Supplemental Material

Supplemental Methods

Exclusion criteria

Exclusion criteria for the patients were the following: any contraindication to PET or MRI, alcohol or illicit drug abuse, active malignancy, significant pre-morbid cognitive impairment, current or recent (within past week) benzodiazepine use, significant neurological disease e.g. epilepsy, multiple sclerosis, or major organ failure that may complicate imaging studies e.g. significant cardiac, renal or liver disease, or inadequate co-operation for scanning.

Exclusion criteria for the healthy subjects: only subjects with no significant health problems and with similar exclusion criteria as for the patients above (apart from the stroke) were recruited. In particular, they had no prior history of cerebrovascular or other disease that could confound imaging studies e.g. alcohol abuse, and were taking no medication that could interfere with FMZ binding.

CT perfusion procedure

Patients were scanned using the hospital helical CT scanner (Siemens Sensation 4 model, Siemens GmbH, Erlangen, Germany, 120kVp, 258 mA). CTp involved the injection of 50ml of iodinated contrast (iopamidon 300) at a flow rate of 8ml/s using an automated injection pump through a 16-18 gauge intravenous cannula placed in the antecubital fossa. The protocol consisted of a 40s cine acquisition with a repetition time of 1s on two contiguous axial slices (voxel dimensions: 0.41 x 0.41 x 10mm) manually positioned at the level of the basal ganglia and the level above. This produced 2 x 10mm thick slices of perfusion data. Two patents were scanned using a CT scanner with extended head coverage (Siemens SOMATOM Definition Flash scanner), but to match the other patients the same two slices as above were extracted from the dataset and used for further analysis.

PET scanning procedures

PET scans were performed on a GE Advance scanner (General Electric, Milwaukee, WI, USA). Immediately prior to tracer injection, a 15 minute $^{68}Ge/^{68}Ga$ rotating rod source transmission scan was obtained for attenuation correction. Subjects received an intravenous bolus injection over 30 seconds of high specific activity PK or FMZ followed by acquisition

of a dynamic emission scan (52 frames over 60 minutes for PK and 55 frames over 75 minutes for FMZ). The mean (SD) specific activity for FMZ was 101 (± 63) GBq/μmol, and mean (SD) activity injected 390 (± 84) MBq. The corresponding values for PK were 61.0 (± 39.9) GBq/μmol and 246.3 (± 86.8) MBq.

Images were reconstructed using 3D filtered back projection into a 128 x 128 x 35 array with voxel dimensions 2.34 x 2.34 x 4.25mm, with a Hann filter applied transaxially to result in an approximately isotropic resolution of 6.8mm FWHM. The emission data were acquired in 3D mode, with corrections applied for randoms, dead time, normalisation, scatter, attenuation and sensitivity.

MRI sequences

Scans were acquired on a Siemens Magnetom Tim Trio 3T whole body magnet. Sequences acquired in patients included: i) high resolution 3D T1W volumetric MPRAGE (9m14s, voxel size 1mm isotropic; no. of slices = 176; slice thickness = 1mm; TR/TE = 2300ms/2.98ms,; FoV=240x256mm) for image co-registration; ii) T2 volumetric SPACE (6m13s, voxel size 0.9mm isotropic, no. of slices = 160; slice thickness = 0.9mm; TR/TE = 3200ms/540ms; FoV = 224x256mm; and iii) T2 Fluid-Attenuated Inversion Recovery (T2-FLAIR) (4m28s, voxel size 0.7x0.7x4mm; no. of slices = 27; spacing beween slices = 5mm; TR/TE = 7840ms/95ms; FoV = 256x320mm) to map the final infarct;

In control subjects, only the high resolution 3D T1W volumetric MPRAGE and T2 FLAIR (as above) as well as an axial PDT2 dual echo (02m47s, voxel size 0.7x0.7x4.0, spacing between slices = 5mm, no. of slices = 27, TR = 4600ms, 2 TE values = 12ms, 104ms; FoV = 240x320mm) were acquired.

CT perfusion data analysis

Images were processed using in-house software implemented with Matlab version 7.3 (The Mathworks, Inc.). The methodology closely followed that used by Wintermark *et al* in order to apply their validated CT perfusion threshold for penumbra and core (Wintermark *et al.*, 2006). The slice containing the clearest ACA (first choice) or MCA branch (second choice) on the unaffected hemisphere was chosen and a voxel with the expected arterial input function shape was manually selected (Wintermark *et al.*, 2007). The cerebral blood volume (CBV) was calculated from the area under the curve; the enhancement curve from the superior sagittal sinus was used as reference. The vascular mean transit time (MTT) was

derived for each voxel from the convolution of the arterial input function with a box-shaped residue function. CSF voxels, based on the non-contrast CT, and voxels representing blood pool, based on the CBV map (> 8%) were excluded. Cerebral blood flow (CBF) was calculated following the central volume principle as (CBF = CBV / MTT). Penumbral tissue was defined as any voxel with MTT affected/unaffected (A/U) ratio >1.45 and CBV >0.82 ml/100g (Wintermark *et al.*, 2006, Murphy *et al.*, 2008, Alawneh *et al.*, 2011, Carrera *et al.*, 2013). The unaffected side mask used to calculate the MTT ratio included all voxels in the cerebral hemisphere contralateral to the stroke.

Infarct map and PET-MR image co-registration

For patients who had both PK and FMZ (see Results) and therefore two MR sessions, the infarct mask was defined on the MR images acquired together with the FMZ scan, i.e., at the second MR session, as late scans would be expected to best represent the final infarct, while having the same infarct mask for both studies was required by design (see below).

The first MR session in conjunction with the PK study was necessary in case the FMZ scan could not be obtained, which effectively happened in one patient (see Results), and also for coregistration purposes. Indeed, the late MR dataset used to define the infarct ROI was coregistered to the initial MR dataset prior to coregistration with the PET image, eventually bringing the infarct ROIs in spatial alignment with both the PK and FMZ binding potential maps.

PET data post-processing

Prior to running this routine, head motion during each PET scan was corrected by image re-alignment within each dynamic image series using SPM8 (Wellcome Trust Centre for Neuroimaging, London, UK, http://www.fil.ion.ucl.ac.uk/spm). To define the reference region for each individual subject, the T1-weighted volume MRI was co-registered to the respective mean realigned PET image. For FMZ images, the pons was used as the reference region (Lucignani *et al.*, 2004, Guadagno *et al.*, 2008). Three elliptical ROIs were drawn on the pons over 3 contiguous slices on the T1-weighted MRI, and were placed posteriorly in order to avoid the grey matter nuclei. For PK images, as per previous stroke studies (Gerhard *et al.*, 2005, Price *et al.*, 2006, Hughes *et al.*, 2012), the reference region was the cerebellum ipsilateral to the stroke; the reference ROI was delineated in the superior cerebellar cortex using a 90% threshold on the grey matter probability maps produced by SPM8, smoothed to

PET resolution. The reference ROIs were transferred to the coregistered dynamic PET images to produce the reference time-activity curve, and then binding potential maps were generated for each subject.

Mapping SNL and MA within the ultimately non-infarcted MCA territory

FMZ: voxel-based single-subject analysis

Image processing was performed using Statistical Parametric Mapping software (SPM8, Wellcome Trust Centre for Neuroimaging, London, UK, http://www.fil.ion.ucl.ac.uk/spm) and Matlab version 7.3 (Mathworks, USA). First, the MR T1 images were spatially normalised to the MNI152 T1 template using the unified segmentation-based normalisation (Ashburner and Friston, 2005), thereby obtaining both the forward and inverse normalisation parameters. In patients this was performed using infarct masking to reduce incorrect warping (Brett *et al.*, 2001). These normalisation parameters were then applied to the coregistered FMZ BP_{ND} maps and the infarct ROI (patients only). MCA templates generated from a combination of Damasio and Phan's maps (Damasio, 1983, Phan *et al.*, 2005) were used, and done previously by our group (Guadagno *et al.*, 2008). The FMZ BP_{ND} maps were proportionally scaled to the mean of the unaffected hemisphere (patients) or randomly to the right or left hemisphere in controls. To reduce the variance of the data and permit inter-subject comparison, the FMZ BP_{ND} maps were then smoothed using a 12mm kernel. An explicit grey matter mask was finally applied defined as that part of the MCA mask with grey-matter probability >0.3 in the SPM average a priori grey matter template (Carrera *et al.*, 2013). This was considered necessary because benzodiazepine receptor density, and hence FMZ $BP_{ND,}$ is very low in non-cortical areas and not accurately assessable (Guadagno *et al.*, 2008). To account for partial volume effects (PVE) of PET imaging and additional SPM-based smoothing, and thereby ensure that only non-infarcted tissue was examined to assess SNL, the final infarct ROI was dilated by 14mm for each patient (Guadagno *et al.*, 2008). This ROI was then mirrored onto the unaffected hemisphere.

SPM analysis was then applied on all voxels within this MCA grey matter mask minus dilated infarct ROI, using small-volume correction. As per Guadagno et al (Guadagno *et al.*, 2008), each individual patient was compared with the group of 12 healthy controls according to established methodology (Signorini *et al.*, 1999). The patient < controls contrast was

applied using a threshold previously determined by permutation analysis conducted on the control dataset (n=12) so that no voxel exceeding this cut-off was present in any control (Signorini *et al.*, 1999). This pragmatic approach yielded a cut-off of $p<0.1$ voxel-level FDR-corrected, which was then applied separately to each patient's affected and unaffected hemisphere, retaining for further analysis only clusters ≥ 5 voxels. The total number of significant voxels so identified on the affected and unaffected hemispheres was then determined for each patient. Although more stringent cut-offs or cut-offs derived from different methods could be applied, this would unlikely affect the statistical significance of the difference in number of computed significant voxels between the non-infarcted MCA mask and its mirror mask across patients (see methodology below).

PK: voxel-based single-subject analysis

The number of voxels with significantly increased PK BP_{ND} in the non-infarcted MCA mask was determined for each hemisphere using a voxel-wise cut-off determined from the 10 aged-matched controls. To this end, the BP_{ND} values for all voxels belonging to the left and right MCA ROIs were obtained for each control (n=10). The 99% upper confidence limit for BP_{ND} values for each of the 20 control MCA ROIs so obtained was then calculated, and the mean of these values was computed (Price *et al.*, 2006). The resulting cut-off was 0.273. As expected, applying this cut-off to the MCA ROIs from the controls showed ~1% of voxels exceeding it (mean value: 1.3%). For each patient, the voxels within the search volume (MCA mask - infarct ROI) with significantly increased BP_{ND}, i.e., lying above this cut-off, were then identified, and their total number determined on each hemisphere. To account for PVE and avoid contamination from high PK uptake within the infarct, the Infarct ROI was dilated by 9mm; the lesser dilatation applied to PK as compared to FMZ was because, unlike for FMZ, no additional smoothing (required for the SPM voxelwise analysis) was necessary for the PK data. To express inflammation, the ratio of the number of significant voxels divided by the total number of voxels within the non-infarcted ipsilateral MCA mask (or its mirror mask on the unaffected hemisphere), to be referred to as PK-ratio, was then calculated and expressed as a percentage.

Mapping SNL and MA within the Non-Infarcted Penumbra (NIP)

The NIP mask was defined as follows. First, as previously described in detail elsewhere (Guadagno *et al.*, 2008, Carrera *et al.*, 2013), the CTp dataset was resampled in the transaxial plane to the PET voxel size (i.e. 2.34 x 2.34 mm) with preservation of the acquisition z voxel dimension (~10 mm), and all BP_{ND} maps were smoothed over 9.7 mm using a box kernel in the z dimension only. Then the T1-weighted MRI was co-registered to the acute plain CT and hence to the two-slice CT perfusion scan. These procedures helped to preserve original CT perfusion spatial resolution as much as possible. The resulting matrix transformation file was subsequently applied to the individual PK and FMZ BP_{ND} maps, MCA regions for affected and unaffected hemispheres, dilated infarct ROI and mirror dilated infarct ROI. As these images had previously already been co-registered to the structural T1-weighted MR acquired at that PET scan session, the acute CTp maps, outcome MRI and FMZ BP_{ND} maps, and all ROIs were ultimately in the same space. At the end of this process, the FMZ and PK BP_{ND} maps, CTp maps, structural MR images, MCA regions and infarct ROIs were therefore in native PET space and voxel dimension in the x,y plane (2.34 x 2.34mm). The NIP mask was then defined as the Penumbra mask (see above) minus the Infarct mask, dilated by 9mm for both FMZ and PK since this time no smoothing was applied to the FMZ BP_{ND} maps; a mirror NIP mask on the unaffected hemisphere was then generated.

In addition, the non-penumbral part of the non-infarcted MCA mask (determined using dialted Infarct ROIs) was also determined by default, and mean FMZ and PK BP_{ND} values were obtained for this tissue compartment and its mirror region.

Relationship between SNL and MA

For both FMZ and PK we computed across the sample: i) the correlation between the difference in the number of significant voxels between the affected and unaffected non-infarcted MCA masks; and ii) the correlation between the difference in mean BP_{ND} between the NIP and mirror masks. In order to address the possibility of a significant relationship in the non-infarcted MCA mask being present in only some subjects but diluted across the sample, we also computed for each patient the correlation between the affected/unaffected side (A/U) ratios for PK and FMZ BP_{ND} determined in pairs of 14mm diameter circular grey matter ROIs sampling the entire affected and unaffected hemisphere (apart from the infarct ROI dilated by 14mm for both tracers), according to a previously described procedure

(Guadagno *et al.*, 2008). Corresponding within-subject analysis was not possible for the NIP given the discontinuous nature of the voxel-based defined tissue compartment.

Supplemental Results

SNL and MA in the non-infarcted MCA territory

There was no significant correlation between the difference in significant FMZ voxel numbers between the affected and unaffected hemispheres and i) age of patients (Kendall's tau, $\tau = .145$, $p = .472$); ii) time interval from stroke onset to FMZ PET (Kendall's tau, $\tau = -.144$, $p = .473$); or iii) volume of final infarct ($\tau = .123$, $p = .538$). Likewise, there was no significant relationship between the interhemispheric PK ratio difference and age, time since stroke onset or infarct volume.

Cited literature

Alawneh JA, Jones PS, Mikkelsen IK, Cho TH, Siemonsen S, Mouridsen K, et al. Infarction of 'non-core-non-penumbral' tissue after stroke: multivariate modelling of clinical impact. Brain. 2011;134(Pt 6):1765-76.

Ashburner J, Friston KJ. Unified segmentation. NeuroImage. 2005;26(3):839-51.

Brett M, Leff AP, Rorden C, Ashburner J. Spatial normalization of brain images with focal lesions using cost function masking. NeuroImage. 2001;14(2):486-500.

Carrera E, Jones PS, Morris RS, Alawneh J, Hong YT, Aigbirhio FI, et al. Is neural activation within the rescued penumbra impeded by selective neuronal loss? Brain. 2013;136(Pt 6):1816-29.

Damasio H. A computed tomographic guide to the identification of cerebral vascular territories. Archives of neurology. 1983;40(3):138-42.

Gerhard A, Schwarz J, Myers R, Wise R, Banati RB. Evolution of microglial activation in patients after ischemic stroke: a [11C](R)-PK11195 PET study. Neuroimage. 2005;24(2):591-5.

Guadagno JV, Jones PS, Aigbirhio FI, Wang D, Fryer TD, Day DJ, et al. Selective neuronal loss in rescued penumbra relates to initial hypoperfusion. Brain. 2008;131(Pt 10):2666-78.

Hughes JL, Jones PS, Beech JS, Wang D, Menon DK, Aigbirhio FI, et al. A microPET study of the regional distribution of [11C]-PK11195 binding following temporary focal cerebral ischemia in the rat. Correlation with post mortem mapping of microglia activation. Neuroimage. 2012;59(3):2007-16.

Lucignani G, Panzacchi A, Bosio L, Moresco RM, Ravasi L, Coppa I, et al. GABA A receptor abnormalities in Prader-Willi syndrome assessed with positron emission tomography and [11C]flumazenil. NeuroImage. 2004;22(1):22-8.
Murphy BD, Fox AJ, Lee DH, Sahlas DJ, Black SE, Hogan MJ, et al. White matter thresholds for ischemic penumbra and infarct core in patients with acute stroke: CT perfusion study. Radiology. 2008;247(3):818-25.
Phan TG, Donnan GA, Wright PM, Reutens DC. A digital map of middle cerebral artery infarcts associated with middle cerebral artery trunk and branch occlusion. Stroke. 2005;36(5):986-91.
Price CJ, Wang D, Menon DK, Guadagno JV, Cleij M, Fryer T, et al. Intrinsic activated microglia map to the peri-infarct zone in the subacute phase of ischemic stroke. Stroke. 2006;37(7):1749-53.
Signorini M, Paulesu E, Friston K, Perani D, Colleluori A, Lucignani G, et al. Rapid assessment of regional cerebral metabolic abnormalities in single subjects with quantitative and nonquantitative [18F]FDG PET: A clinical validation of statistical parametric mapping. NeuroImage. 1999;9(1):63-80.
Wintermark M, Flanders AE, Velthuis B, Meuli R, van Leeuwen M, Goldsher D, et al. Perfusion-CT assessment of infarct core and penumbra: receiver operating characteristic curve analysis in 130 patients suspected of acute hemispheric stroke. Stroke. 2006;37(4):979-85.
Wintermark M, Meuli R, Browaeys P, Reichhart M, Bogousslavsky J, Schnyder P, et al. Comparison of CT perfusion and angiography and MRI in selecting stroke patients for acute treatment. Neurology. 2007;68(9):694-7.