

A 3D whole-brain cellular atlas of MRI-mapped human ischemic stroke: Injury beyond the lesion defined by neuroimaging.

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Running title: Cellular Characterization of the Ischemic Penumbra

Key words: Human – Cerebrovascular disease – Stroke – Axonal regeneration – Blood-brain-barrier – Neuroimaging

Abstract

Background: Ischemic stroke is a leading cause of death and disability, yet decades of animal modelling and clinical trials have failed to deliver effective neuroprotection. A central obstacle is that even cutting-edge brain MRI cannot resolve the cellular state of tissue: what is lost, what is salvageable, and how far injury extends. Yet MRI guides patient selection, treatment windows and trial endpoints. No capability has yet mapped the adult human brain MRI to the ground truth of cellular pathology across the 3D whole organ in a scalable manner.

Methods: Case 1 had a 16-day course after middle cerebral artery occlusion. We acquired *in vivo* and post-mortem MRI, and serially sectioned the fixed brain at 20 μm (11,009 sections). Sections were stained with Nissl, H&E and silver (myelin), and by immunohistochemistry for fibrinogen (blood–brain barrier), HIF1 α (hypoxia), APP (axonal injury), GAP43 (axonal sprouting), GFAP (astrocytes), CD68 (microglia/macrophages) and CD34 (vasculature). Sections were then reconstructed in 3D and registered to the *in vivo* MRI. Case 2 (3-day course, posterior cerebral artery occlusion; 9,130 sections) provided qualitative histological corroboration. AI-based cell and pathology detection ran across the ~ 1.5 -petabyte dataset. This enabled derivation of quantitative cellular pathology measures, spatially stratified by MRI-defined lesion type, arterial territory and neuroanatomical region.

Findings: Clinical MRI underestimated the histologically defined injury, which exceeded the DWI lesion by 39%. APP-labelled axonal injury and GAP43-labelled sprouting extended through subcortical white matter beyond the infarct boundaries. They reached peri-infarct and distant tissue up to 40 mm from the core edge, revealing a potentially novel population of regenerating axons in the peri-infarct zone. Blood–brain barrier (BBB) disruption was confined to the acute infarct and was negligible in distant and contralateral tissue. HIF1 α revealed hypoxic changes in distant cortex not evident on MRI, and covert (possibly agonal) pathology was present in the contralateral hemisphere. Case 2 showed similar qualitative features.

Interpretation: This is the first whole-brain, cellular-level resolution histological reconstruction of a human ischemic stroke registered to ante-mortem clinical MRI. Cellular injury of ischemic stroke is more extensive, distributed and dynamic than advanced brain MRI indicates. Hypoxic, inflammatory and reactive/reparative processes extend well beyond the MRI-defined penumbra.

These findings pave the way to elucidation of the histological truth of target tissue pathology and potential therapeutic windows in stroke, while exposing the limits of MRI-based trial endpoints. This work also initiates a human-tissue benchmark biobank for novel molecular target identification/validation, including BBB protection/restoration, hypoxia signalling, neuroinflammation and neural tissue repair. This single-donor atlas is a proof-of-concept. The necessary next step is to build a multi-donor MRI–histology 3D whole-brain atlas database, spanning stroke subtypes, territories and time from onset. It is planned to become a large enough data corpus to pre-train multi-dimensional imaging foundation models of stroke neuropathology, as well as to advance specialized generative AI models that may infer cellular pathology from routine clinical MRI, making every scan a virtual biopsy. We call for a coordinated multi-centre and international brain donation and cellular imaging programme aiming to accelerate the growth of this next-generation of reverse translational discovery platforms, to enable drug development targeting novel and pathologically-validated molecular pathways in stroke, with far-reaching applications to other neurological disorders where the biology remains incompletely understood.

Funding

Fairfax India Charitable Foundation

Data Availability

An annotated subset of 1,139 brain MRI-registered histological sections is openly available for viewing at C-STROKE (c-stroke.humanbrain.in). Data will soon be made queryable and downloadable.

Introduction

Our brain commandeers as much as one-fifth of our heart's output of blood with every heartbeat, despite representing only about one-fiftieth of our body mass. Any interruption to this delivery of blood, and the oxygen and nutrients it carries, can result in ischemic stroke – a leading cause of disability and death around the world (1).

Despite decades of basic scientific as well as clinical research on ischemic infarcts, and on emphasis on preventive measures for those at risk, meaningful therapies are limited and require immediate implementation to be effective even partially (2). For example, the threading of catheters into the occluded brain artery in ischemic stroke, whether to administer tissue plasminogen activator or to mechanically retrieve a clot, must be done within a short time of the onset of symptoms and still does not guarantee success in preserving life or brain function (2). Moreover, this technique is only available at certain tertiary care centres. Immediate surgery to evacuate any accumulated blood in the event of reperfusion hemorrhage of an infarct is likewise of limited availability. Otherwise, treatment consists mainly of “supportive” measures, often necessitating acute intensive care such as mechanical ventilation, sedation, and complex medical management, followed by rehabilitation in those surviving the early stages (2,3).

Predicting a given patient's recovery following ischemic stroke is notoriously challenging for several reasons: a) whether the stroke has had secondary reperfusion hemorrhage (“hemorrhagic conversion”); b) the specific functional region(s) of the brain affected and the extent of damage; c) the degree of swelling that occurs in the surrounding brain tissue, which is in part related to factors 1 and 2 as well as to the rapidity with which medical care can be administered; and d) the overall health of the individual experiencing the event (4). If the interruption of blood flow lasts

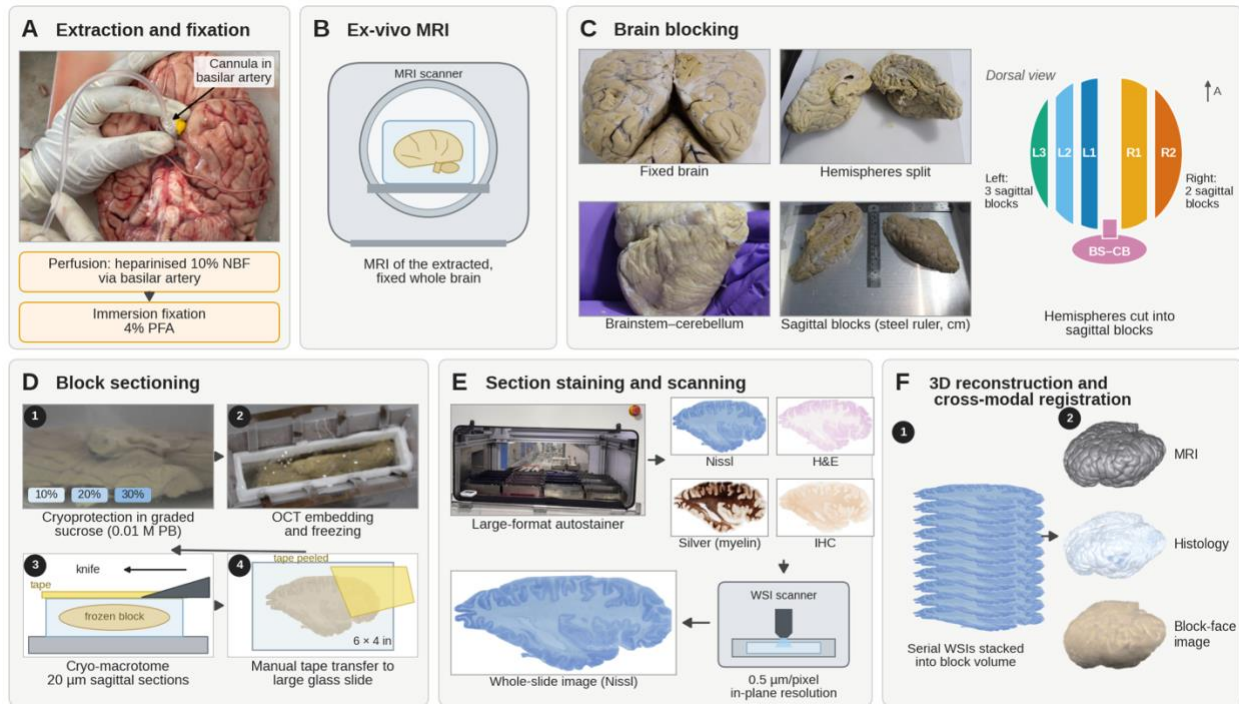
for more than a few minutes, irreversible injury to brain tissue ensues in the central zone called the “core”. As far as we know, this core has little or no capability to recover or regenerate and will later become a scar or cavity in a surviving individual. The area surrounding the core, however, termed the “penumbra”, is postulated to have a lesser degree of oxygen deprivation and is potentially recoverable. Currently the penumbra is defined by neuroimaging features in living patients, whether they go on to die or survive (5).

The penumbra is of great interest to researchers and clinicians attending patients with ischemic stroke, since minimization of any damage to this region is considered a key determinant of their eventual recovery. Despite characterization of the core and penumbra in the brains of experimental animal models of ischemic stroke (6), much about the penumbra has not been adequately studied *directly* in postmortem human brain. The consensus in the field is that progress in optimizing the outcome after ischemic stroke requires understanding the cellular and molecular changes in the penumbra (7).

Our recently developed multimodal quantitative cellular pathology technology was used to characterize the ischemic stroke penumbra in 2 cases with acute and evolving infarcts. We used both established and novel methods of registration of antemortem and postmortem imaging followed by comprehensive tissue analysis of specific markers of acute oxygen deprivation and blood-brain barrier disruption, cellular responses to ischemia and tissue damage, and axonal injury and regeneration across the core region of stroke as well as its penumbra, and more distant regions. This 3-dimensional whole-organ reconstruction of cellular-level histology is foundational to larger studies of human brain ischemia, which require bridging of *in vivo* to *ex vivo* MRI and neuropathology. This approach pioneers the emergence of a scalable reverse

translational platform for pathological discovery to advance transformative therapeutic strategies for stroke neuroprotection and regeneration.

Materials and methods



Supplemental Figure 1. Workflow from brain extraction to whole-brain 3D multimodal histology. (A) After extraction, the brain was perfused with heparin with 10% neutral-buffered formalin (NBF) through a cannula placed in the basilar artery, then immersion-fixed in 4% paraformaldehyde (PFA). (B) The fixed whole brain was imaged by *ex vivo* 3T MRI before blocking, as a 3D reference for registration. (C) The brainstem–cerebellum (BS–CB) was separated as a single block and the cerebral hemispheres were divided at the midline. The left hemisphere was cut into three sagittal blocks (L1–L3) and the right into two (R1–R2). (D) Blocks were cryoprotected in graded sucrose (10%, 20%, 30%) in 0.01 M phosphate-buffered saline (PBS), embedded in optimal cutting temperature (OCT) compound and frozen, and cut into 20 µm sagittal sections on a large-format cryo-macrotome. Each section was manually tape-transferred onto a glass slide. (E) Sections were stained serially with Nissl, H&E, silver for myelin, and immunohistochemistry (IHC). Slides were digitised on a whole-slide scanner at 0.5 µm/pixel in-plane resolution. (F) Serial whole-slide images (WSIs) were stacked to reconstruct each block as a 3D histology volume. Block volumes were registered to the *ex vivo* MRI and

combined into a whole-brain 3D histology reconstruction, co-registered with MRI and block-face image volumes.

Our multimodal approach has been described previously in the developing human brain and is schematized in Supplemental Figure 1 (8). Here, we tailored it to accommodate the adult brain, as well as to optimize our analysis of the ischemic penumbra, as originally defined by neuroimaging parameters, in 2 cases with ischemic onset between 3 and 16 days prior to death, respectively. We present first the more subacute case, as it had penumbral characteristics on *in vivo* MRI. We then applied what we learned to the acute case without *in vivo* MRI, to extrapolate possible penumbral features.

Clinical case details

Case 1 was a 69-year-old man with poorly controlled diabetes mellitus and ischemic infarcts. Briefly, at 31 days prior to death he had acute COVID-19 infection, and developed sinonasal inflammation, left visual loss and ptosis, requiring antibiotics, antifungals and steroid therapy. Clinical neuroimaging at 16 days prior to death demonstrated foci of restricted diffusion in the left frontal lobe and in the parieto-occipital lobe (distal branches of left middle cerebral artery territory). Angiogram at 11 days prior to death disclosed filling defects of the left A1 and M2 cerebral artery segments, with cross-filling of the left A2 via the anterior communicator, and focal anastomotic filling of some distal left MCA branches. Repeat MRI at 11 days prior to death highlighted evolution of ischemic changes to a larger infarct in the parieto-occipital lobe and a more limited infarct in the left frontal lobe, with features suggestive of border-zone ischemia. Tissue changes in the infarct cores as well as in the peri-infarct regions (i.e., the penumbra), were annotated over this time interval.

Case 2 was a 74-year-old woman with type 2 diabetes mellitus and chronic obstructive pulmonary disease, who presented with speech difficulty, followed by stupor, with motor response to pain only. Head CT showed diffuse confluent hypodensity of the bilateral frontoparietal and periventricular white matter, consistent with hypertensive small vessel changes, as well as moderate diffuse atrophy. Her family declined further care, including MRI, and she died the following day, 3 days after symptom onset.

Postmortem specimen accrual, handling, and feature annotation

The postmortem examinations were conducted at CMC, Vellore, India, with due consent from each family for specialized analysis of the brain tissue at Sudha Gopalakrishnan Brain Centre at Indian Institute of Technology Madras. The interval from death to internal carotid and basilar artery perfusion with heparinized saline, followed by 20% neutral buffered formalin, and subsequent immersion fixation of the brain in 10% formalin, was 14 hours for Case 1 and 6 hours for Case 2 (9).

Following fixation, *ex vivo* imaging of both specimens was performed as previously described in the developing human brain (8). The cerebellum and brainstem were separated from the hemispheres at the midbrain level, and the hemispheres were divided into sagittal slabs, with notation of any macroscopic areas of softening, focal hemorrhage or other changes. After cryoprotection and embedding, the brain slabs were serially sectioned sagittally at 20 μ m thickness, as described previously. The total number of sections was 11,009 for Case 1 and 9,130

for Case 2. Histochemical and immunohistochemical staining was performed on sections at regular intervals (Table 1).

Table 1. Immunohistochemical stains and rationale for use

Phenomenon	Immunohisto-chemical stain	Target (source, type and catalog #)
Hypoxia	HIF1 α	Hypoxia-inducible factor alpha (hypoxic cells of all types) (ThermoFisher Scientific, mouse monoclonal, MA1-16504)
Vascular integrity	Fibrinogen	Leakage of serum protein fibrinogen across blood-brain barrier (Abcam, mouse monoclonal, Ab58207)
Inflammatory response	CD68	Lysosomal protein in macrophages/microglia (ThermoFisher Scientific, mouse monoclonal, 14-0688-82)
Astrogliosis	GFAP	Glial fibrillary acidic protein (astrocytes) (Cell Signaling Technology, rabbit monoclonal, 80788)
Axonal injury	APP	Amyloid precursor protein trafficked in axons (ThermoFisher Scientific, mouse monoclonal, 14-9749-82)
Axonal regeneration	GAP43	Growth-associated protein 43 expressed in growth cones (Proteintech, rabbit polyclonal, 16971-1-AP)
Vasculature	CD34	Endothelial cells of vessels (Abcam, rabbit monoclonal, EP373Y)

All antibodies were used at a dilution of 1:1000. The color-coding allows correlation with the timeline graphs presented in Figure 8.

Digital image analysis and 3D registration

MRI acquisition, pre-processing, histology reconstruction, multi-modal registration and histology feature detection methods are elaborated in Supplementary Material 1.

Results

Given that ischemia is a process, a particular subject may have both established irreversible tissue necrosis as well as ongoing compromise. Here we attempt to discern subacute or organizing phases (in Case 1), and acute changes (in Cases 1 and 2), using our panel of standard histology and focused immunohistochemistry. Overlap of findings, both spatially and temporally, is to be expected, and is an advantage of our multimodal platform.

Determination of core and penumbra (peri-infarct zones) by co-registration of imaging and histology

Because the *in vivo* MRI in Case 1 allowed definition of core and penumbra, and even some temporospatial evolution between the 2 imaging time points prior to death, we focused our efforts initially on using the findings from this case to then extrapolate the defining features in the acute ischemic and peri-ischemic zones in Case 2, for which *in vivo* imaging was too early and of insufficient resolution.

Standard histologic stains

In Case 1, the ischemic core, as defined in the parieto-occipital lobe by *in vivo* MR imaging 16 days prior to death consists, on H&E, Nissl, and myelin stain (silver impregnation), of established regions of necrosis of brain tissue elements (neurons, glia, axons, blood vessels), with “mummification” and patchy beginning fragmentation and cavitation (Figure 1). In the additional areas in the posterior frontal lobe best seen in the second *in vivo* neuroimaging set (11 days prior to death), the cores show an irregular topographic distribution, suggesting coalescence of smaller into larger, more “geographic” zones of ischemic necrosis.

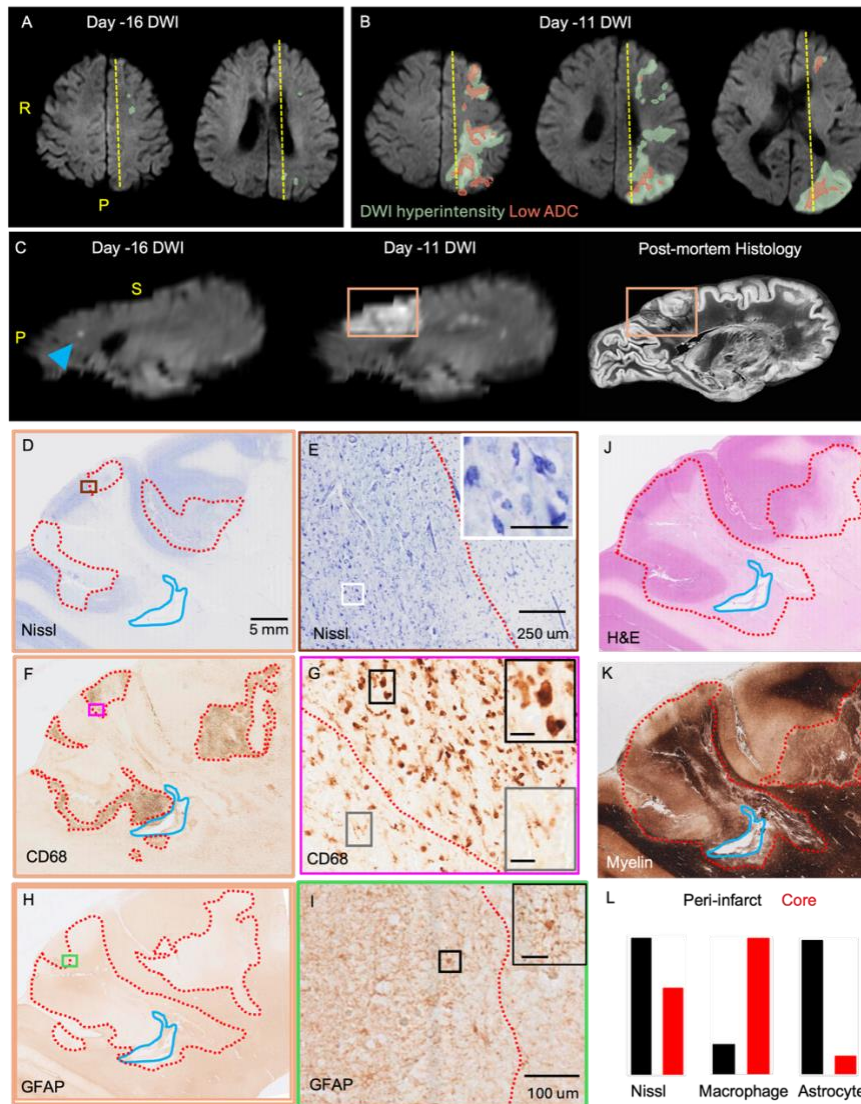


Figure 1. Imaging and cellular correlates of subacute ischemic stroke (Case 1). A) Axial diffusion-weighted imaging (DWI) at 16 days before death (day -16) reveals subacute focal ischemic injury in the left hemisphere white matter tracts. Dotted lines, registered plane of histologic images. B) At 11 days before death (day -11), DWI shows an enlarged ischemic lesion involving the left middle cerebral artery territory, including the frontal, parietal, and occipital cortices, with low apparent diffusion coefficient values marking ischemic core regions. Dotted line, plane of histologic images. C) Focal lesion at day -16 (blue arrowhead) evolves to larger infarct at day -11 (orange box). D-L) Cellular-resolution analysis of histological sections for characterization of the MRI-defined ischemic regions within the orange boxed area in panel C (all sections in this figure are within 840 μ m sagittal range). D) On Nissl stain, the region corresponding to the focal lesion identified on day -16 MRI is a cavitated zone (blue outline), whereas the regions newly lesional at day -11 on MRI shows markedly reduced Nissl staining (red dashed outline). Scale 5 mm, also applies to panel F, H, J and K. E) Higher magnification of the boxed region in D shows loss of Nissl-positive cells within the core (right) and ischemic neurons in the peri-infarct region (left). Scale 250 μ m, also applies to panel G; inset scale 50 μ m, also

applies to insets in G and I. F) In CD68-immunostained sections, the infarct core corresponds to a region of dense macrophage infiltration. G) The magnified region shows abundant macrophages in the core and reactive microglia in the peri-infarct zone (scale as in E, inset scale as in E inset). H) In GFAP-immunostained sections, the ischemic core is characterized by loss of astrocytes. I) The magnified region shows astrocyte depletion in the core and reactive astrocytes with swollen somata and thickened, branched processes in the peri-infarct region (scale as in E, inset scale as in E inset). J) On H&E-staining, changes are less pronounced, but the ischemic region appears as pale staining bordered by rarefied and vacuolated rims (scale as D). Lesional region from day -11 overlaid with red dashed lines. K) On Gallyas myelin staining sections, alterations in both gray and white matter are visible. L) Quantitative analysis using stain-specific cell-detection models confirm these regional differences, demonstrating reduced Nissl-positive cells (normalized to peri-infarct), increased macrophage density (normalized to core), and loss of astrocytes (normalized to peri-infarct) in the infarct core relative to the peri-infarct zone (1 cm tissue region surrounding the core).

In Case 2, and focally in Case 1, acute neuronal ischemia appeared as irregular lines of vacuolization bounding classical shrunken neurons lacking fine cytologic detail. In Case 2, these changes were conspicuous in the temporo-occipital cortical ribbon and hippocampus (posterior cerebral artery territory) (Figure 2). On H&E, Nissl, and myelin silver impregnation, these acute ischemic zones are not easy to distinguish clearly from the peri-infarct zones at this early time-point.

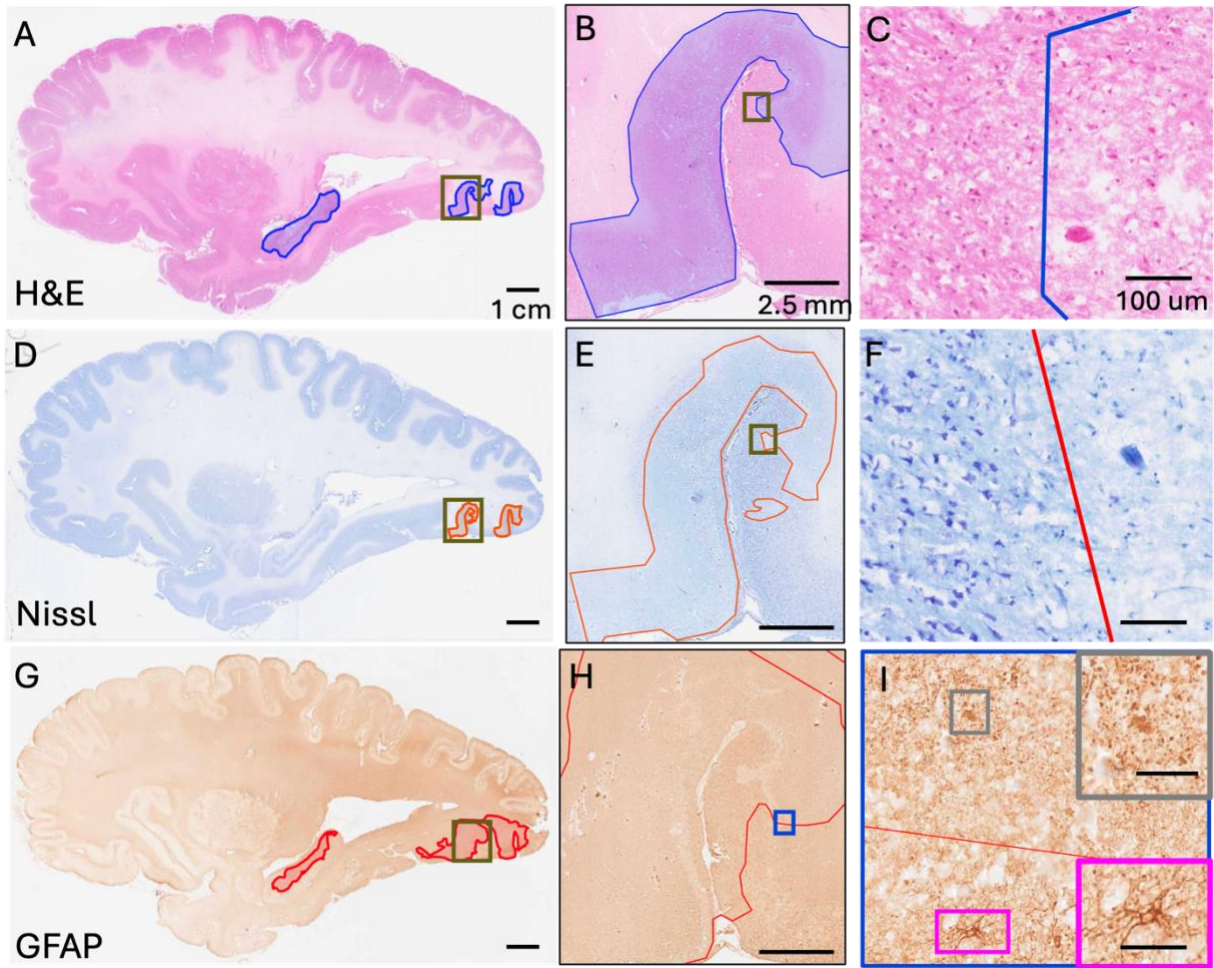


Figure 2. Cellular signatures of acute ischemic injury precede lesions on neuroimaging in acute ischemic stroke case (Case 2). (A–C) Histological characterization of acute ischemic injury from a second case in the study, representing an acute stroke patient who died three days after symptom onset, despite computed tomography (CT) showing no signal changes of acute ischemia two days post-onset. A) H&E-stained sagittal section from left hemisphere shows distinct regions of acute ischemic injury within the left hippocampus and temporo-occipital cortices (delineated by blue outlines). Scale 1 cm, also applies to D and G. B) Higher magnification of the temporo-occipital cortical lesion corresponding to the black boxed area in A details structural boundaries of the ischemic core. Scale 2.5 mm, also applies to E and H. C) High-power magnification of the black boxed area in B demonstrates increased tissue vacuolation and rarefaction within the ischemic core (right side of blue boundary line) relative to the adjacent peri-infarct region (left side of boundary line). Scale 100 μ m, also applies to F and I. (D–F) Analysis of neighboring Nissl-stained sections maps cellular density. D) Low-power overview demonstrates corresponding regions of pale Nissl staining and structural cell loss (delineated by orange outlines). E) Magnified view of the black boxed region in D illustrates the microstructural boundaries of a zone marked by Nissl pallor. F) High-magnification architecture of the black boxed region in E shows marked gray matter cell depletion inside the ischemic core (right side of red boundary line) compared to the peri-infarct zone (left side of boundary line).

(G–I) Evaluation of astrocytic pathology using GFAP immunohistochemistry. G) Low-power overview maps regional astrocytic changes, with the region of prominent astrocyte loss delineated by a red outline. H) Medium-power magnification of the black boxed region in G bridges the core boundary. I) High-power resolution of the regional boundary zone highlighted by the blue box in H. Within the ischemic core (above the red boundary line), clasmatodendritic astrocytes display fragmented, punctate processes (red box, top inset; inset scale bar 50 μ m). Conversely, within the neighboring peri-infarct region (below the red boundary line), reactive astrocytes exhibiting hypertrophic processes and swollen cell bodies predominate (black box, bottom inset; inset scale bar 50 μ m).

Delineation of cellular inflammatory responses

Macrophages, highlighted by CD68 immunostain, are abundant in the necrotic cores in both the frontal and parieto-occipital infarcts in Case 1 (Figure 1 F, G; Figure 3). Reactive-appearing microglia, with fine cell processes, surround the necrotic cores and are scattered in the more-distant peri-infarct zones. Cells bounding the necrotic zone comprised hypertrophic astrocytes with abundant perikaryal and process staining for GFAP. Similar cells are seen, although in a more scattered and less concentrated distribution, in microscopic fields progressively further from the core, and into “normal-appearing” brain parenchyma (as noted on H&E or Nissl); semi-automated quantification of GFAP-immunoreactive cells reflected these qualitative findings (Figure 4).

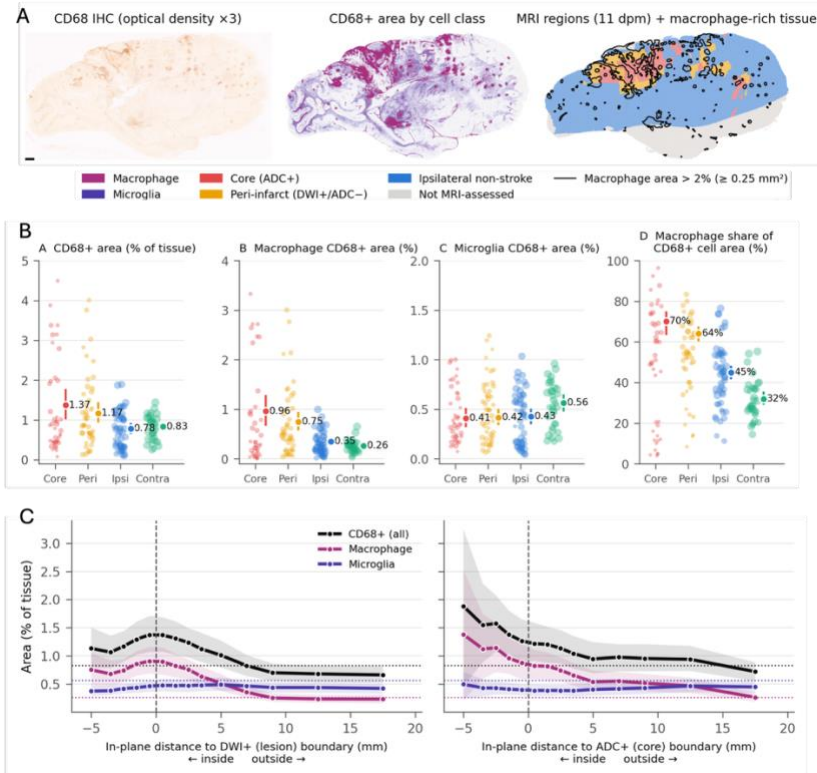


Figure 3. CD68+ microglia and macrophages relative to MRI-defined lesion compartments.

(A) Left: representative section with CD68 immunohistochemistry from the stroke affected hemisphere. Middle: CD68+ area classified as macrophage (plum) or microglia (violet); colour intensity is the local CD68+ area fraction. Right: compartments from the 11-dpm *in vivo* MRI projected onto the section. Red, ischemic core (ADC+); amber, peri-infarct (DWI+/ADC-); blue, ipsilateral non-stroke (DWI-/ADC-); grey, tissue outside the MRI-assessed volume. Black outlines mark macrophage-rich tissue. Scale bars, 5 mm. (B) Total CD68+ area, CD68+ area classified as macrophage, CD68+ area classified as microglia, and macrophage share of classified CD68+ area in the MRI-defined compartments. Large symbols with bars: pooled estimate with 95% CI (block-stratified section bootstrap). Small symbols: individual sections, sized by compartment tissue area. Core, ischemic core; Peri, peri-infarct; Ipsi, ipsilateral non-stroke; Contra, contralateral. (C) CD68+ area (% of tissue) in 1–5 mm bins of in-plane signed distance to the DWI+ lesion boundary (left) and the ADC+ core boundary (right); negative values lie inside the lesion. Lines, pooled estimates. Shading, 95% CI (section bootstrap). Dotted lines, contralateral levels. Ipsilateral MRI-assessed tissue only.

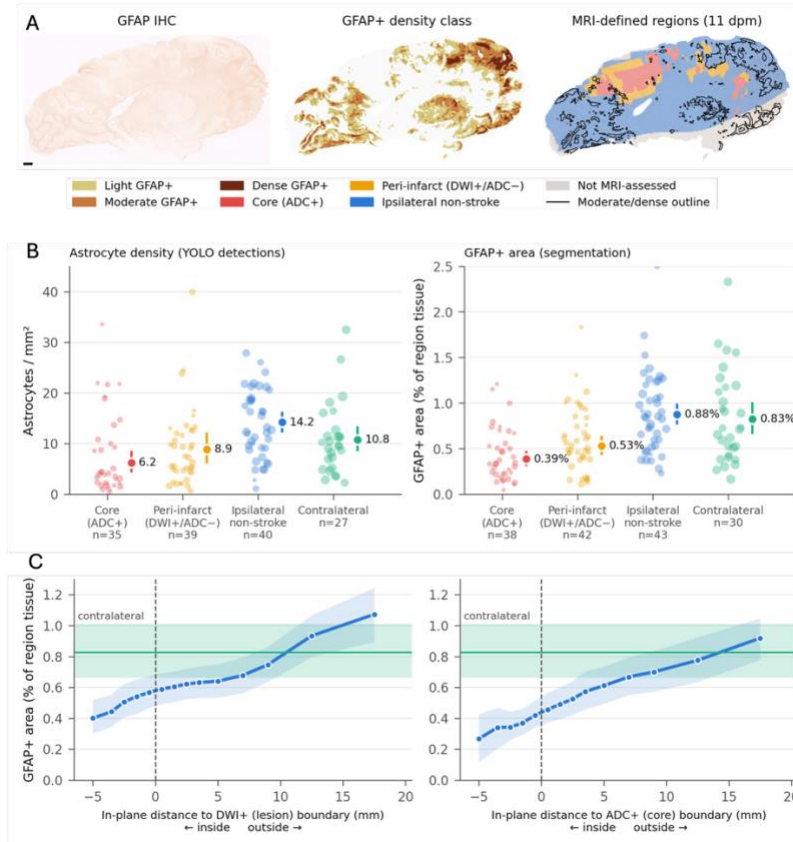


Figure 4. GFAP immunoreactivity in MRI-defined lesion compartments in representative sections. (A) Left: Representative section from stroke-affected hemisphere with GFAP immunohistochemistry. Middle: GFAP+ density classes (light, moderate, dense). Right: compartments from the 11-dpm in-vivo MRI projected onto the section. Red, ischemic core (ADC+); amber, peri-infarct (DWI+/ADC-); blue, ipsilateral non-stroke (DWI-/ADC-); grey, tissue outside the MRI-assessed volume. Black outlines show moderate and dense GFAP+ tissue. Scale bars, 5 mm. (B) Left: astrocyte density (detections per mm² of compartment tissue), Right: GFAP+ area (% of compartment tissue) in MRI-defined lesion compartments. Large symbols with bars: pooled estimate with 95% CI (block-stratified section bootstrap). Small symbols: individual sections, sized by compartment tissue area. n, sections contributing ≥ 5 mm² of tissue to the compartment. (C) GFAP+ area in 1–5 mm bins of in-plane signed distance to the DWI+ lesion boundary (left) and the ADC+ core boundary (right); negative values lie inside the lesion. Line, pooled estimate. Shading, 95% CI (section bootstrap). Green line and band, contralateral estimate with 95% CI. Ipsilateral MRI-assessed tissue only.

Detection of blood-brain barrier compromise and acute cellular hypoxia

In both cases, fibrinogen has leaked into the acutely ischemic zones, and persists in the necrotic regions, indicating acute and persistent loss of blood-brain-barrier function; staining is negligible outside the ischemic areas (Figure 5).

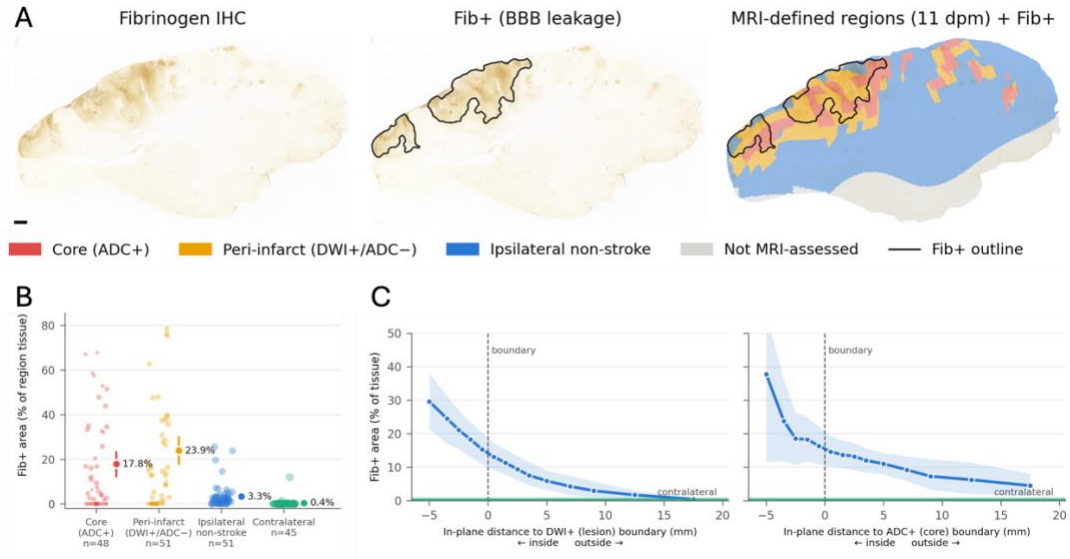


Figure 5. Fibrinogen extravasation in MRI-defined lesion compartments. (A) Left: Fibrinogen immunohistochemistry in a representative section from the stroke-affected left hemisphere. Middle: delineated Fib+ regions. Right: compartments from the 11-dpm in-vivo MRI projected onto the section. Red, ischemic core (ADC+); amber, peri-infarct (DWI+/ADC-); blue, ipsilateral non-stroke (DWI-/ADC-); grey, tissue outside the MRI-assessed volume. Black outlines show Fib+ regions. Scale bars, 5 mm. (B) Fib+ area as a percentage of compartment tissue. Large symbols with bars: pooled estimate with 95% CI (block-stratified section bootstrap). Small symbols: individual sections, sized by compartment tissue area. n, sections contributing $\geq 5 \text{ mm}^2$ of tissue to the compartment. (C) Fib+ area (% of tissue) in 1–5 mm bins of in-plane signed distance to the DWI+ lesion boundary (left) and the ADC+ core boundary (right); negative values lie inside the lesion. Line, pooled estimate. Shading, 95% CI (section bootstrap). Green line and band, contralateral estimate with 95% CI. Ipsilateral MRI-assessed tissue only. Open-ended bins ($> 6 \text{ mm}$ inside, $> 20 \text{ mm}$ outside) are omitted.

Of note, HIF1 α immunoreactivity is essentially nil within the necrotic cores in Case 1, but is widely expressed in numerous cells, including neurons, glia, and endothelium, at some distance from the ischemia (Figure 6). Similarly, in Case 2, although we do not have well-defined cores as in Case 1, the extent of labelling with HIF1 α is far greater than anticipated based on the classical histologic phenotype of acutely ischemic neurons, i.e., pyknosis, hypereosinophilia, and vacuolated background neuropil on H&E and Nissl.

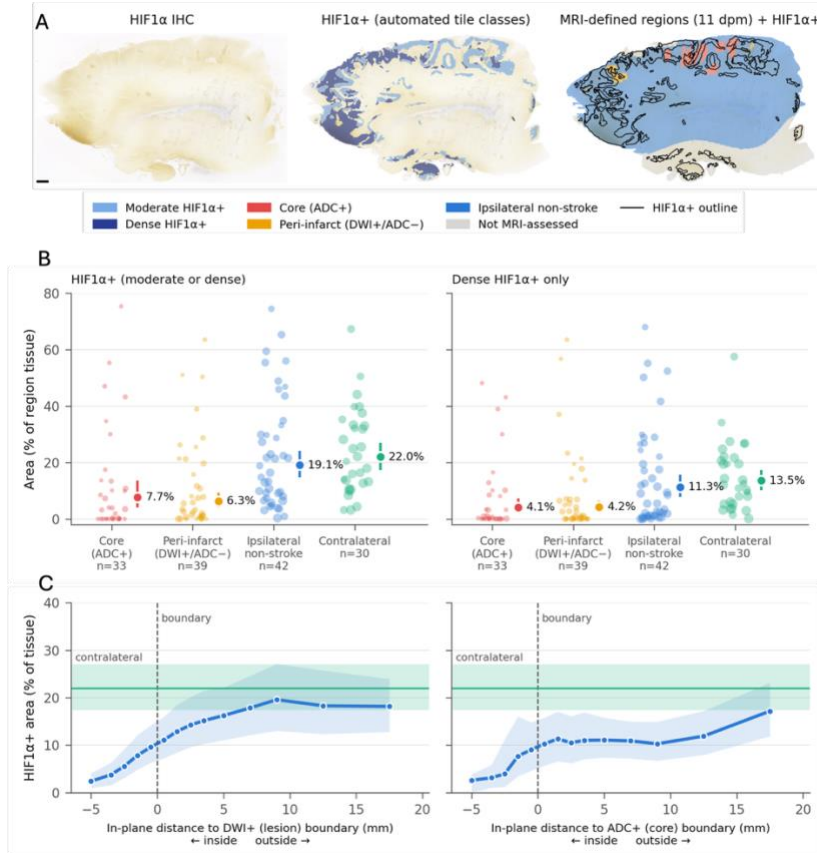


Figure 6. HIF1 α immunoreactivity in MRI-defined lesion compartments. (A) Left: Representative section from stroke-affected hemisphere with HIF1 α immunohistochemistry. Middle: automated HIF1 α + classes (light blue, moderate; dark blue, dense). Right: compartments from the 11-dpm *in vivo* MRI projected onto the section. Red, ischemic core (ADC+); amber, peri-infarct (DWI+/ADC-); blue, ipsilateral non-stroke (DWI-/ADC-); grey, tissue outside the MRI-assessed volume. Black outlines show HIF1 α + tissue. Scale bars, 5 mm. (B) HIF1 α + area as a percentage of compartment tissue, for moderate-or-dense HIF1 α + (left) and dense HIF1 α + only (right). Large symbols with bars: pooled estimate with 95% CI (block-stratified section bootstrap). Small symbols: individual sections, sized by compartment tissue area. n, sections contributing ≥ 5 mm² of tissue to the compartment. (C) HIF1 α + area (% of tissue) in 1–5 mm bins of in-plane signed distance to the DWI+ lesion boundary (left) and the ADC+ core boundary (right); negative values lie inside the lesion. Line, pooled estimate. Shading, 95% CI (section bootstrap). Green line and band, contralateral estimate with 95% CI. Ipsilateral MRI-assessed tissue only.

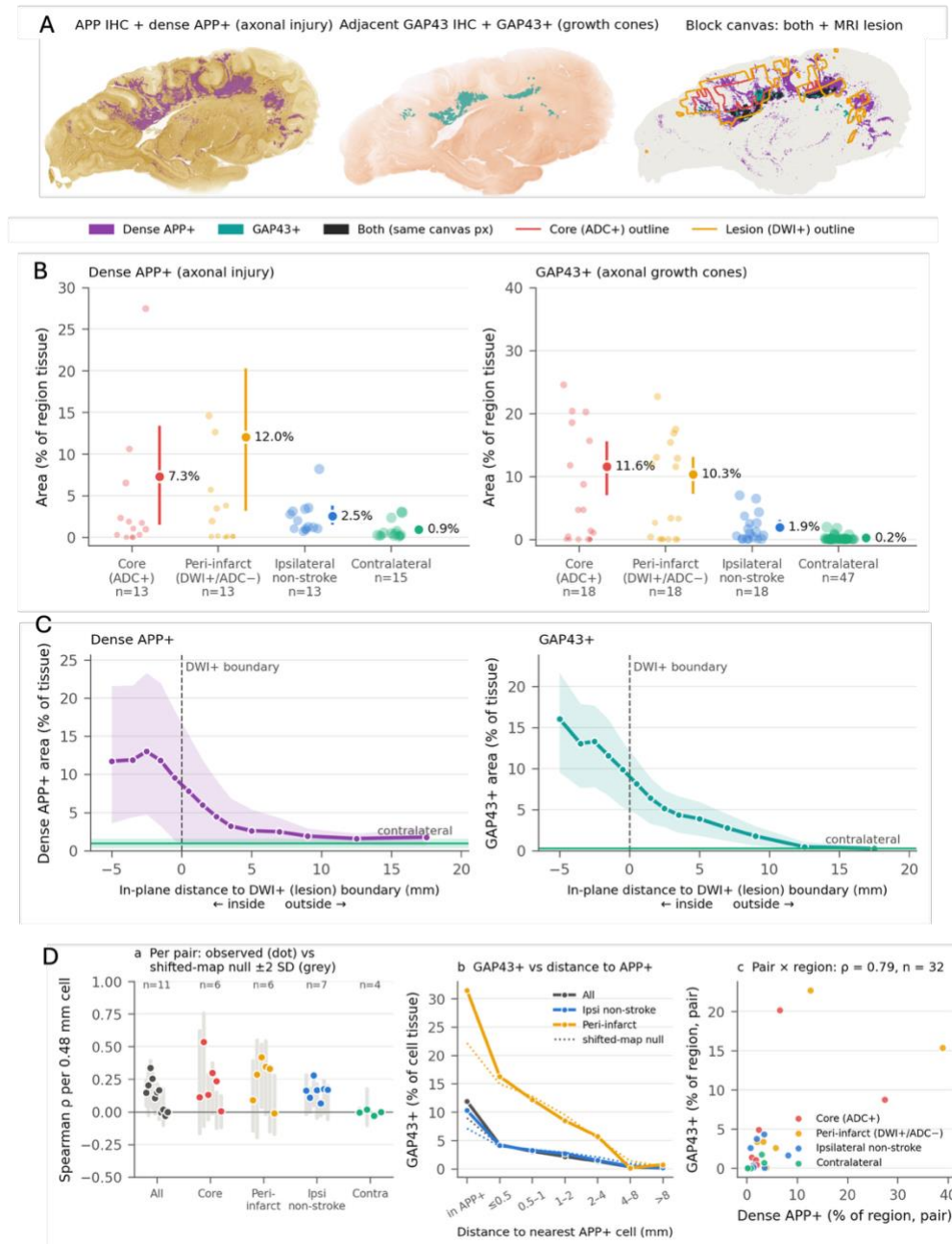


Figure 7. Axonal injury and regrowth. (A) Representative section from stroke-affected hemisphere with APP (left) and GAP43 (middle) immunohistochemistry. Right: both sections placed in the common block space, with the 11-dpm lesion outlines. Red, ischemic core (ADC+); amber, DWI+ lesion. Dark grey marks pixels positive in both sections. (B) APP+ (left) and GAP43+ (right) area as a percentage of compartment tissue. Large symbols with bars: pooled estimate with 95% CI (block-stratified section bootstrap). Small symbols: individual sections, sized by compartment tissue area. n, sections contributing ≥ 5 mm² of tissue to the compartment. (C) APP+ (left) and GAP43+ (right) area (% of tissue) in 1–5 mm bins of in-plane signed distance to the DWI+ lesion boundary; negative values lie inside the lesion. Line, pooled estimate. Shading, 95% CI (section bootstrap). Green line and band, contralateral estimate with

95% CI. Ipsilateral MRI-assessed tissue only. (D) Left: Spearman correlation between APP⁺ and GAP43⁺ fractions across 0.48 mm cells (dots) for adjacent APP–GAP43 section pairs ≤ 0.5 mm apart. Grey bars show the mean ± 2 SD of the null from 200 random 2–8 mm shifts of the GAP43 map. Middle: GAP43⁺ burden (% of cell tissue) by distance to the nearest APP⁺ cell. Solid lines, observed; dotted lines, shifted-map null. Right: Pooled dense-APP⁺ against GAP43⁺ burden per pair and compartment.

Identification of axonal injury and regeneration

Amyloid precursor protein immunohistochemistry marks numerous swollen axons, visible as bulbs, thick cords, and beaded profiles, in large geographic zones within and along the irregular margins of the ischemic zones in both cases, indicating more widespread axonal injury than suggested on standard H&E and Nissl stains (Figure 7). APP-immunoreactive bundled axons are also noted in the internal capsule, putaminal pencils, and basal forebrain/anterior commissure in Case 1. Distant from the cores and peri-infarct zones are rare, scattered APP-immunoreactive profiles, of uncertain significance.

Evidence of axonal growth cones by GAP43 immunohistochemistry is widespread in many of the same regions highlighted by APP immunostain. Scattered single GAP43-immunoreactive profiles are present within the necrotic cores, while at the margins of the infarcts, they are more densely aggregated. Rare GAP43-immunoreactivity is also seen distant from the cores and peri-infarct regions.

Discussion

In this study, we applied, for the first time to the adult human brain, a novel methodology successfully established to characterize the 3D whole-organ histology in the developing human nervous system (8–15). This work demonstrates several important hallmarks: 1) the ability to “scale up” a sophisticated multi-dimensional tissue and image processing platform computing 3D reconstructed atlases of the postmortem human brain from fetal to adult volumes; 2) the application of this platform to investigate quantitative cellular pathology in human neurologic diseases directly from whole-brain tissue samples; and 3) a first-in-kind mapping that bridges structural MRI and the ground truth of the underlying cellular-level neuropathology, which may allow spatially-defined translational discovery of cell-specific molecular pathways, thereby enabling the identification and/or validation of pathology-proven targets for neuroprotection drug development.

The index cases processed here provide a means of addressing the biologic underpinnings of an especially pervasive cause of global morbidity and mortality, that of cerebral ischemic injury or stroke, which has been persistently refractory to many experimental and empiric therapeutic advances (16). This work contributes to increase our understanding of the nature and extent of cellular changes in the peri-infarct regions of penumbra as well as more distant tissue areas directly from a deep histopathological characterization of the 3D whole-brain in two distinct cases of ischemic stroke.

Key findings contributed by this study

Precise 3D spatial localization of cellular changes

While the millimetric voxel-wise resolution of MR imaging, whether *in vivo* and *ex vivo*, allows distinction of signal intensity changes across acquisition modalities in differentially affected brain regions, the whole-mount 3D reconstructed histological sections represent a leap in resolution by multiple orders of magnitude. This work unravels the micrometric cellular neuropathology of ischemic stroke, including reactive glial and axonal changes in the peri-infarct zones and beyond, that cannot be revealed by the most advanced MR imaging techniques. Prior histologic studies of human brain infarcts have relied for the most part upon standard ~1x3cm histologic sections, which can in no way reflect the complete tissue pathology we can document using our whole-brain histology technique, further stacked digitally in 3 dimensions and incorporating immunohistochemical mapping for cell-specific biomarkers of interest. This whole-organ resolution of quantitative cellular neuropathology correlated with brain MRI appears to be unprecedented.

Deep phenotyping of the extent and character of cellular responses to brain ischemia

A projected time-course of reactive cellular events including macrophage, microglia, and astrocyte responses in the ischemic core and peri-infarct penumbra regions is outlined diagrammatically in Figure 8.

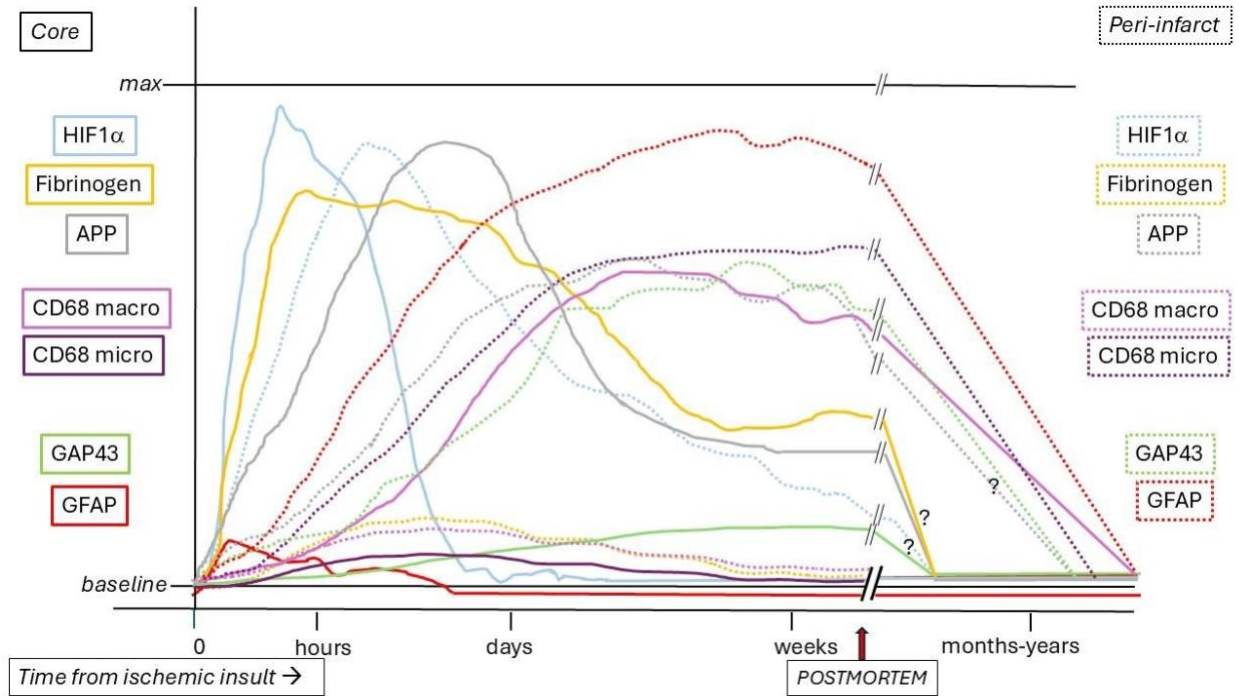


Figure 8. Hypothetical timeline of quantified reactive cellular events following ischemic stroke. Qualitative protein expression highlighted by immunostains (color-coded as listed in Table 1) in the core (solid lines) and peri-infarct regions (dotted lines) (as outlined by H&E and Nissl stains and mapped onto whole-brain sections). Curves between time 0 and postmortem are estimated on the basis of established neuropathologic features as well as direct observation of Case 1 (subacute case). CD68 macro=macrophages; CD68 micro=microglia; baseline=expression in absence of pathology; max=maximum density of immunopositive cells.

Widespread evidence of cellular hypoxia despite relatively localized blood-brain barrier disruption

Whole-brain regions with acute hypoxia as outlined by HIF1 α expression are far more prevalent than recognized on either MRI or even standard (H&E, Nissl) histology, suggesting a larger volume of potentially reversible/salvageable tissue injury available for potential therapeutic modification of outcome in peri-infarct as well as otherwise “normal-appearing” brain tissue. Not only neurons are affected by ischemic reactions, as glial and vascular wall cells also showed HIF1 α expression in peri-infarct and distant areas. Given that HIF1 α is rapidly broken down upon return to normoxic conditions, and that, if high levels persist, they lead to cell death, the

changes detected postmortem in peri-infarct and more distant areas may represent agonal phenomena (17). In fact, low levels of hypoxia may stimulate regenerative (protective) responses. The temporal pattern of HIF1 α expression post stroke onset remains complex to interpret retrospectively from the postmortem timepoint, since fluctuations in blood oxygenation may be occurring in intensive care. This further complicates extrapolation across the clinical time course in our two index cases, as many recurrent “waves” of non-lethal hypoxia very likely occurred. The hypothetical timeline and relative extent of hypoxia as well as BBB disruption, as determined from HIF1 α and fibrinogen immunoreactivity, respectively, is synthesized from prior human neuropathologic studies of brain ischemia (18,19) combined with observed data from this study, and is presented in schematic form in Figure 8.

3D spatial quantification of the interplay between axonal injury and regeneration within and outside of the core infarct region

This work provides a comprehensive 3D representation of axonal injury (by APP immunohistochemistry) generally overlapping with axonal regeneration (by GAP43 immunohistochemistry), even in regions considered acute/recent on standard histology – another window of opportunity for supporting tissue salvageability in peri-infarct and “normal appearing” zones. We are unable to discern whether those axons expressing APP (i.e., disrupted axonal transport along the axis cylinder) are the same, a subset of, or entirely different from those expressing GAP43 in (regenerating) growth cones. Nevertheless, we clearly observed that these axonal events are co-occurring in the same regions at the same time, a phenomenon never demonstrated in the human brain, to the best of our knowledge.

Our GAP43 findings in brain tissue are of interest in the perspective of the clinical detection of GAP43 in the cerebrospinal fluid (CSF) of stroke patients. In one study, CSF was collected longitudinally from 28 individuals with stroke and 19 controls, and was found to be elevated in the former at day 7-9 and up to 3 weeks after stroke, with return to baseline levels at 3-5 months (20). Levels did correlate with infarct severity and size. While we do not have CSF measures of GAP43 in our cases, the quantitative tissue expression patterns observed in this study support this proposed timeframe and further suggest that GAP43 in the CSF may also originate from peri-infarct penumbra and even more distant regions. Figure 8 schematizes the timelines of these processes insofar as is known from the literature and from the present work.

Finally, we acknowledge that heterogeneity of distribution in tissue injury and reactive responses in brain ischemia and hypoxia, including changes in areas distant from the focal core lesion, is not a new concept, and has been suggested by Koukalova et al. in 2024, in their review of diverse human and animal works (7). However, we demonstrate and further characterize this important observation for the first time directly from the whole human brain reconstructed in three dimensions.

Limitations

All postmortem human studies have inherent limitations, including tissue autolysis, which were minimized by short postmortem intervals, and perfusion of the brains with fixative immediately after removal. Loss of antigenicity during immersion fixation did not appear to be a significant limitation in these cases. The use of fixed tissue also precludes some molecular analyses, although we are exploring means of modifying our approach to permit this in future cases.

Finally, inferences derived from two brains with different ischemic time courses must be interpreted with caution, although we principally emphasize the value of the capability to deeply phenotype stroke cases through 3D whole-brain multi-dimensional characterization of evolving tissue ischemia. Thus, we primarily consider this work as a “proof-of-principle” of the power of this technology for quantitative cellular neuropathology.

Future directions

Expanding our histochemical analyses to include markers of ischemia-induced neurogenesis (21) is ongoing. In future ischemic brain samples, we expect to explore spatial transcriptomic signatures of the stroke penumbra, in keeping with the growing corpus of knowledge indicating the involvement of a number of pro- and anti-inflammatory and neuroprotective factors in the peripheral blood of patients with stroke (22).

Acknowledgments

We thank the following for their contributions:

Review of results and/or manuscript drafts: Shibeshih Belchew, Brian Edlow, Taylor Kimberly, Hannah Kineey

Tissue processing: Karthikeyan S, Dilli Priya, Hema Priya, Karthika Pandurangan

Histological staining: Jivitha Jyothi Ramesh, Jansy Ethiraj, Dhanamathi, E. Thamarai Selvi, Monisha. M, Pandiyaraj. M, Vanishree, Parthipan. E, Dhinesh Tharan, Abirami, Navamani, Divya

Data production for online viewer: Prabhu, Nagoor Meeran, R. Ghouse Baig, Lalithasree, Hariharan, Kanisha Sivakumar, Alice Teddy Fernandes, Vaishnavi Reghunath, Nachammai. V, Bashpa Vijayan, Subhashini Lakshmanan, Easha Doulgar, Pooja. V. S, Srilakshmi Narayanan

Histology image registration: Abinaya S, Gayathri T, Madhuhasan L, Priyadarshini M, Sivaprasad S, JayaVinisha, Vinoth Kanna

Software & visualization: Ramanan S, Nitheshkumar Sundhar, Jigneshkumar Mistry, Thangaraj Lamech, Mohd Asfar, Meena Vishali G, Divya D, Joshua Vedanayagam, Sivani Krishna T, Yashavi Upadhyay, Nitya Prakash Pandey, Azhar Bihari, Mani Durga, Manju Gnanakavi G, Muskan Periwal, Omm Ray

Mold Preparation and Surface Scanning: Prithviraj, Satheskumar V

Scanning Team: Mohamed Riyaz A, Chandru, Lavanya, Nithya Murugavel

We express our sincere gratitude to Mr. Prem Watsa for his generous intellectual and material encouragement of this project.

This work was presented in part at the 101st and 102nd Annual Meetings of the American Association of Neuropathologists. We thank the AANP for honoring Dr. Rakesh Nanjappa with a Travel Award to support his attendance.

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