny pomiar stężenia mleczanu w mózgu in vivo jest możliwy przy użyciu spektroskopii rezonansu magnetycznego (MRS) zaś nieinwazyjny pomiar temperatury dowolnego punktu mózgu w przypadku obecności zmian niedokrwiennych został wykonany w niniejszym opracowaniu oraz już opublikowanych pracach będących fragmentem tego doktoratu, po raz

pierwszy, także z użyciem MRS (pierwsza „mapa" temperatury mózgu u chorych z ostrym udarem niedokrwiennym).

Uzyskane dane dla różnych regionów niedokrwionego mózgu u 40 pacjentów zostały zbadane na obecność ich związku i korelacji z obrazem klinicznym choroby w okresie do 3 miesięcy od zachorowania (National Institutes of Health Stroke Scale – NIHSS), ostatecznym stanem funkcjonalnym pacjentów (modified Rankin scale – mRS), zmianami wielkości ogniska niedokrwiennego (DWI - obrazowanie oparte na dyfuzji) a także innymi cechami klinicznymi i neuroradiologicznymi (PWI - obrazowanie oparte na perfuzji, CBF - mózgowy przepływ krwi, CBV - mózgowa objętość krwi, MTT - tzw. średni czas przejścia krwi przez krążenie mózgowe w określonym obszarze).

Dodatkowym elementem rozprawy doktorskiej było opisanie wpływu temperatury ciała (głęboka systemowa hipotermia jako skuteczna metoda leczenia udarów niedokrwiennych oraz gorączka – częsty wtórny objaw udarów mózgu istotnie pogarszający rokowanie) na stężenie mleczanu w niedokrwionej tkance na zwierzęcym modelu udaru niedokrwiennego mózgu.

U każdego z 40 pacjentów z ostrym niedokrwiennym udarem mózgu wykonano badanie kliniczne (w tym NIHSS oraz zmodyfikowana skala Rankina) oraz panel badań neuroradiologicznych (spektroskopia protonowa

rezonansu magnetycznego – MRS, obrazy w sekwencjach T2-zależnych, oraz, jak wspomniano wcześniej, DWI, PWI, CBF, CBV i MTT).

Na obraz DWI mózgowia każdego pacjenta została komputerowo naniesiona siatka spektroskopowa (MRS), podzielona na voxele o wymiarach 10 x 10 mm (Rycina 1). W ten sposób każdy voxel mógł zostać przypisany do jednego z 5 utworzonych regionów mózgu ze świeżym ogniskiem niedokrwiennym: trzon ogniska niedokrwiennego (DAL), brzeg ogniska niedokrwiennego (PAL), normalnie wyglądająca tkanka otaczająca ognisko niedokrwienne (PAL+) (PAL oraz PAL+ mają stanowić obszar potencjalnej strefy półcienia – „penumbry"), normalnie wyglądająca tkanka półkuli ipsi- (INL) i kontralateralnej (CNL). Dla każdego voxela (ok. 2000 voxeli) uzyskano spektrum, z którego, po odpowiednich obliczeniach otrzymano temperaturę (zależną od różnicy stężeń N-acetyloasparaginianu i wody w tkance) i stężenie mleczanu dla odpowiadającej danemu voxelowi objętości tkanki (10 mm^3). Do liczbowego wyrażenia stężenia każdego ze związków chemicznych, uzyskanych pierwotnie w formie pików spektrogramu, użyto oprogramowania AMARES (http://www.mrui.uab.es/mrui).

Na modelu zwierzęcym ostrego udaru niedokrwiennego mózgu, polegającym na permanentnej okluzji (mikroelektrokoagulacja) proksymalnej części tętnicy środkowej mózgu myszy, określono wpływ fizjologicznych, patologicznych (gorączka) i terapeutycznych (hipotermia) temperatur ciała

na mózgowe stężenie mleczanu. Do określenia stężenia mleczanu w tkance użyto dehydrogenazy mleczanowej.

Mapa i schematyczny rozkład temperatury mózgowia w ostrej fazie udaru niedokrwiennego u ludzi zostały przedstawione graficznie (Ryciny 2 i 3). Najwyższą temperaturę miały tkanki położone w najbliższym sąsiedztwie ogniska niedokrwiennego oraz tworzące jego brzeg, a zatem obszar mózgu potencjalnie odpowiadający tzw. strefie półcienia („penumbra"). Aż 64% pacjentów miało podwyższoną (>37,5°C) temperaturę tkanek tego regionu mózgu. Temperatura wzrastała najpierw w obszarze ogniska niedokrwiennego a dopiero później w pozostałych regionach mózgowia. Temperatura ogniska niedokrwiennego jak i półkuli kontralateralnej była większa u chorych z ogniskami ocenionymi jako średnie lub duże oraz u tych, u których obserwowano tzw. „DWI/PWI mismatch" lub zmniejszoną perfuzję (CBF) w obszarze niedokrwienia. Nie znaleziono prostych korelacji pomiędzy temperaturą regionów mózgowia a wyjściowym stanem neurologicznym czy progresją objawów klinicznych. Pogłębiona analiza wykazała jednak, że u chorych w cięższym stanie neurologicznym średnia temperatura ogniska niedokrwiennego była istotnie wyższa niż temperatura tkanki półkuli kontralateralnej („zdrowej"), zaś u chorych z niewielkimi objawami neurologicznymi takiej tendencji nie obserwowano. Może to sugerować, że wzrost temperatury w obszarze zawału tkanki mózgu (a nie jej

bezwzględna wartość) jest związany ze złym stanem klinicznym chorych. Patogeneza wzrostu temperatury tkanki mózgu po udarze niedokrwiennym jest wieloczynnikowa (wpływ egzo- i endotermicznych reakcji biochemicznych, miejscowa odpowiedź układu immunologicznego, zmiana stosunków wymiany ciepła krew-tkanki, czynniki genetyczne).

Najwyższe stężenie mleczanu obserwowano w centrum ogniska niedokrwiennego (Rycina 4), ale jego wartość nie miała wpływu na przedmiotowy stan kliniczny chorych ani na progresję objawów neurologicznych. Natomiast stężenie mleczanu w obszarze „penumbry" i pozostałej tkance półkuli ipsilateralnej korelowało istotnie z wyjściowym stanem neurologicznym chorych oraz z dużym prawdopodobieństwem (wskaźniki korelacji > 0,6) pozwalało przewidzieć progresję objawów neurologicznych w okresie do 3 miesięcy od zachorowania. Wykazano, że wzrost stężenia mleczanu w tkance, która w badaniach neuroradiologicznych wyjściowo odpowiada zdrowemu obszarowi, może poprzedzać destrukcję (zawał) tej tkanki.

W towarzyszącym projektowi głównemu badaniu na zwierzęcym – eksperymentalnym modelu ostrego udaru niedokrwiennego wykazano, że stężenie mleczanu w tkance mózgu jest zależne od temperatury ciała w sposób bliski funkcji liniowej (Rycina 5), a głęboka systemowa hipotermia (metoda leczenia udarów niedokrwiennych mózgu) lub wyidukowana

hipertermia (gorączka jest częstym objawem wtórnym udarów mózgu, istotnie pogarszającym rokowanie) znacznie redukowały lub zwiększały (odpowiednio) stężenie mleczanu w tkance (Rycina 6). Oznaczać to może, że znany od dawna wpływ temperatury ciała na stan kliniczny chorych w ostrej fazie udaru niedokrwiennego mózgu może być realizowany bezpośrednio poprzez modulację metabolizmu obszaru mózgu otaczającego dokonane ognisko zawałowe (ścisły związek pomiędzy produkcją mleczanu w tkance i rokowaniem potwierdzono w uprzedniej części opracowania).

W rozprawie przedyskutowano patofizjologię charakterystycznej dystrybucji temperatury mózgu po udarze niedokrwiennym, patofizjologiczne podstawy dla prezentowanych ścisłych związków pomiędzy metabolizmem „zdrowo-wyglądającej" tkanki otaczającej ognisko zawałowe mózgu i dalszą progresją choroby (tj. wpływ procesów w komórkach regionu niedokrwienia, możliwy wpływ procesów auto-neuronaprawczych podnoszonych w ostatnich badaniach nad komórkami macierzystymi na przykładzie danych z badań autora) oraz znaczenie tych zjawisk w praktyce klinicznej: diagnostyce (spektroskopia rezonansu magnetycznego) i leczeniu (hipotermia).

Podsumowując, w rozprawie doktorskiej podjęto próbę wyjaśnienia fenomenu „DWI–clinical mismatch" – niezupełnej korelacji pomiędzy obrazem radiologicznym (anatomicznym) ogniska niedokrwiennego a obrazem klinicznym i przebiegiem choroby, stawiając hipotezę popartą wynikami badania o istotnej roli niektórych czynników metabolicznych w

tym zakresie. W opracowaniu przedstawiono, wg naszej wiedzy, pierwszą mapę dystrybucji temperatury tkanki mózgu u chorych w ostrej fazie udaru niedokrwiennego oraz dodatkowo, na modelu eksperymentalnym choroby, wykazano, jaki wpływ na metabolizm tkanki mózgu ma leczenie udarów niedokrwiennych głęboką hipotermią lub gorączka - częste powikłanie choroby pogarszające rokowanie. Zaproponowano, że pomiar stężenia mleczanu w ostrej fazie udaru niedokrwiennego mózgu w otoczeniu widocznego ogniska niedokrwiennego przy użyciu techniki spektroskopii rezonansu magnetycznego mógłby być badaniem stosowanym rutynowo w praktyce klinicznej dla określenia kierunku dalszego rozwoju choroby (szacowanie prawdopodobieństwa powiększania się obszaru zawałowego tkanki) i na tej podstawie kwalifikacji chorego do dalszego postępowania (np. endowaskularnej hipotermii).

Wnioski końcowe – podsumowanie:

- zmiany metaboliczne w „zdrowo-wyglądających" w badaniach neuroobrazowych regionach półkuli ipsilateralnej (nie tylko w tzw. strefie półcienia) są przyczyną tzw. kliniczno-radiologicznej rozbieżności. Zaburzenia metaboliczne tkanki mózgu mogą poprzedzać jej zawał (powiększanie się ogniska niedokrwiennego) i pogarszanie się stanu klinicznego pacjenta. Dlatego oznaczanie stężenia mleczanu przy pomocy technik spektroskopii rezonansu magnetycznego można rozważać jako

praktyczno-kliniczną metodę istotnie wspomagającą szacowanie rokowania u chorych z ostrym udarem niedokrwiennym mózgu

- w pierwszej dobie udaru niedokrwiennego temperatura mózgu jest najwyższa w obszarze tzw. strefy półcienia

- hipotermia (metoda leczenia udarów mózgu) oraz gorączka (częsty objaw we wczesnej fazie udarów mózgu, istotnie pogarszający rokowanie) mogą modulować stan kliniczny chorych poprzez wpływ na metabolizm tkanek półkuli ipsilateralnej.

1. Introduction

In acute ischemic stroke, anticipating early disease progression (of symptoms, lesion enlargement, and most importantly, the final clinical outcome) is complicated in the first hours after onset. Patients with similar history, symptoms, conventional CT or MR imaging features and general background may experience very different disease courses and outcomes. [1-4] Similarly, it is very difficult to predict whether and how much the visible ischemic lesion on DWI will enlarge within the days following stroke. The mismatch between the lesion extent as suggested by the clinical stroke severity and the visible lesion extent on DWI has become known as the "clinical-DWI mismatch". Exploration of this problem has quickly became an important challenge for stroke researchers as information about the potential disease progression is crucial in management decision making.

In looking to explain stroke clinical-DWI mismatch, we hypothesized that progression of damage in ischemic brain was associated with baseline metabolic events in tissue that is anatomically and "radiologically" healthy-looking at baseline. We examined two targets, one "specific" (human brain lactate concentration), and one "unspecific" (brain tissue temperature), both measured within 24 hours from stroke, using various MR techniques. Both can be measured non-invasively in brains of severely ill acute stroke patients, [5,6] and simultaneously may provide important information about energy-

metabolism processes in DWI-"healthy" looking brain tissue. The parameters were in turn examined for the association with clinical course of the disease from its first day up to three months from onset and with other characteristics. The sufficiency of the cells of this area, and thus progression to apoptosis or necrosis and poor functional outcome, might be estimated by assessing the key by-products of metabolism.

1.1 Introduction – brain tissue lactate concentration and ischemic stroke pathophysiology

For many years lactate has been regarded as a marker of cerebral anaerobic metabolism and a probable marker of tissue destruction secondary to acidosis. [7-9] However, a recent theory [10], known as the astrocyte-neuronal lactate shuttle (ANLS), suggests that neurons, thought to be activated via glutamate receptors, change their metabolism to be able to use astrocyte-produced lactate as their energy substrate. Therefore the ANLS might be particularly active in ischemic but still viable brain tissue, where astrocyte lactate production and glutamate release are increased. [11-14] Lactate might be a marker of early ischemia in tissue at risk of infarction in DWI-healthy brain beyond the edges of the DWI-visible lesion at baseline.

1.2.1 Introduction – a novelty of noninvasive measurement of brain tissue temperature in ischemic stroke

Temperature can be measured non-invasively with magnetic resonance (MR) spectroscopy (MRS). [15-17] Experimental studies in phantoms [18,19] and experimental models [18-23] show very close correlation between temperature measured by MRS and implanted probes. MRS has been used to measure temperature in normal adult human volunteers, [16] including during head cooling, [24] in infants, [15] and in patients with brain tumours, [17] but not in patients with ischemic stroke.

We used proton MRS chemical shift imaging (MRSI), in which spectra are collected from across a slice of brain rather than just one small single voxel, to measure sub-regional brain temperature within and around the ischemic lesion on a voxel-by-voxel basis in patients with an acute ischemic stroke as visualized on diffusion-weighted imaging (DWI).

1.2.2 Introduction – body and brain temperatures and ischemic stroke pathophysiology

Changes in energy metabolism might be suggested by a change in heat production. Elevation in body temperature above 37.5 degrees centigrade (°C), or pyrexia, is common in acute ischemic stroke. [25-27] Pyrexia in the first 24 hours after stroke is associated with a worse outcome, [25] possibly

by association with severe stroke. [25-27] In experimental models, pyrexia is associated with more extensive infarction than normothermia, [25,28] hypothermia reduces histological neuronal damage [25,29,30] and may improve functional outcome, [31] and was associated with a reduction in ischemic lesion volume on diffusion-weighted imaging (DWI) in one patient. [32] Preliminary trials of hypothermia in stroke patients are promising. [33] Pyrexia in the first few hours after stroke is thought to result from processes in the brain, [34,35] rather than from complications of the stroke (e.g. deep venous thrombosis) which occur later. [26,27] The need to place an invasive sensor through a burr hole or craniotomy, [34-36] and sampling from only one small region of brain have limited studies of brain temperature after stroke in patients to date, but have shown that brain temperature was 1°C higher than body temperature in a small study of patients with extensive middle cerebral artery (MCA) ischemic stroke. [34]

We hypothesised that temperature would be elevated in the ischemic lesion more than normal brain, and that, if related to the ischemic process itself, temperature might vary between tissues within and around the ischemic lesion. We compared differences in temperatures between tissues categorized according to the appearance on DWI, and tested associations with various patient and lesion characteristics.

1.3 Introduction – substudy in an animal ischemic stroke model

From the human study [performed prior to the experimental part of the thesis] we concluded that an increase in lactate concentration within healthy looking ipsilateral brain tissue (and not "just" in potential "penumbra") may precede progression of symptoms, tissue infarction (and thus lesion enlargement) and neurological deterioration.

There is a strong association between raised body or brain temperatures (in animal studies) and raised body temperatures (in patients) and ischaemic lesion progression and poor functional outcome after stroke, [37-39] and induced hypothermia shows promise as a treatment for acute ischemic stroke (lesion enlargement prevention and improvement of neurological outcome). [23]

We hypothesized that hypothermic treatment of ischemic stroke is effective due to its influence on brain energy metabolism and its product lactate. Therefore, in an animal model of acute focal ischemic stroke, we investigated whether body temperature is correlated with lactate concentration in ipsilateral brain tissue (not "just" in potential "penumbra") and whether induced deep systemic hypothermia (stroke treatment measure) may decrease and hyperthermia (pyrexia is a frequent condition after stroke significantly deteriorating disease severity) increase lactate concentration in this tissue.

2. Aims of the Doctoral Dissertation

- to explore whether the "clinical-DWI mismatch", stroke neurological deterioration and progression of damage in ischaemic brain are associated with metabolic disturbances in tissue that is anatomically and "radiologically" healthy-looking at baseline

- to explore the distribution of brain tissue temperatures and their associations with imaging and patient characteristics in acute ischemic stroke

- to explore whether body temperature is correlated with brain lactate concentration and whether induced deep systemic hypothermia (an effective stroke treatment measure) or pyrexia (a frequent condition after stroke significantly deteriorating disease severity) modulate stroke severity via brain metabolism changes in an animal model of ischemic stroke

3. Material and methods

3.1 Patient recruitment

We recruited patients prospectively with symptoms of moderate to severe acute cortical ischemic stroke without contraindications to MRI. A stroke physician obtained a detailed history and examination, measured the stroke severity according to National Institutes of Health Stroke Scale (NIHSS) and determined the stroke type on the Oxfordshire Community Stroke Project (OCSP) classification. [40] NIHSS was used to monitor changes in the patients' clinical condition and, with the Rankin scales, outcome at three months after stroke. The time of onset was taken as the time when the symptoms were first observed, or if the patient awoke with a stroke, then the time last known to be well. Body temperature was recorded by tympanic thermometer around the time of scanning. Patients underwent MRI as soon as possible after stroke onset (within a maximum of 24 hours), at 2-6 days, 9-15 days, 1 - 3 months after stroke. The study was approved by the local Research Ethics Committee, and informed consent was obtained for all patients.

3.2 MRI and MRSI technique

We used a GE Signa Echospeed LX 1.5T (General Electric, Milwaukee, WI, USA) MR scanner with self-shielding gradients (22 mT/m maximum) and

'birdcage' quadrature head coil. We performed axial T_2-weighted fast spin echo imaging (T2W); axial DWI (tensor) with field-of-view (FOV) 240 mm, 15 axial slices of thickness 5 mm, slice gap 1 mm, acquisition matrix 128 × 128, echo time (TE) 97.4 ms, repetition time (TR) 10 s and diffusion sensitizing gradients with scalar b-values of 1000 s/mm^2 were applied in six non-collinear directions; axial PWI using the dynamic signal change following a bolus injection of a gadolinium-based contrast agent (over 85 seconds, thirty-four volumes of 15 axial slices were obtained using the same FOV, acquisition matrix and slice locations as the DWI data, but with a TE of 30 ms and TR of 2.5 s), and single slice PRESS proton MRSI centered on the slice showing the maximum ischemic lesion extent on DWI. The MRSI voxel grid was carefully placed within brain to include as much of the visible ischemic lesion and contralateral normal brain as possible and to avoid any contamination of the spectra with lipid signal from bone marrow or subcutaneous fat. The imaging parameters for MRSI were: FOV 320 mm, slice thickness 10 mm, acquisition matrix 24×24, TE 145 ms and TR 1000 ms. Automatic shimming and water suppression were applied. For each phase encoding, 512 complex data points were acquired with a sampling interval of 1 ms. We did not monitor body temperature during scanning as all sequences operated well within the specific absorption rate (SAR) limits, only two sequences were echo-planar, and did not last long enough to

influence body temperature in adults. The image acquisition took 20 minutes, not including patient settling time.

3.3 Image processing

DWI and PWI processing (as described recently by our group [5]). We removed bulk patient motion and eddy current-induced artifacts from the DWI and PWI data using a 3D computational image alignment program to register thecomponent echo-planar imaging volumes to the T2W volumes acquired as part of the DWI protocol. Maps of the average DWI signal, (<DWI>), were obtained from the six DW images acquired for each slice. Co-registered maps of CBF, CBV and MTT were calculated from gamma-variate functions fitted on a voxel-by-voxel basis to concentration-time curves obtained from the dynamic signal change following injection of the contrast agent. [41]

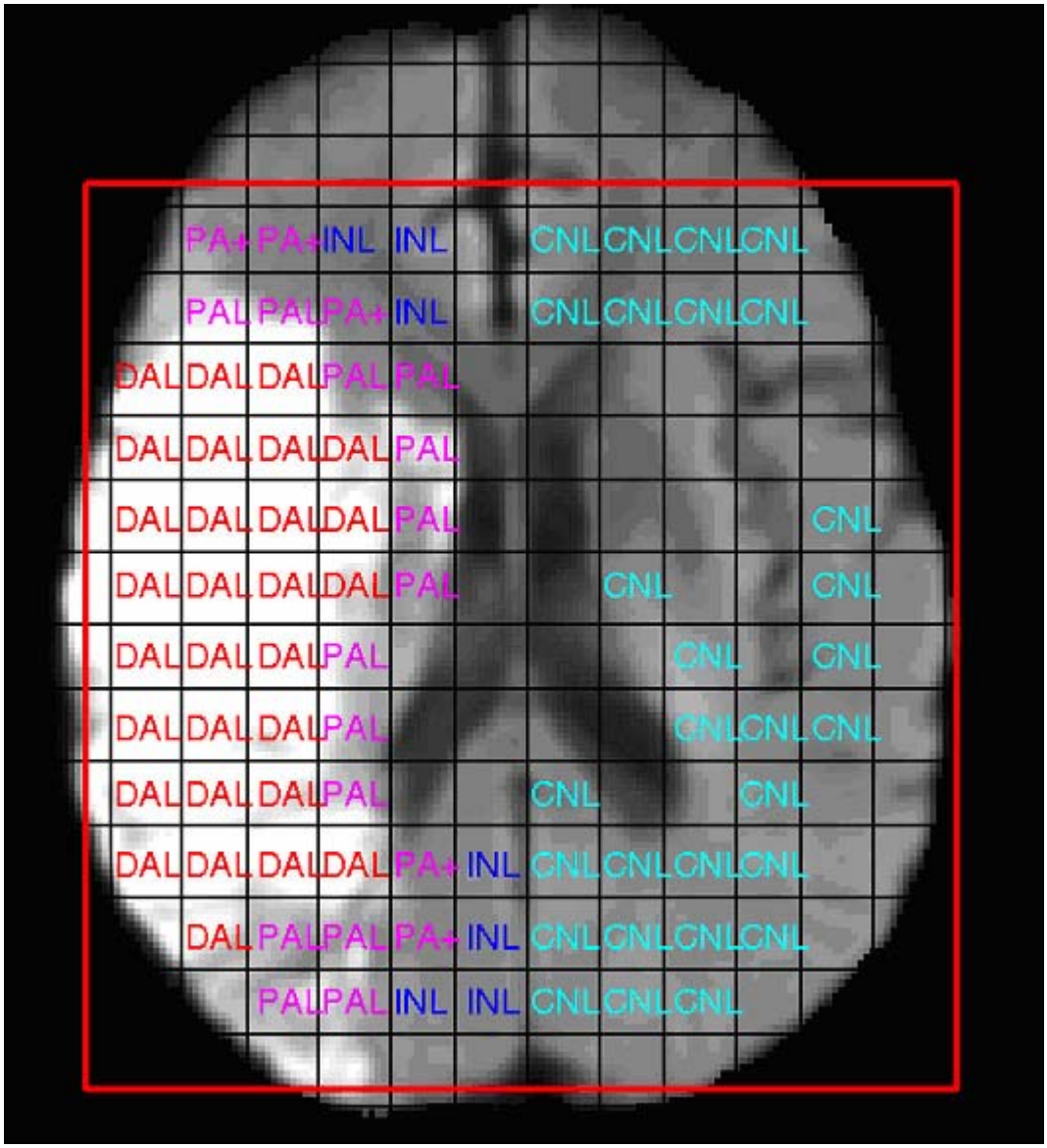
MRS processing. Spectroscopic images were interpolated to a 32×32 matrix, yielding 10 mm^3 voxels. Subsequent processing consisted of zero-order phase correction using the residual water signal (effectively bringing water to a chemical shift of 4.70 ppm) and removal of the residual signal using the Hanckel-Lanczos Singular Value Decomposition (HLSVD) method. [42] Spectroscopic data were Fourier transformed for display and editing purposes, but were modeled in the time domain by five Gaussian components

(corresponding to choline, creatine, N-Acetyl aspartate (NAA) containing compounds and lactate) using the AMARES algorithm within the MRUI package (http://www.mrui.uab.es/mrui). The chemical shifts (i.e. frequency) of the fitted metabolite peaks were reported to a precision of 0.001 parts per million (ppm). Spectra were automatically discarded if fitted line widths were less than 1 Hz or greater than 10 Hz, if the metabolite peaks were more than 0.1 ppm offset from their expected values, if the voxels lay on the edges of the PRESS excitation region or came from voxels falling on CSF. All spectra were also inspected visually, and discarded if judged to be of poor quality, e.g. having a badly elevated baseline or containing spurious peaks. Cerebral temperature (T) for each remaining voxel was calculated from the apparent chemical shift of the NAA peak (cs_{NAA}), using the relation $T=37°+100(cs_{NAA}-2.035)$, where a chemical shift of 2.035 ppm was found in 20 healthy control subjects with an assumed brain temperature of 37°C, and a sensitivity 0.01 ppm/°C was taken from the literature. [43,44] As reported previously, the technique is capable of detecting very small differences in temperature within subjects. [5] Lactate was identified by its characteristic appearance at TE=145ms: namely, an inverted doublet with peak separation of 7Hz corresponding to the J-coupling.

MRSI and DWI/PWI parameters and patient characteristics. MRS, DWI and PWI images were co-registered using an affine transformation. We superimposed the MRS voxel grid on the DWI image, and the voxels were

coded according to the DWI visual appearance only, blind to all other information (Figure 1). We used an operational tissue classification based on the DWI visual appearance to avoid imposing apparent diffusion coefficient (ADC) or PWI thresholds that are currently of uncertain validity. [44,45] Voxels including a majority (>75%) of obviously hyperintense tissue (bright white) on DWI were categorized as "definitely abnormal" ("DAL") and taken to represent the infarct "likely core" (tissue in the DWI lesion that was most probably permanently damaged at the time of imaging). [47] Voxels including mostly tissue that was only slightly hyperintense on DWI were coded as "possibly abnormal" ("PAL"). Note these voxels could also include <25% normal tissue and <25% definitely abnormal tissue. Voxels forming the rim of normal-appearing tissue immediately outside the DWI lesion were coded as "possibly abnormal plus" ("PAL+"). The possibly abnormal and possibly abnormal plus voxels were taken to represent the "likely penumbral" tissues, as it is known that milder DWI hyperintensities may resolve to leave no permanent damage, [46-48] the "metabolic" penumbra includes tissue which is initially normal on DWI, [49] the precise margin of the "at risk tissue" is not yet defined. [44,45] A similar definition applied previously to T2-weighted images at 12-36 hours after stroke found significant differences in the normal neuronal marker NAA between definitely and possibly abnormal tissue, [50] and the experimental stroke data indicate that the strongest correlation between histological evidence of neuronal death and

Figure 1. Exemplar of the method of categorizing spectroscopy voxels according to the appearance of the brain on diffusion-weighted imaging (DWI). The grid is the spectroscopy acquisition and is co-registered to the slice from the DWI sequence showing the maximum ischemic lesion extent. DAL = definitely abnormal tissue; PAL = possible abnormal tissue; PAL+ = tissue one voxel thick immediately outside the definitely or possible abnormal tissue; INL = ipsilateral normal brain; CNL = contralateral normal brain. Voxels containing CSF, with poor quality spectra, or falling outside the brain were excluded from the final analysis.



imaging is the intensity of the DWI signal (ie "brightness"), not the ADC. [46] While our approach may be slightly novel and not a conventional definition of "penumbra", we used this approach because we wished to avoid definitions based on DWI/PWI mismatch (which may over or underestimate tissue at risk), [1,51-52] and use an "operational" definition that was independent of perfusion variables. Normal-appearing brain located beyond the "possibly abnormal plus" rim of the DWI lesion in the ipsilateral hemisphere, and in the contralateral hemisphere, was coded "ipsilateral normal" ("INL") or "contralateral normal" ("CNL") respectively. Additionally we used a code "PNIL" to describe all ipsilateral tissues except the core of the ischemic lesion ("PNIL" = "INL" + "PAL" + "PAL+"). Voxels covering cerebrospinal fluid or which lay outside the brain were excluded.

3.4 Statistical analysis

Brain temperature map. For each patient, we calculated the mean and standard deviation of the temperature readings for each tissue voxel category. We used RevMan software (http://www.cc-ims.net/RevMan) for the primary analysis of temperature differences between tissue types using odds ratios by the fixed effects method to calculate weighted mean differences between tissue types within each patient. This avoided potential confounding by

fluctuations in scanner performance or other factors that might influence apparent between-patient differences and enabled us to examine subtle differences in temperature between tissue types. Analysis of temperature differences between tissue types within each patient also avoided any subtle and inadvertent effects of whole body heating or cooling while in the scanner. Note that due to the constraints of the MRS grid size, the variability of the shape of ischemic lesions, and the need to exclude voxels with poor spectra, not all patients contributed voxels of all five types to the analysis, but all available voxels have been used. The secondary analysis was of average (+/- SD) absolute temperatures of each tissue type for all patients which were calculated from the mean of the readings for each tissue type, but note that this analysis may be less reliable because it is less able to avoid potential confounding effects (e.g. no voxels of a particular type) between patients. We compared the difference between lesion and contralateral normal voxel temperatures using RevMan software or Mann-Whitney U tests as appropriate.

Tissue lactate concentrations. We calculated mean lactate concentration for each of these brain regions (definitely, possibly, probably abnormal+ and normal tissue) by calculating means from all voxels that were coded as DAL, PAL, PAL+, INL, CNL respectively and compared them using ANOVA and T-tests.

Association with other clinical and imaging characteristics. We examined mean lactate concentrations and tissue temperatures for any association with NIHSS on admission, in the 2nd to 6th day, the 9th to 15th day, and at 1 to 3 months from stroke and tested associations with the modified Rankin scale at 3 months using Pearson correlation analysis, ANOVA, Tukey, Shapiro-Wilk and T-tests. Any non-normal data was transformed to normal by logarithmic transformation.

To test associations with local brain temperature changes or lactate concentrations, we defined lesion and patient characteristics as follows: ischemic lesion size (small <6; medium 6-16; and large >16 definitely/possibly abnormal voxels); blood flow in the infarct (lesion CBF normal or increased vs. decreased relative to contralateral normal brain); PWI/DWI mismatch (PWI lesion larger than the DWI lesion = mismatch present vs. PWI lesion the same as or smaller than the DWI lesion =no mismatch); time from stroke to scanning (<10 hours vs. >10 hours); and body temperature (with vs. without pyrexia). To explore whether the possible associations between brain metabolic changes and stroke clinical course and outcome were mediated by the growth of the ischemic lesion we additionally performed our analysis within subgroups, for which we defined lesion growth as the visible lesion expansion on DWI between baseline and subsequent imaging.

We examined the influence of temperature on brain lactate formation in two ways. First, we checked whether mean lactate concentration varied with temperatures within each tissue (DAL, PAL, PAL+, INL, CNL); then we explored whether lactate concentration varied with temperature across the whole brain using Pearson correlation tests.

3.5 Experimental (animal) procedures

Experimental procedures in general

All experimental procedures were approved by the Local Ethical Commission for Animal Experiments in Gdansk (permit number 8/05) and performed according to the National Institute of Health guidelines for the care and use of laboratory animals. C57BL/j mice were used for all experiments. All animal procedures were performed under isoflurane anesthesia (initially 2%, followed by 1.5% to 1.8% in 75% N_2O, 25 % O_2). A servo-controlled homoeothermic blanket, developed in-house, was used to maintain core temperature at 37 °C when needed.

Animal model of acute focal cortical ischemic stroke

Permanent occlusion of left middle cerebral artery (MCA) main (proximal) trunk was performed by topical microelectrocoagulation after craniotomy as

in our previous studies (Figures 7a and 7b) [53,54] in 15 mice in total. 5 healthy mice were used as a control group.

Temperature-dependent brain lactate concentration

Within several hours of MCA occlusion (performed on servo-controlled homoeothermic blanket), 5 mice were placed in different environmental temperatures to obtain a different core temperature in each animal (rectal temperatures: 32.4-35.1-37.8-38.9-39.9 °C). In these conditions animals were sacrificed and contralateral (CNL) and ipsilateral (PNIL) hemispheres quickly collected and frozen. Enzymatic methods were used for lactate concentration determination in each hemisphere.

Modulation of brain lactate concentration by deep hypothermia or hyperthermia

Within several hours of MCA occlusion, in anesthetic conditions, 5 mice were placed in a low-temperature environment to obtain deep systemic hypothermia (rectal temperatures range: 25.8 – 27.9, mean 27.12 °C). The remaining 5 mice were placed in a high-temperature environment to induce hyperthermia (rectal temperatures range: 37.1 to 42.2, mean 39.72 °C). In these conditions 5 “hypothermic” and 5 “hyperthermic” animals were sacrificed and contralateral and ipsilateral hemispheres quickly collected and

frozen. Enzymatic methods were used for lactate concentration determination in each hemisphere of these 10 animals.

Tissue lactate concentration assay

The obtained brain samples were used for protein concentration and lactate assay using lactate dehydrogenase (LDH), both as described previously. [56,57]

4. Results

We recruited 40 patients with acute ischemic stroke, mean age 78 (range 58-95) years. Seventeen patients had a total anterior circulation stroke (TACS), 22 a partial anterior circulation stroke (PACS) and one a lacunar stroke (LACS). [40] The mean NIHSS score was 11.1 (mean 11), range 1-29. Baseline MRI was obtained at a median of 7 hours, (mean 10.34 hours) after stroke, range 1.05 – 25.05 hours. Note in this cohort, there were no patients who awoke with a stroke, therefore time of onset was known in all patients. One patient was scanned after the 24 hour deadline due to inadvertent delays to scanning, but were retained in the analysis as they had been recruited into the study.

Baseline NIHSS and Rankin scores were obtained for all 40 patients, second, third and fourth examination NIHSS scores for 21, 17, and 20 patients

respectively. Baseline tissue lactate concentrations were obtained for 39 patients.

4.1 Intracerebral temperatures

The temperature in the DWI definitely abnormal tissue ("likely core") was on average 0.50°C higher (weighted mean, range -1.45° to +1.9°) than in ipsilateral normal voxels (Table 1 and Figure 2), and 0.38°C higher (weighted mean, range -2.17° to +2.91°) than contralateral normal voxels (both $p<0.001$). Direct comparison of ipsilateral and contralateral normal voxels confirmed that contralateral normal brain temperature was on average 0.22°C higher than ipsilateral normal brain temperature ($p<0.001$). In 12/29 patients (41%), the definitely abnormal voxel temperature was above 37.5°C. Temperatures in the possibly abnormal and possibly abnormal plus ("likely penumbral") voxels were on average higher than in definitely abnormal voxels. Specifically, possibly abnormal voxel temperature was 0.36°C higher than definitely abnormal ($p<0.001$) and possibly abnormal plus was 0.17°C higher than definitely abnormal ($p=0.01$). 25/39 patients (64.1%) had a mean temperature in possibly abnormal or possibly abnormal plus tissue above 37.5°C.

Averaging all the patients, the mean temperatures of each tissue group were: definitely abnormal 37.30°C, possibly abnormal 37.63°C, possibly abnormal plus 37.66°C, ipsilateral normal 37.16°C, and contralateral normal 37.25°C (Table 1, Figures 2 and 3).

Table 1. Comparison of temperature differences and absolute mean temperatures in acute ischemic stroke in different brain tissues as defined by diffusion-weighted imaging (DWI). DAL = definitely abnormal tissue; PAL = possibly abnormal tissue; PAL+ = tissue one voxel thick immediately outside the definitely or possibly abnormal tissue; INL = ipsilateral normal brain; CNL = contralateral normal brain.

Comparison between:	Weighted mean temperature differences with 95% CIs. (°C)	Test for overall effect	Maximum temperature differences °C	Mean of patients' temp. means °C	
DAL vs. CNL	**0.38** (0.27,0.49)	p<0.00001	2.91, -2.17	37.30	37.25
DAL vs. INL	**0.50** (0.34,0.66)	p<0.00001	1.90, -1.45	37.30	37.16
DAL vs. **PAL**	**-0.36** (-0.45,-0.26)	p<0.00001	1.28, -2.17	37.30	37.63
DAL vs. **PAL+**	**-0.17** (-0.30,-0.04)	p=0.01	1.66, -2.23	37.30	37.66
PAL vs. INL	**0.29** (0.14-0.44)	p=0.0002	1.92, -0.84	37.63	37.16
PAL vs. CNL	**0.14** (0.05,0.23)	p=0.004	2.68, -1.77	37.63	37.25
PAL+ vs. INL	**0.37** (0.24,0.51)	p<0.00001	2.43, -1.50	37.66	37.16
PAL vs. **PAL+**	**-0.15** (-0.26,-0.04)	p=0.007	2.50, -1.66	37.63	37.66
INL vs. **CNL**	**-0.22** (-0.32,-0.12)	p<0.00001	1.56, -1.08	37.16	37.25
(DAL+PAL) vs. (INL+CNL)	**0.17** (0.07,0.27)	p=0.0007	2.45, -2.17	-	-

Figure 2. Schematic showing the distribution of the temperatures within the brain following acute ischemic stroke as defined on diffusion-weighted imaging (DWI). DAL = definitely abnormal tissue on DWI; PAL = possible abnormal tissue; PAL+ = tissue one voxel thick immediately outside the definitely or possible abnormal tissue; INL = ipsilateral normal brain; CNL = contralateral normal brain.

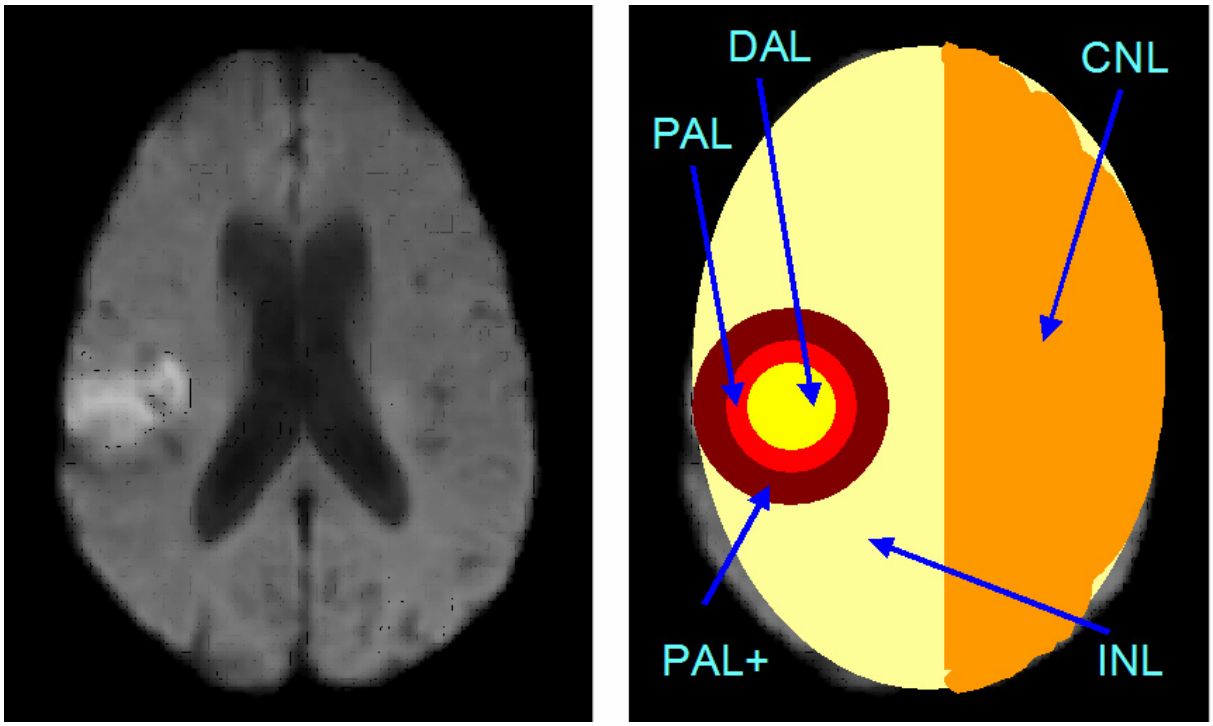
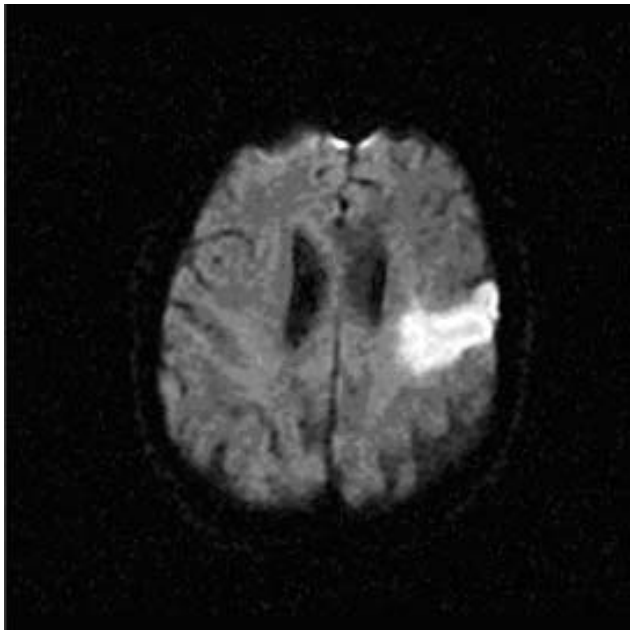
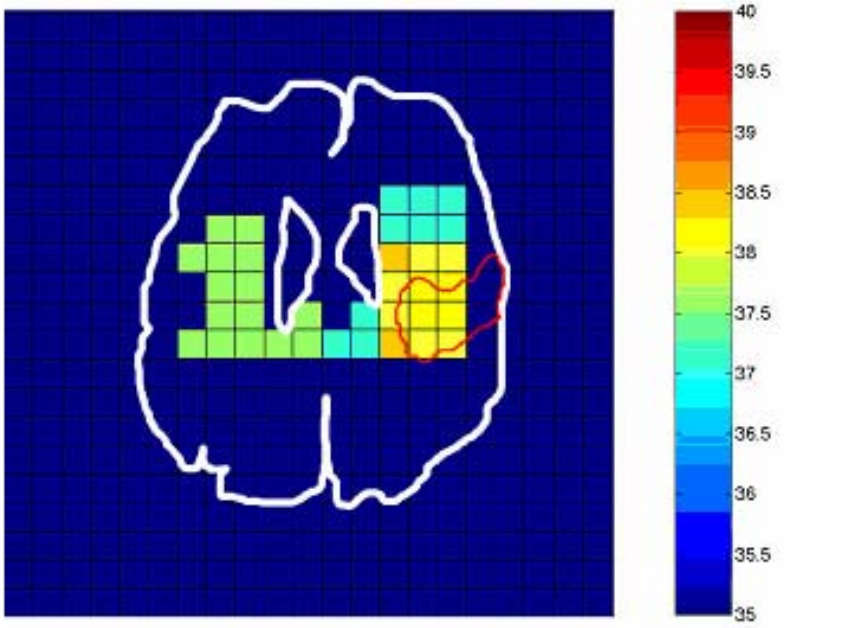


Figure 3. Map of distribution of actual voxel temperatures relative to the DWI image for an individual patient. A) DWI image showing a recent hyperintense ischemic lesion in the left tempo-parietal cortex. B) Color map of distribution of temperature (°C) per voxel superimposed on a line drawing of the DWI lesion outline for the same patient as in A.

A.



B.

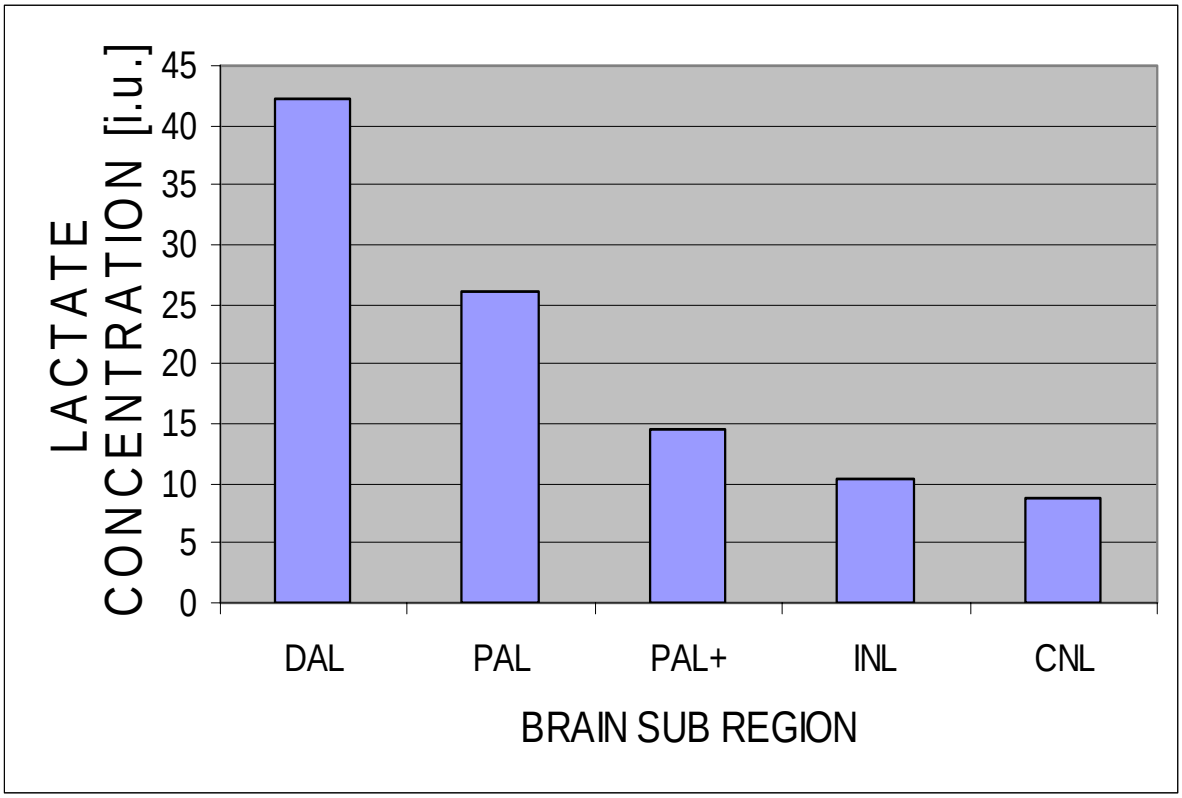


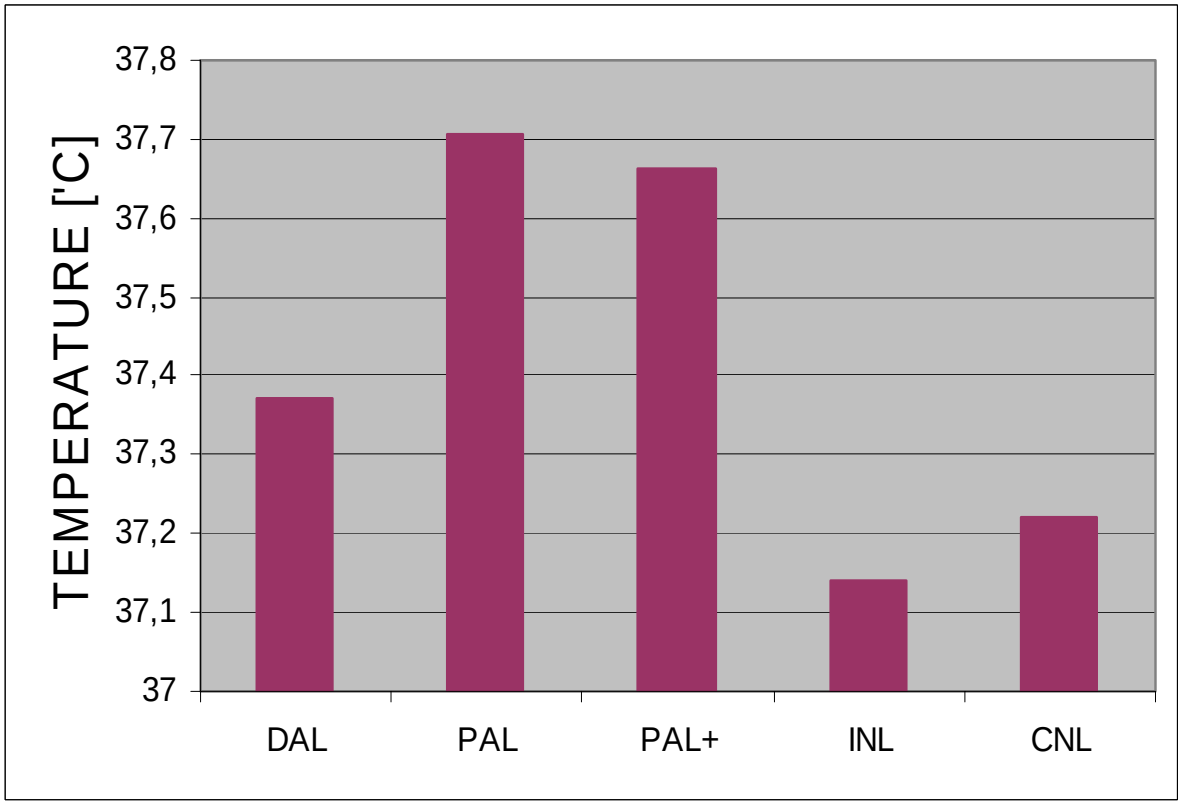
4.2 Brain lactate concentrations

At baseline, mean lactate concentration was highest in the core of the ischemic lesion ("DAL" 42.1), then in the potential penumbral tissues ("PAL" 26.0, "PAL+" 14.5), in ipsilateral normal brain ("INL" 10.4) and contralateral normal brain ("CNL" 8.7), $p<0.05$ (Figure 4). Lactate concentrations are given in arbitrary units, but corrected for scanner gain settings to enable comparisons between patients. Such units are usually referred to as "institutional units" (i.u.).

The highest lactate levels and highest temperatures occurred in different parts of the focally ischaemic brain (Figure 4). There was no association between lactate and temperature in any of the individual subregions.

Figure 4. Brain lactate and temperatures by subregion (DAL = definitely abnormal tissue on DWI; PAL = possible abnormal tissue; PAL+ = tissue one voxel thick immediately outside the definitely or possible abnormal tissue; INL = ipsilateral normal brain; CNL = contralateral normal brain) at baseline. Lactate concentrations are given in arbitrary units, but corrected for scanner gain settings to enable comparisons between patients. Such units are usually referred to as "institutional units" (i.u.).


LACTATE CONCENTRATION [i.u.]
45
40
35
30
25
20
15
10
5
0
DAL
PAL
PAL+
INL
CNL
BRAIN SUB REGION


TEMPERATURE ['C]
37,8
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37,3
37,2
37,1
37
DAL
PAL
PAL+
INL
CNL

4.3 Brain lactate concentrations or temperatures and clinical outcome

4.3.1 Brain lactate and stroke severity at baseline

There was a significant correlation between baseline NIHSS and tissue lactate concentration within PAL, PNIL and CNL regions ($r=0.33$; 0.39; 0.32 respectively, $p<0.05$). In INL this association did not quite reach statistical significance ($r=0.34$, $p=0.053$). In the remaining tissue types such association was not found.

4.3.2 Baseline brain lactate and clinical deterioration

There was no association between the 2nd, 3rd and 4th examination NIHSS and lactate concentration within any of the brain subregions. However, to explore whether elevated tissue lactate concentration preceded further clinical deterioration (anaerobic metabolism intensity corresponds to lack of energetic substrates and therefore may precede tissue destruction), we compared baseline lactate and subsequent changes in NIHSS.

There was a strong correlation between clinical deterioration measured as increase in NIHSS from baseline to 1 and 3 months and baseline lactate concentration within the PAL and PNIL brain subregions ($r=0.60$ and 0.65 respectively, $p<0.01$). There was no association between other tissues and change in NIHSS.

4.3.3. Baseline brain lactate and functional outcome

There was no association between baseline lactate concentration in any tissue and 3 months modified Rankin scale (Table 2).

Table 2. Associations between baseline lactate concentrations (i.u.) in brain subregions (DAL = definitely abnormal tissue; PAL = possibly abnormal tissue; PAL+ = tissue one voxel thick immediately outside the definitely or possibly abnormal tissue; INL = ipsilateral normal brain; CNL = contralateral normal brain, PNIL = "PAL"+"PAL+"+"INL") and modified Rankin scale scores.

Brain subregion	DAL	PAL	PAL+	INL	CNL	PNIL
Rankin 0,1,2	43,202	25,04684	15,92842	10,35778	7,836842	15,30842
Rankin 3,4,5,6	41,1125	26,91895	12,93235	10,48889	9,442	18,961
p value	0.8148	0.7277	0.4126	0.9627	0.2791	0.7056

4.3.4 Brain temperature and stroke severity at baseline

There was no straightforward association between baseline NIHSS and tissue temperature within any of the brain subregions. However, in 13 patients with mild strokes (NIHSS score 0-8), there was no difference between lesion and contralateral normal brain temperatures (mean 36.93 and 37.41°C respectively, p=0.11), whereas in the 16 patients with more severe strokes (NIHSS >8), the average lesion temperature was 0.70° higher than contralateral normal tissue (mean 37.73°C and 37.26°C respectively, p<0.001). (Table 3) Note this temperature difference was not simply a function of lesion size as the distribution of small and large lesions was similar in patients with NIHSS 0-8 (number with small/medium/large lesions =11/7/0) as in those with NIHSS >8 (number with small/medium/large lesions =10/4/4, Chi-squared 0.99, p=NS).

4.3.5 Baseline tissue temperature and clinical deterioration

To explore whether tissue temperature elevation preceded further clinical deterioration, we compared baseline temperature of brain subregions and changes in NIHSS between baseline and 1 – 3months after stroke. However, non of these associations were significant.

Table 3. Temperatures in the ischemic lesion (abnormal tissue as seen on diffusion-weighted imaging - DAL) compared with contralateral normal brain (CNL) in patient subgroups.

Analyses in italics had too small a sample size for reliable results. *a "negative" value indicates DAL cooler than CNL, a "+" value indicates DAL hotter than CNL.

Patient or lesion group – characteristics	Weighted mean temp. differences + 95% CIs (°C), DAL vs CNL*	Test for overall effect	Mean of patient temp. means DAL, CNL (°C)
Small lesion	**0.66** (0.51,0.81)	p<0.00001	37.20, 37.08
Medium lesion	**0.06** (-0.12,0.24)	p=0.51	37.39, 37.47
Big lesion	***0.14*** *(-0.20,0.47)*	*p=0.43*	*37.85, 37.60*
CBF decreased	**0.31** (0.19,0.43)	p<0.00001	37.56, 37.27
CBF normal or increased	***-0.68*** *(-0.39,-0.97)*	*p<0.00001*	*36.84, 37.49*
Mismatch present	**0.55** (0.39,0.71)	p<0.00001	37.73, 37.31
Mismatch absent	**-0.22** (-0.07,-0.37)	p=0.004	36.93, 37.35
Time from onset to MR<10 h	**0.40** (0.25,0.55)	p<0.00001	37.25, 37.18
Time from onset to MR>10 h	**0.33** (0.17,0.48)	p<0.0001	37.46, 37.43
NIHSS =< 8	**0.12** (-0.03,0.27)	p=0.11	36.93, 37.41
NIHSS > 8	**0.70** (0.53,1.36)	p<0.00001	37.73, 37.26

4.3.6 Baseline tissue temperature and functional outcome

There were no significant associations between baseline temperature and functional outcome.

Mean tissue baseline temperature in DAL, PAL and INL regions was lower in patients who were independent at 3 months (mRs 0, 1 or 2) compared with those who were dependent or dead (mRs 3, 4, 5 or 6, p=NS). Mean baseline temperature in the tissue immediately outside the lesion (PAL+) was higher by 0.56 degrees (p=NS) in those who were independent (mRs 0,1 or 2) compared with those who were dependent or dead (mRs 3-6) – Table 4.

Table 4. Associations between baseline tissue temperatures (°C) of brain subregions (DAL = definitely abnormal tissue; PAL = possibly abnormal tissue; PAL+ = tissue one voxel thick immediately outside the definitely or possibly abnormal tissue; INL = ipsilateral normal brain; CNL = contralateral normal brain) and modified Rankin scale score.

Brain subregion	DAL	PAL	PAL+	INL	CNL
Rankin 0,1,2	37.39867	37.61211	37.79684	37.09944	37.25737
Rankin 3,4,5,6	37.48882	37.6995	37.23412	37.27526	37.18905
p value	0.8321	0.7882	0.1246	0.4994	0.7256

4.4 Ischemic lesion growth and brain lactate or temperature

To explore whether the association between brain metabolic changes and stroke clinical course and outcome were mediated by the simple growth of the ischemic lesion (as measured by visible lesion expansion on DWI between baseline and subsequent imaging), we compared the lactate concentration in different subregions between patients with DWI lesion growth and those without. Although mean tissue lactate concentration in brain subregions did not differ significantly between those with and those without lesion growth, it was higher in all subregions in those who developed lesion growth. The difference was particularly pronounced in PAL+ region (Table 5).

Table 5. A comparison of the lactate mean concentrations (i.u.) in different brain subregions (DAL = definitely abnormal tissue; PAL = possibly abnormal tissue; PAL+ = tissue one voxel thick immediately outside the definitely or possibly abnormal tissue; INL = ipsilateral normal brain; CNL = contralateral normal brain) between patients with DWI ischemic lesion growth and those without.

Brain subregion	DAL	PAL	PAL+	INL	CNL
DWI growth	47,3375	27,76	20,16111	10,63778	8,421111
No DWI growth	43,28632	25,28667	11,449	8,744	8,357273
p value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

The same comparison for the temperature mean values did not reveal significant differences between subgroups however these related to 9 patients only whose DWI lesion grew (Table 6).

Table 6. A comparison of the temperature mean values (°C) in different brain subregions (DAL = definitely abnormal tissue; PAL = possibly abnormal tissue; PAL+ = tissue one voxel thick immediately outside the definitely or possibly abnormal tissue; INL = ipsilateral normal brain; CNL = contralateral normal brain) between patients with DWI ischemic lesion growth and those without.

Brain subregion	DAL	PAL	PAL+	INL	CNL
DWI growth	37,3225	37,77333	37,11857	37,04222	36,82222
No DWI growth	37,38588	37,63429	37,66294	37,13632	37,22364
p value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

4.5 Brain temperatures: relationship to other lesion and patient characteristics

In the following analyses, we compared the DWI visible lesion with contralateral normal tissue.

Lesion size (Table 3). Temperatures in the DWI ischemic lesion and normal brain were higher in patients with medium/large lesions (lesion 37.51°C, contralateral normal tissue 37.50°C, p=NS), than in patients with small lesions (lesion 37.2°C,and contralateral normal brain 37.08°C, p<0.001).

Cerebral blood flow (CBF) and PWI/DWI mismatch (Table 3). In patients with decreased CBF in the DWI ischemic lesion (n=23), the ischemic lesion was on average 0.31°C hotter than contralateral normal tissue (p<0.001). In patients with normal or increased CBF in the ischemic lesion (n=7), lesion temperature was 0.68°C cooler than contralateral normal brain (p<0.00001). However there were rather few patients with normal or increased lesion CBF. In patients with any PWI/DWI mismatch (on either or both of CBF or mean transit time, MTT, n=16), the ischemic lesion temperature was higher than contralateral normal brain (mean temperature difference 0.55°C, p<0.001): in those without mismatch (n=13), the lesion temperature was lower then in contralateral normal brain (mean difference 0.22°C, p=0.004). The difference in temperature profile between patients with and without mismatch was statistically significant (p=0.01, Mann-Whitney U). Thus, ischemic lesions in patients with DWI/PWI mismatch were hotter by almost 1°C than in those without mismatch.

Time from stroke to scanning (Table 3). Temperature in the DWI ischemic lesion rose before that in contralateral normal brain: patients scanned within ten hours of stroke, 37.25°C and 37.18°C respectively, mean difference 0.40°C, $p<0.001$; patients scanned after ten hours, temperatures in ischemic lesion and normal brain were both elevated, although the lesion temperature was still higher than in normal brain (37.46°C and 37.43°C respectively, mean difference 0.33°C, $p<0.001$).

Body vs. brain temperature. Mean body temperature was 36.2°C. Only one patient was pyrexial at the time of scanning (body temperature 37.8°C). This subject, imaged nearly 23 hours after the stroke, had a relatively mild stroke (PACS, NIHSS 4), and had mean temperatures above 37.5°C in possibly abnormal, possibly abnormal plus and contralateral normal tissues.

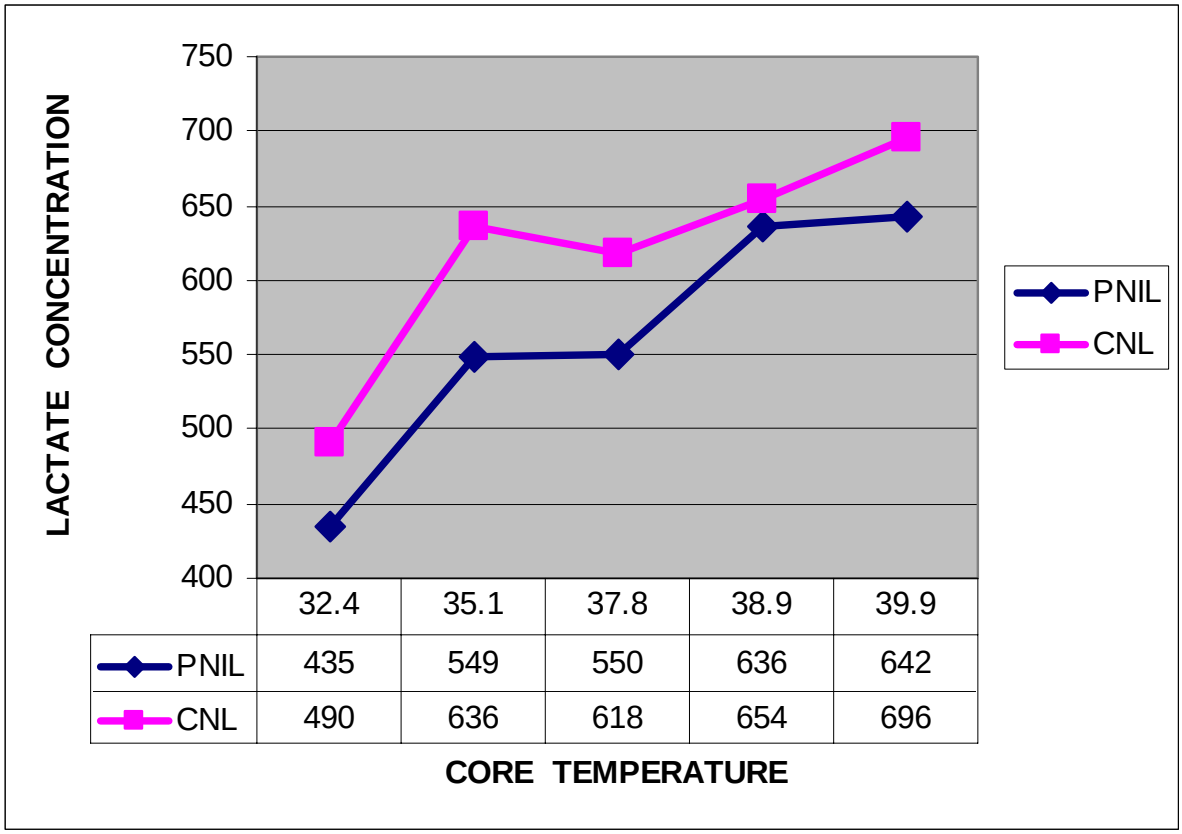
4.6 Brain lactate and body temperature relationship in an animal ischemic stroke model

In all 15 stroke animals, mean brain lactate concentration was higher in "ischemic" ("PNIL") than in contrlateral ("CNL") hemisphere although this difference did not reach statistical significance (mean lactate concentrations: 664 and 647 nmol/mg protein respectively, $p>0.05$).

Temperature-dependent brain lactate concentration

The association between lactate concentration in brain regions (focally ischemic – "PNIL" and contrlateral – "CNL" tissues) and core temperature (°C) is presented in figure 5.

Figure 5. The association between lactate concentration (nmol/mg protein) in brain regions (focally ischemic – "PNIL" and contralateral – "CNL" hemisphere) and core temperature (°C).

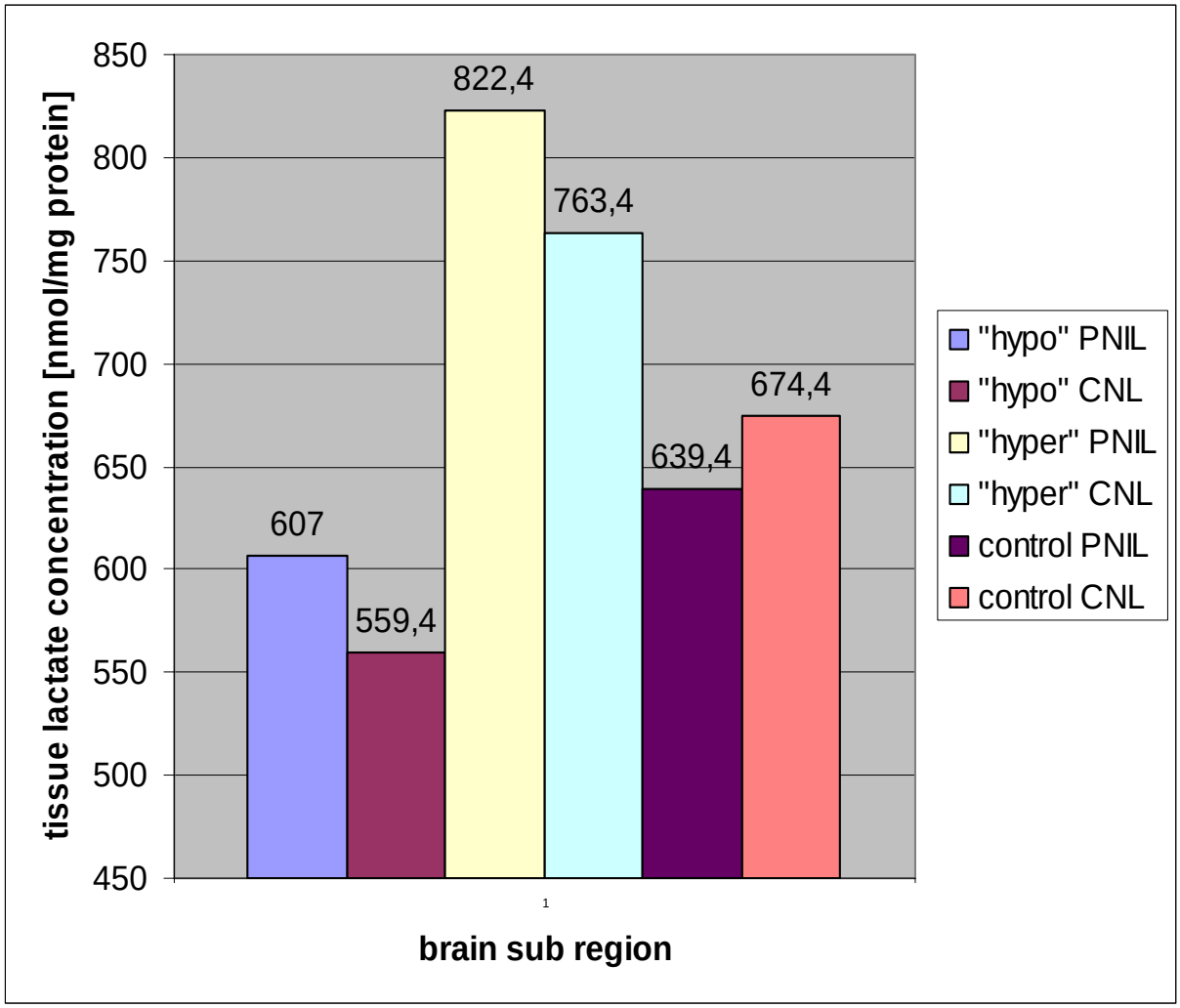


Modulation of brain lactate concentration by hyper- or deep hypothermia

Core temperatures of hypothermia-treated mice ranged between 25.8 and 27.9, mean 27.12 °C, whereas for hyperthermic animals the range was 37.1 to 42.2, mean 39.72 °C.

Ischemic hemisphere tissue lactate concentration in hypothermia-treated individuals was lower than in “hyperthermic” individuals ($p < 0.05$) and was not significantly lower than in healthy animal brains. Mean lactate concentration in ischemic brains of “hyperthermic” animals was higher than in healthy brains ($p < 0.05$) (Figure 6).

Figure 6. Modulation of tissue lactate concentration (nmol/mg protein) by deep hypothermia (“hypo”) or hyperthermia (“hyper”) in focally ischemic hemisphere (“PNIL”) and in contralateral brain (CNL).



5. Discussion

5.1 Ischemic brain temperature distribution

We found elevated temperatures in the ischemic lesion as seen on DWI, particularly in brain tissues that we operationally classified as "potential penumbra" (those that appeared only marginally abnormal on DWI or just outside the edge of the DWI lesion). [47-49] These tissues were hotter than the operationally classified lesion "likely core" (definitely abnormal on DWI), which in turn were hotter than normal-appearing brain (Figure 2 and 3). Patients with large lesions and those scanned later had higher lesion and normal brain temperatures than patients with small lesions or scanned earlier. Patients with DWI/PWI mismatch or reduced perfusion in the ischemic lesion had higher lesion temperatures than those without mismatch or with increased perfusion. Although only one patient was pyrexial at the time of scanning, more than 41% of patients had raised lesion "likely core" ischemic lesion temperature, and more than 64% had raised "likely penumbral" temperatures. Thus measurement of regional brain temperature with MRSI is feasible in patients with acute ischemic stroke, could be used to assess the effectiveness of cooling in trials of hypothermia to treat stroke, and avoids the need for invasive and potentially risky monitoring.

We used an operationally-defined and perhaps slightly novel classification of "likely core" and "likely penumbral" tissue based on the DWI lesion appearance to avoid confounding by using unconfirmed perfusion or ADC

thresholds in the definition. Tissue which is moderately abnormal on DWI can recover [46-48] and normal-appearing tissue at the edge of the DWI lesion may be metabolically abnormal. [49] While the tissue falling in the DWI/PWI mismatch has commonly been referred to as “penumbra”, mismatch volumes vary widely depending on which perfusion parameter is used, how the PWI data are processed, and do not reliably define final infarct extent. [44,45,51,53] Furthermore, 50% of patients with no DWI/PWI mismatch (by any definition) have infarct growth, [1] therefore the tissue immediately outside the DWI-visible lesion is likely to be “potentially penumbral” in many cases.

In the present study, despite many patients having elevated ischemic lesion temperatures, only one patient was pyrexial (body temperature >37.5°C) at the time of scanning. Perhaps we assessed our patients too early to find elevated body temperature. We did not measure body temperature serially to see whether any changes in body temperature were related in any way to brain temperature. However, our finding of higher brain temperatures in severe strokes, large lesions and at later time points is consistent with the known associations between body temperature elevation and severe stroke, so would be consistent with the elevated ischemic lesion temperature occurring due to intrinsic cerebral events and possibly leading, in due course, to elevated body temperature after stroke. We measured body temperature with a tympanic thermometer. These may read temperature as incorrectly

elevated if the patient has recently been lying on the ear from which the measurement is taken. [58] As only one of our patients was pyrexial, our concern is more that we under-read body temperature. Rectal temperatures may be a more valid and reliable measure of body temperature, but are not used in our stroke unit.

The temperature increase in the contralateral compared with ipsilateral normal brain may be an apparent difference resulting from relatively few ipsilateral normal voxels to contribute to analysis in patients with large lesions, and further work is required to characterize this better. However it is also possible that the contralateral temperature elevation reflects true abnormalities in response to the ischemia, such as increased neuronal activity in areas subserving the mirror-image functions on the non-paretic side as those affected by ischemia on the paretic side. We do not believe that temperature elevation was secondary to a general increase in body temperature (only one patient was pyrexial), or to the heating effects of MR scanning (as all sequences were operated well within the SAR limits and we have no reason to expect that any external heating effect, however subtle, would affect tissues unequally). Furthermore, previous normal volunteer MRSI repeatability data showed that average brain temperature actually fell by 0.09ºC per scan ($p<0.001$), presumably due to the cool air flow in the MR scanner bore (or reduced brain activity while resting in the scanner). [5] Also

the analysis of within-patient tissue temperature differences minimized any external factors which might influence MRSI. [5]

5.2 Ischemic brain temperature elevation pathophysiology

The brain tissue temperature-elevating process could be caused by biochemical reactions in ischemic cells, or due to mediators released by infiltrating inflammatory cells, or occur because altered CBF affects heat exchange, or all three, possibly varying with the time lapse after stroke. Our data suggest that the larger the lesion, or the longer the time after stroke, the more heat generated and passed to normal brain. However, this is unlikely to be a simple direct process of heat conduction as otherwise the ipsilateral normal brain would be hotter than the contralateral and it was not. Failure of heat exchange through loss of blood flow could contribute to elevated temperature, but if this were so, the tissue with the most reduced blood flow ("core") should be hotter than the "penumbra", and this was not the case. The association between normal/increased CBF and lower lesion than normal brain temperature suggests that some of the observed regional temperature changes result from altered blood-brain heat exchange (i.e. increased flow removes heat more rapidly than reduced flow), but this does not explain where the excess heat comes from in the first place.

Little is known of the thermal effects of chemical reactions occurring in normal or ischemic brain, the relative role of blood as a heat-dispersing agent, the heat conductance of brain, or temperature control in normal brain. Cellular processes that might drive temperature elevation in ischemic tissue [25,59-61] include stimulation of microglial cells and production of free radicals, peroxidation of cellular membrane lipids, increased inappropriate release of excitatory amino acids, increased metabolic kinetics with cytoskeleton destruction by calcium cations prior to energy depletion, increased anaerobic instead of aerobic glycolysis, and intense release of inflammatory mediators. [31,62-67] However there was no association between glucose, lactate, pyruvate or glutamate measured with a microdialysis probe and brain temperature measured invasively in acutely head injured patients, although this may reflect a lack of sensitivity from very localized invasive measurements. [68] Cerebral uncoupling protein 2 (UCP2) status may influence the tissue temperature response as well as potentially being neuroprotective. [69-71] Temperature elevation early after stroke might result from reactions in ischemic but viable brain, and later (as the viable tissue dies) from other inflammatory responses. Serial studies at different times after stroke are required to elucidate the precise time course of temperature changes, and further research is required to study the temperature-generating effects of biochemical reactions in the ischemic brain.

5.3 Brain metabolism and clinical outcome

This study revealed that metabolic changes taking place within 24 hours from stroke onset in the DWI-healthy-looking tissue of the focally ischemic hemisphere (importantly, not only in potential penumbral tissues) were associated with stroke severity, further clinical deterioration and 3 month functional outcome.

Lactate concentrations in potential penumbral and ipsilateral normal-looking tissues were associated with stroke severity (as measured by NIHSS) at baseline and predicted neurological deterioration within the next 1 to 3 months (as measured by an increase in NIHSS). Mean lactate concentration was highest in the ischemic core tissues, but core lactate was not associated with clinical severity either at baseline or at later examination points.

We did not find any direct associations between tissue temperatures and multiple NIHSS measurements or Rankin scales. However, in the case of the latter, the tissue forming the healthy looking rim immediately outside the lesion (PAL+) was much hotter in the group with better functional outcome. Additionally, patients with more severe strokes had larger differences between lesion and contralateral tissue temperatures than those with milder strokes. Assuming that contralateral brain temperature does not increase early

after stroke, this might account for the fact that relative lesion temperature increase rather than its absolute reading is associated with stroke severity.

Post-stroke brain temperature is likely to be modulated by multiple pathomechanisms. Among them, local metabolic reactions, local and systemic inflammatory response, local blood supply changes and up- or down-regulation of specific genes taking part in tissue protection and simultaneously modulating environment temperature (for example uncoupling proteins - UCPs) [59-72] seem the most evident. Exploration of this complicated pattern requires further studies.

We urge the caution that the association between the mentioned metabolic factors and clinical condition, in some cases as strong as above 0.6, was evaluated for the whole, heterogeneous patient group. The sample size was not large enough to perform multivariate analyses to correct for confounders. This means that these correlation coefficients must have been confounded by as obvious stroke severity indicators as lesion size, presence of cerebral oedema or coexistence of other diseases. Therefore the shown correlations are likely to be stronger in reality.

5.4 Ischemic brain lactate, temperature and lesion growth

Patients with DWI lesion growth had higher lactate concentrations in all brain subregions than those without lesion growth. It was especially obvious within a healthy looking tissue immediately outside the lesion (PAL+), although still not significant statistically due to a small number of patients in subgroups. This would mean that increased lactate formation in brain tissue precedes infarction (and thus precedes the expansion of the lesion in conventional neuroradiological images). Confirmation of this finding, however, needs further studies on a bigger patient sample.

5.5 Lactate in ischemic brain

Our study confirms the thesis of lactate, as a marker of tissue metabolism impairment or of its harmful effect on para-ischemic brain, a theory that has been acknowledged for some decades until the ANLS hypothesis description in 1994. [7-14,73-75] However, the results are not necessarily contradictory to the ANLS assumptions because the corresponding expression and disposal changes of lactate membrane transporters (MCTs) and lactate dehydrogenase (LDH) isoforms on ischemic human astrocytes and neurons, as well as the subsequent lactate distribution within the tissue compartments, remain unknown.

5.6 Reliability of MRS temperature measurement

We developed and validated a magnetic resonance proton spectroscopic imaging (MRSI) technique to measure regional brain temperatures in acute ischemic stroke in humans, as described in detail recently. [5]

5.7 MR spectroscopy brain lactate concentration measurement remarks

Our metabolite measurements are in arbitrary units, but corrected for scanner gain settings to enable comparisons between patients. Such units are usually referred to as "institutional units". Because of the unknown relaxation times (T1 and T2) in pathological tissues, we are not able to "normalize" the measurements and interpret them in terms of absolute millimolar concentrations. A limitation of the spectroscopic imaging technique is the difficulty of achieving a satisfactory shim over the whole slice, especially on scanners (like the one used in this study) that are not fitted with high-order shimming coils. Several spectra from frontal regions had to be discarded because of the susceptibility effects from the nearby sinuses. Another concern is that the RF excitation is nonuniform near the edges of the PRESS localised volume, leading to several poor quality spectra from cortical regions of interest. Inevitably, patients with small lesions will have contributed more INL measurements than patients with large lesions, and this may influence the statistical analysis.

5.8 Pyrexia, hypothermia and lactate in focally ischemic mouse brain. Consequences for clinical neurology

Brain lactate formation was associated with core temperature. This relationship was similar in ischemic and contrlateral hemispheres and was close to linear correlation. Tissue lactate formation was significantly reduced by induced deep systemic hypothermia compared to hyperthermia. Ischemic brain lactate concentration in hypothermic conditions was similar to healthy brain whereas induced hyperthermia caused a dramatic increase in lactate formation in ischemic and healthy hemispheres.

In the previous ischemic stroke patients study [previous part of the thesis] we revealed that peri-lesion ipsilateral hemisphere brain lactate concentration is correlated to neurological outcome and further symptoms progression. On the other hand, there is a strong association between raised body temperature (pyrexia is a common condition early after stroke onset) and ischaemic lesion progression or poor functional outcome after stroke, [37-39] and induced hypothermia shows promise as a treatment for acute ischemic stroke. [33] Therefore we conclude that hypothermia or pyrexia might influence stroke patients' clinical condition by modulation of ipsilateral brain tissue energy metabolism (including one of its products – lactate). Lactate peri-ischemic brain tissue concentration, which it is possible to measure non-invasively in stroke human brains [previous part of the thesis], might constitute a tool for qualification of stroke patients for deep systemic hypothermia treatment.

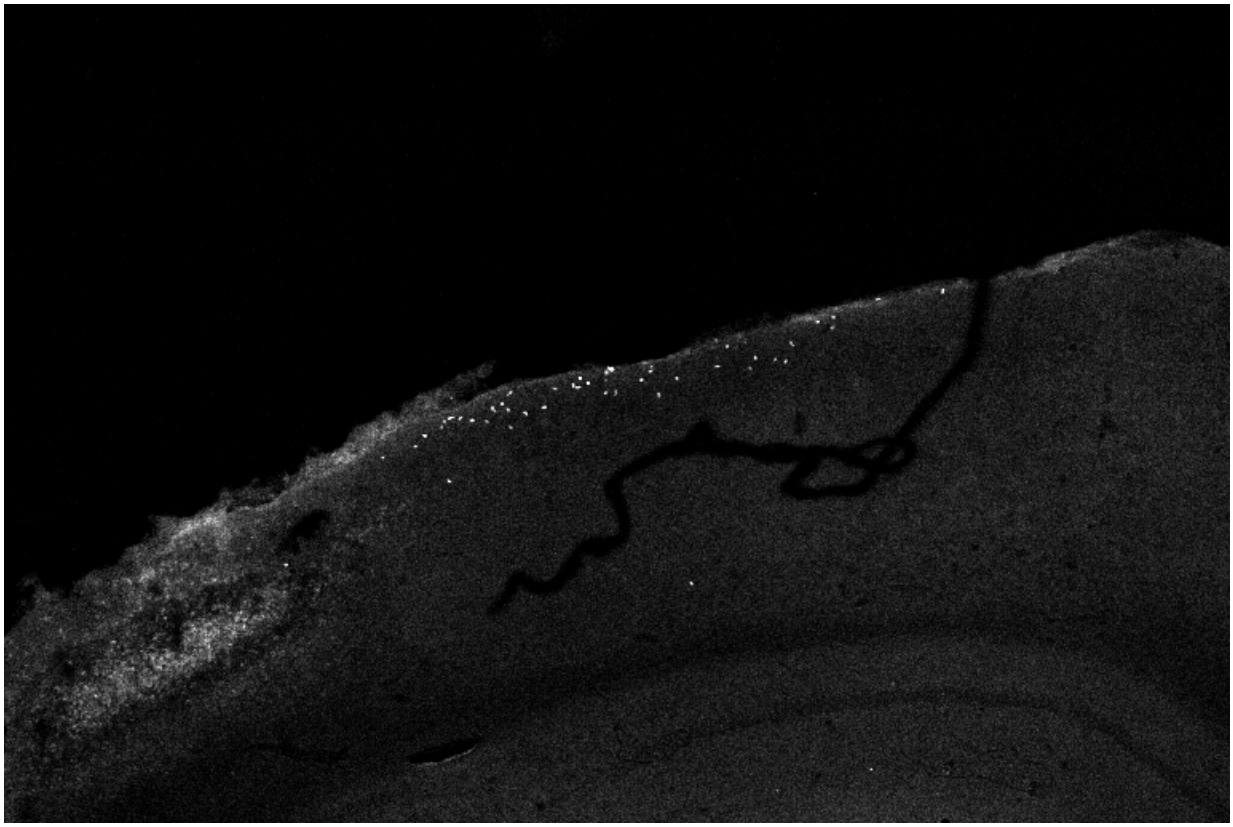
5.9 Brain lactate concentration and stroke severity pathophysiological links – examples

This study revealed that an increase in lactate concentration in potential penumbral and ipsilateral normal-looking tissues was associated with stroke severity at baseline, further neurological deterioration and possibly with ischemic lesion expansion. We proposed that this association might be caused by the fact that an increase in tissue lactate concentration mirrors anaerobic metabolism intensity, which corresponds to lack of energetic substrates and therefore may precede tissue destruction.

There are however other mechanisms that may account for this association, at least confirmed in animal models. For example, migration of native neural stem cells towards the nervous tissue lesion and subsequent cell proliferation, which corresponds to milder disease symptoms and better clinical outcome, is also associated with local lactate concentration decrease. [53,76-81] Ischemic stroke may initiate migration of neural, embryonic or bone marrow stem cells towards the ischemic lesion from different, often distant, brain regions or from blood and their further proliferation and differentiation within the ischemic locus. This phenomenon has been described previously in some independent studies [77-81] and one from our group [53] Briefly, we observed the fate of bone marrow (BM) cells collected from 5-week-old transgenic mice ubiquitously expressing green fluorescent protein (GFP) after their transcranial transplantation into the brain of focally ischemic (MCAo) wild mice 24 h of stroke. The transplanted

BM cells migrated towards the ischemic lesion (Figures 7a and 7b – post mortem section confocal microscope scans with visible ischemic lesion in MCA territory and GFP-BM cells between transplantation site and the lesion), proliferated (Figure 8 – in vivo confocal microscope scans performed via implanted cranial window show an increase in the number of GFP-BM cells in the 1^{st}, 5^{th} and 7^{th} day from transplantation in/around MCA territory) and changed their morphology within 7 – 14 days from transplantation. [53]

Figures 7a and 7b. Confocal microscope post-mortem sections with visible ischemic lesion in MCA territory and GFP-BM cells between transplantation and lesion sites. Unpublished photographs from our previous project in an animal model of ischemic stroke listed in the references. [53]



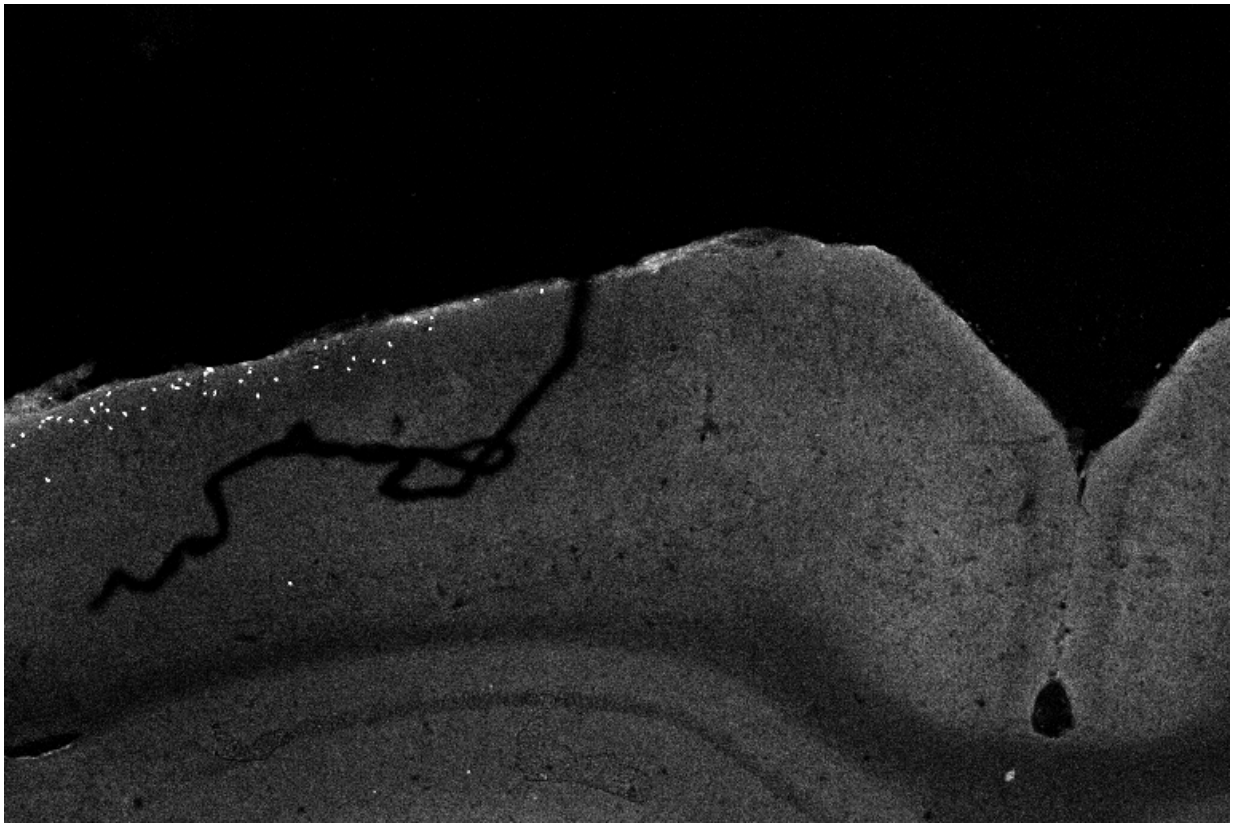
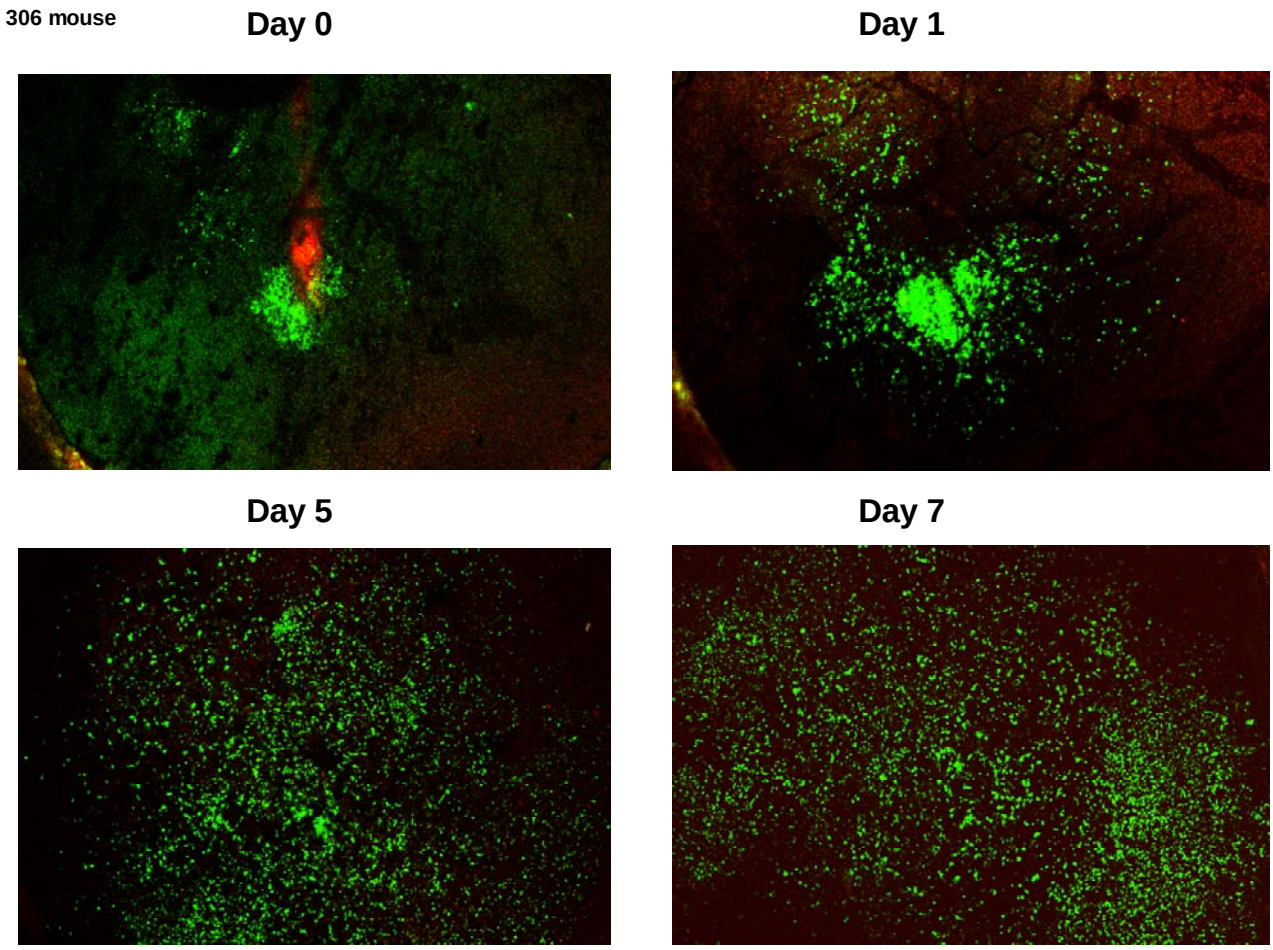


Figure 8. Confocal microscope *in vivo* scans performed via implanted cranial window show an increase in the number of GFP-BM cells in the 1st, 5th and 7th day of transplantation in/around MCA territory. Unpublished photographs from our previous project in an animal model of ischemic stroke listed in the references. [53]



6. Final conclusions of the Doctoral Dissertation

We conclude that changes in baseline "energy metabolism" within the healthy looking ipsilateral brain tissue (and not "just" in potential "penumbra") could account for the recently described ischemic stroke "DWI-clinical mismatch". They may precede progression of symptoms, lesion enlargement and the final clinical outcome. MR spectroscopy techniques could be used to predict ischemic stroke deterioration, perhaps performed routinely with DWI, and therefore be important information for management decisions.

We found elevated temperatures in the ischemic brain, particularly in tissues that we classified as "potential penumbra".

Hypothermia (an effective stroke treatment measure) or pyrexia (a common condition early after stroke onset, associated with ischaemic lesion progression or poor functional outcome) might influence stroke severity by modulation of ipsilateral brain tissue energy metabolism.

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