Rapid Activation of the Neuroinflammatory Cascade Following Acute Stroke: Plasma and Extracellular Vesicle Factors Obtained on a Mobile Stroke Unit.

Supplemental Materials

Supplemental Methods

Study Population and Data Collection

Demographics, premorbid status and stroke onset clinical characteristics were recorded from the electronic medical record. Premorbid data included history of stroke, and disability status with the modified Rankin Scale (mRS) score. Stroke data included the National Institutes of Health Stroke Scale (NIHSS) score obtained acutely on the mobile stroke unit (MSU) and upon arrival to the University of Colorado Hospital (UCH) emergency department (ED). Stroke onset clinical lab values included the complete blood count (CBC) with differential, basic and complete metabolic panel, and coagulation tests. Stroke treatment data included administration of recombinant tissue plasminogen activator (tPA), mechanical thrombectomy, and medical and surgical interventions for intracranial hemorrhage. Radiological data included computed tomography (CT) of head obtained on the MSU, CT in the ED, and CT and magnetic resonance imaging (MRI) obtained during acute hospitalization.

Healthy adult controls

For the single molecule array (SiMoA) analyses, 21 healthy adult controls were used, all from a cohort of healthy college athletes enrolled in a separate study of traumatic brain injury. For the enzyme-linked immunosorbent assay (ELISA) analyses, a group of 5 older healthy adults was used. These were enrolled in a separate study of cognitive function. Informed written consent was obtained for all participants engaged in these two studies, which were approved by the Institutional Review Board of the University of Denver. Stroke history and cardiovascular risk factors were not available for these healthy controls.

Biosample collection and processing

Peripheral blood was collected for each patient from an upper extremity venipuncture site on the MSU, during the acute pre-hospital stroke evaluation. The first tube of blood drawn was set aside for the research study. Approximately 2-5 mL of blood was obtained in an ethylenediaminetetraacetic acid (EDTA) tube which was transported to the hospital on the MSU at room temperature as is done with the clinical blood sample used for patient care, and then placed in a 4°C refrigerator in the UCH emergency department (ED) upon arrival of the MSU to UCH. The blood was then transported to the Department of Neurosurgery (Graner lab) and processed. Aliquots of whole blood, plasma and peripheral blood mononuclear cells (PBMC) were prepared and stored in a -80°C freezer.

Plasma quantification of neuroinflammatory and neuronal biomarkers

Plasma levels of IL-6 were determined using a sandwich enzyme-linked immunosorbent assay (Quantikine HS Elisa, #HS600C, R&D Systems) following manufacturer's instructions. Briefly, plasma samples were thawed on ice before being diluted one-third with the supplied RD5-4 diluent and assayed against a standard curve with IL-6 concentrations ranging from 0.156 pg/mL to 10 pg/mL. All samples were tested in duplicate. Sample IL-6 concentrations were determined by non-linear regression from the standard curves using GraphPad Prism V8 (GraphPad, La Jolla, CA).

The Neurology-4-plex A assay was used, according to the manufacturer's instructions, on the single molecule array (SiMoA) platform (SR-X™ biomarker detection system, Quanterix, MA) to measure plasma levels of neurofilament-light (NfL), UCH-L1 and GFAP. Briefly, plasma samples were thawed on ice then centrifuged 5 minutes at 10,000 x g. The resulting supernatants were diluted one-fourth in provided sample diluent and loaded to a 96-well plate alongside standards and quality controls (one analog and one digital). The NF-light calibration curve ranged from 0.646 to 514 pg/mL and the GFAP from 9.63 to 8,405 pg/mL. Then antibody-coated magnetic beads and

biotinylated detector were added to the wells. After a wash step, streptavidin-ß-galactosidase is added. The fluorescent signal values generated from the calibration curves of known concentrations were fitted to a 4-parameter logistic curve with $1/y^2$ weighting, and sample concentration were extrapolated by the Quanterix software. All samples were run in duplicates on one occasion.

The MSD S-plex proinflammatory panel 1 kit (MesoScale Discovery, Rockville, MD) uses a sandwich immunoassay format coupled to signal-enhanced electrochemiluminescence (ECL) detection to achieve femtograms per milliliter sensitivity. This assay kit was used to measure plasma levels of the following cytokines with known association with stroke that then underwent additional analysis (IL-6, IL-17, IL-1β, TNFα and IFNγ). Briefly, plasma samples were thawed on ice then centrifuged 3 minutes at 2,000 x g. The clarified supernatants alongside 8 standards were loaded onto a 96-well plate with integrated screen-printed carbon ink electrodes on the bottom of each well that are used as solid-phase supports for binding reactions. The assay kit was used according to the manufacturer's instructions. The plate was read on a MESO QuickPlex SQ 120 intrument, which measures the intensity of ECL emitted, which is proportional to the amount of analyte present in the sample.

Extracellular vesicle isolation and quantification of neuroinflammatory factors

The HSP pull down method was conducted with Vn96 PSQGKGRGLSLSRFSWGALTLGEFLKL (pep1) and the reverse analogue sequence (pep2); both synthesized by *Vivitide* (Gardener, MA). Two hundred µL of plasma sample was centrifuged at 17,000 x g for 10 minutes. Supernatant was transferred to a new tube and the remaining pellet discarded. Ten µL of HSPA peptides PEP1 and 10 µL of PEP2 were added to each sample (vivitide ME-020p-kit; vivitide, Gardner, MA). Samples were rotated for 30 minutes, and then centrifuged at 17,000 x g for 15 minutes. Supernatant was carefully removed and the pellets were resuspended with 1 mL of PBS. After resuspension, 500 µL of additional PBS was added to the tubes to fill the remaining

volume. Samples were then centrifuged at 17,000 x g for 5 minutes. Supernatant was carefully removed and PBS resuspension was repeated for a total of two washes. After removing the PBS supernatant, 100 µL of RIPA (Radio-Immune Precipitation Assay) buffer (Sigma-Aldrich, St Louis, MO, R0278-50ML) and 100 µL of nuclear extraction reagent (NER) from NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL #78833) were added to each pellet. Each sample was resuspended in solution, vortexed for 5-10 seconds, then rotated for 15 minutes. Samples were then used to probe the Proteome Profiler Human XL Cytokine Array (R&D, Minneapolis, MN #ARY022B). Manufacturer's protocol for the Proteome Profiler Human XL Cytokine Arrays was followed. However, the provided chemiluminescence reagents were substituted with developer from the Super Signal West Femto Kit (Thermo Scientific, 34095). Cytokine arrays were imaged using the FluorChemQ Multi Image 3 (Alpha Innotech/ProteinSimple, Wallingford, CT). Images were optimized using Photoshop and spots were quantified using NIH ImageJ (https://imagej.nih.gov/ij/). Heatmaps were generated using Microsoft Excel (Microsoft 365).

Statistical Analysis

Patients were dichotomized into those with confirmed stroke vs. without confirmed stroke, and confirmed stroke vs. healthy controls. Two patients diagnosed with stroke symptoms, but likely either TIA or averted by tPA, were included with the non-stroke group. Patient premorbid disability status (mRS), clinical acute stroke status (NIHSS), and discharge status (mRS, NIHSS) were recorded individually by patient and as median and range. Stroke neuroninflammatory factors were evaluated by concentration in plasma and by arbitrary units of pixel density in association with extracellular vesicles. Biomarkers were recorded individually by patient, and as median and range.

Supplemental Results

Patient population

Of study patients treated on the MSU and diagnosed as stroke, 2 were eventually diagnosed as likely transient ischemic attack (TIA) or stroke averted by thrombolysis. Of patients with confirmed stroke 2 (25%) were being treated with antiplatelet therapy at the time of stroke onset. The remaining 5 patients were stroke mimics, including: seizures, COPD exacerbation, pulmonary embolism and alcohol withdrawal Among confirmed strokes, 6 (75%) were acute ischemic stroke (AIS) involving middle cerebral artery (MCA) territories, and 2 (25%) were thalamic intracerebral hemorrhage (ICH). Median initial NIHSS score was 11 (range 4-19), and 3 (37%) patients improved before arrival at the emergency department (ED), while 4 (50%) worsened. Six (75%) of confirmed stroke patients received IV tPA, and this was administered on the MSU in the field to 4 (67%), and after arrival at the ED in 2 (33%) patients. Two (33%) AIS patients underwent mechanical thrombectomy. Median length of hospital stay for confirmed stroke patients was 4 (range 2-7) days (Table S1). Of all healthy controls, 14 (54%) were female, and median age was 21 (range 18-86) years. In controls used in SiMoA analyses, 11 (52%) were female, and median age was 21 (range 18-26) years. Among controls in the ELISA analyses, 3 (60%) were female and the median age was 70 (range 65-86) years.

Blood sampling

Peripheral venous blood was obtained at the patients' homes for measurement of neuro-inflammatory markers at a median of 58 (range 36-133) minutes after stroke symptom onset for all 8 confirmed stroke cases. IL-6 is known to have diurnal variation, with a morning trough of approximately 0.5 pg/mL between 6 a.m. and 12 noon.[19] In our cohort, no association was found between the time of day blood was drawn and IL-6 levels. For confirmed stroke patients, median time between the initial blood draw on the MSU and refrigeration at 4°C was 23 (range 12-37) minutes, and median time from the MSU blood draw to freezing at -80 °C was 1,501 (range 339-4,058) minutes. No correlation was found between blood processing times and inflammatory factor levels in any of the blood or extracellular vesicle assays used in the study (Table S10).

Acute Outcomes

At hospital discharge, median NIHSS score for confirmed stroke patients was 4 (range 1-9). NIHSS severity was minor in 4 (50%) patients, moderate in 3 (37%) and moderate to severe in 1 (12%). Six (75%) patients improved from initial evaluation, 1 (12%) was unchanged and 1 (12%) worsened. Four (50%) patients had favorable functional disability (Modified Rankin Scale score ≤2), 3 (37%) were discharged to home, 3 (37%) to inpatient rehabilitation, 1 (12%) to a skilled nursing facility and 1 (12%) to hospice (Table S11).

Plasma Neuroinflammatory Factor Levels

Plasma IL-6 levels were quantified with electrochemiluminescence assay (ECL) in all confirmed stroke patients. The individual IL-6 concentration was elevated in all 7 patients with ischemic stroke, and was at the median normal level in 1 patient who had a thalamic intracerebral hemorrhage. In the ischemic stroke patients, IL-6 concentrations increased consistently with increased sampling time from symptom onset for individual patients for the blood draw on the MSU: 3.82 pg/mL at 36 minutes, 5.29 pg/mL at 48 minutes, 9.25 pg/mL at 83 minutes, 16.80 pg/mL at 102 minutes and 36.76 pg/mL at 133 minutes (Fig. 2). During the first 133 minutes after stroke onset, plasma IL-6 increased at a rate of 0.4 pg/mL per minute (Table S2, Fig. 2). Median plasma IL-6 concentration was 39.74 pg/mL (range 5.64-94.05) at 24 hours after stroke onset. Median increase during the first 24 hours was 12.96 pg/mL (range 3.88-84.97) (Table S4, Fig. S1). No association was found between plasma IL-6 concentration and time of day blood drawn, initial NIHSS score or patient age (Fig. S6). In ECL analyses, IL-10 and IL-12p70 remained at normal levels acutely and during the next 24 hours. We surmise that the IL-10 and IL-12p70 response occurs later in the stroke course, or is not as robust as the IL-6 increase.

Plasma IL-6 was also evaluated with enzyme-linked immunoassay (ELISA) in the 7 confirmed acute stroke patients (5 AIS, 2 ICH) and 5 healthy adult control subjects.

Median age of healthy controls was 70 years (range 65-86) and 3 (60%) were female. In the ELISA analysis, median plasma IL-6 was 7.36 pg/mL (range 2.03-41.05) for stroke patients vs. 2.30 pg/mL (range 0.93-6.22) for healthy controls. In 5 (71%) stroke patients, plasma IL-6 for blood obtained on the MSU was higher than in healthy controls, and in all stroke patients IL-6 was higher in blood obtained on the MSU than the median normal level for healthy adults[12] (Table S5).

In single molecule array (SIMOA) analysis in 6 confirmed stroke patients median NfL was 26.63 pg/mL (range 1.94-130.82) for stroke patients compared to median 4.09 pg/mL (range 1.34-8.84) for controls. Median UCH-L1 for stroke patients was 41.27 pg/mL (range 20-54-85.98) compared with median 17.38 pg/mL (range 2.98-32.91) for controls. Median GFAP concentration for stroke patients was 195.22 pg/mL (range 52.77-1,526.74) compared to median 80.37 pg/mL (range 56.43-132.86) for healthy controls. In stroke patients, median change in plasma protein levels from symptom onset to 24 hours after stroke was increased for NfL and GFAP, and decreased for UCH-L1. For NfL, levels between stroke onset and 24 hours increased in 3 (60%) patients; for UCH-L1, levels increased in 2 (40%); and for GFAP, levels increased in 5 (100%) patients. (Table S6, Figs. S2-S3).

Extracellular Vesicle-derived Neuroinflammatory Factor Levels

Extracellular vesicles were isolated from study blood samples with HSP-pull down,[20-23] and cytokine array analysis was used to quantify EV-derived neuroinflammatory factors in 5 stroke patients (4 AIS, 1 ICH), and 2 pooled samples of blood from healthy adult subjects (Fig. S4). In extracellular vesicle analyses, mean ages of the groups of pooled healthy control subjects were 53 ± 11 years and 54 ± 9.5 years; each group was 50% female. At 24 hours after stroke onset, median levels of all factors increased compared to stroke-onset levels, except for PECAM1. For OPN and CRP, concentrations increased for all patients during this period. For MMP-9, CXCL4, IL-6, and PECAM1,

trajectory of change was mixed, with most but not all patient levels increasing over the first 24 hours of stroke progression (Figs. S4-S5).

Leukocyte concentrations

White blood cells and subtypes were evaluated in clinical UCH laboratory testing as part of the routine clinical care of MSU patients. Results were available for 7 (87%) of patients with confirmed acute stroke (5 AIS, 2 ICH). Blood for these panels was obtained a median of 116 minutes (range 84-242) after stroke symptom onset. White blood count was elevated in 3 (43%) and normal in 4 (57%). Neutrophils were elevated in 3 (50%) of stroke patients with available values. Monocytes were elevated in 1 (17%) patient. Lymphocytes and eosinophils were normal in all patients (Table S8).

Supplemental Discussion

Among proteins and chemokines found to be elevated, GFAP is associated with breakdown of the blood-brain barrier in acute brain injury including trauma and stroke.[24, 25] GFAP has shown promise as a biomarker for ischemic/hemorrhagic differentiation in stroke,[5, 26] but thus far with insufficient sensitivity for clinical application.[27] NfL, a component of the neuronal cytoskeleton, reflects neuronal structural insult in stroke.[28] UCH-L1 is a protein associated with brain self repair after stroke,[29] C-reactive protein may be associated with blood-brain barrier disruption,[30, 31] and CXCL4 induces adhesion of neutrophils to cerebral endothelium after acute stroke.[32]

Table S1. Stroke onset characteristics of patients with confirmed stroke treated on mobile stroke unit

				Acute Clinical Evaluation												
Patient characteristics				Evaluation timing	Stroke onset NIHSS			MSU clinical presentation							Radiological findings†	
Patient no.	Sex	Age	Premorbid mRS	Minutes from stroke symptom onset on MSU	MSU	ED	Δ‡	Gaze deviation Visual field loss	Facial paralysis	Weakness	Sensory loss	Aphasia	Dysarthria	Inattention neglect	Non-contrast head CT (MSU) and Head CTA	
1	M	85	0	83	15	20	+5	X	X	X	X		X		R. M1 occlusion, loss gray-white differentiation R. insula	
2	F	36	0	48	22	26	+4			X	X	X	X		R. M2 occlusion	
3	F	59	0	102	11	6	-5		X	X	X		X	X	R. M2 superior division occlusion, R. MCA hypoattenuation	
4	M	76	1	133	4	5	+1	X		X		X		X	R. M2 occlusion, encasement by petroclival meningioma	
5	M	78	1	40	21	29	+8	X	X	X		X			L. MCA ischemia, possible L. insula infarction	
6	M	45	0	36	6	1	-5			X	X				No acute hemorrhage or territorial infarct	
7	F	86	0	69	4	4	0	X	X				X		R. Thalamic ICH with IVH extension. ACA aneurysm	
8	M	52	0	38	11	9	-2	X	X		X		X		L. Thalamic hemorrhage	

NIHSS, National Institutes of Health Stroke Scale; MSU, mobile stroke unit; mRS, modified Rankin Scale; ED, emergency department; CT, computed tomography; CTA, CT angiography; R, right; L, left; M1, M1 segment of middle cerebral artery; M2, M2 segment of middle cerebral artery; MCA, middle cerebral artery; ICH, intracerebral hemorrhage; ACA, anterior communicating artery skilled nursing facility.

† Derived from clinical radiology reports.

‡ Acute change in NIHSS score from initial evaluation on MSU to first subsequent evaluation in ED.

Table S2. Blood-based neuroinflammatory factor levels of patients with confirmed stroke treated on mobile stroke unit

Patient characteristics				Clinical blood samples - ED					Ultra-early biomarker blood samples - MSU									
					UCH clinical lab testing					ECL	SIMOA			Extracellular vesicles isolated by HSP pull down				
Patient no.	Sex	Age	Stroke Diagnosis	Time from stroke symptom onset (mins)	WBC (10^9/L) Ref: 4-11.1	Abs Neutrophils (10^9/L) Ref: 1.8-6.6	Abs Lymphocytes (10^9/L) Ref: 1.0-4.8	Abs Monocytes (10^9/L) Ref: 0.2-0.9	Time from stroke symptom onset (mins)	IL-6 (pg/mL) Ref: 1.89 (0.3-5.0)	NfL (pg/mL) Control: 4.09 (1.34-8.84)	UCH-L1 (pg/mL) Control: 4.09 (1.34-8.84)	GFAP (pg/mL) Control: 80 (56-133)	CRP (pixel density) Control: 8959	MMP-9 (pixel density) Control: 1765	CXCL4 (pixel density) Control: 702	PECAM1 (pixel density) Control: 1804	IL-6 (pixel density) Control: 502	OPN (pixel density) Control: 2451
1	M	85	AIS	116	4.6	3.0	3.0	0.4	83	9.2↑	40.8↑	52.6↑	156↑	26823↑	4265↑	14781↑	5280↑	510	12432↑
2	F	36	AIS	N/A	N/A	N/A	N/A	N/A	48	5.3↑	1.9	28.0↑	53↑	20729↑	1542	13648↑	5309↑	653↑	7507↑
3	F	59	AIS	242	12.5↑	9.7↑	2.0	0.6	102	16.8↑	130.8↑	44.2↑	234↑	10758↑	4708↑	6771↑	4756↑	1047↑	3409↑
4	M	76	AIS	193	11.2↑	9.1↑	1.0	1.0↑	133	36.8↑	21.3↑	164.2↑	282↑	12875↑	8134↑	9734↑	2739↑	1047↑	4639↑
7	F	86	ICH	107	12.4↑	11.1↑	0.7↓	0.4	69	13.1↑	31.9↑	86.0↑	1527↑	N/A	N/A	N/A	N/A	N/A	N/A
8	M	52	ICH	84	8.9	5.9	2.4	0.5	38	1.8	7.72↑	20.5↑	71↑	11774↑	5863↑	7503↑	4486↑	1859↑	5557↑
5	M	78	AIS	151	10.6	N/A	N/A	N/A	40	3.5↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	M	45	AIS	87	7.3	3.7	2.4	0.7	36	3.8↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

ED, emergency department; MSU, mobile stroke unit; UCH, University of Colorado Hospital; ECL, electrochemiluminescence immunoassay; SIMOA, single molecule array; HSP, heat-shock protein; AIS, acute ischemic stroke; ICH, intracerebral hemorrhage; WBC, white blood count; IL-6, interleukin 6; NfL, neurofilament light chain; UCH-L1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary astrocytic protein; CRP, C-reactive protein; MMP-9, matrix metalloproteinase 9; CXCL4, C-X-C motif ligand 4; PECAM-1, platelet endothelial cell adhesion molecule-1; OPN, osteopontin.

Above reference level or control.
Below reference level of control.

Table S3. Plasma neuroinflammatory factors in MSU stroke patients and stroke mimics stroke

Blood-based markers*	Normal Range†	All Patients (N=15)	Confirmed Stroke (N=8)	Stroke Mimic** (N=7)
Plasma pro-neuroinflammatory factors				
Minutes from stroke symptom onset		69 (35-835)	46 (36-133)	405 (35-835)
IL-17A (pg/mL)	0.091 (0.030-0.90)	0.51 (0.12-1.42)	0.36 (0.12-1.42)	0.56 (0.21-1.30)
Normal		15 (100)	8 (100)	7 (100)
Elevated		0 (0)	0 (0)	0 (0)
IL-1β (pg/mL)	0.070 (ND-5.90)	0.14 (0.05-4.35)	0.11 (0.05-0.29)	0.14 (0.09-4.35)
Normal		14 (94)	8 (100)	6 (86)
Elevated		1 (6)	0 (0)	1 (14)
IL-6 (pg/mL)	1.60 (0.330-5.80)	7.31 (1.76-282.98)	7.27 (1.76-36.76)	7.31 (1.78-282.98)
Normal‡		2 (13)	1 (12)	1 (14)
Elevated‡		13 (87)	7 (88)	6 (86)
TNFα (pg/mL)	0.170 (0.038-1.70)	0.53 (0.34-5.70)	0.47 (0.34-0.74)	0.55 (0.41-5.70)
Normal		14 (94)	8 (100)	6 (86)
Elevated		1 (6)	0 (0)	1 (14)

Data are N (%), median (range). MSU, mobile stroke unit; IL-17A, interleukin 17A; IL-1β, interleukin 1β; IL-6, interleukin 6; TNFα, tumor necrosis factor alpha; ND, not detected.
*Plasma neuroinflammatory factors measured by electrochemiluminescence assay (ECL).
**Includes one patient with suspected TIA, and one patient with a possible ischemic stroke aborted by tPA.
†Mesoscale Discovery S-PLEX Proinflammatory Panel 1 human insert. https://www.mesoscale.com/~/media/files/product%20inserts/s-plex%20proinflammatory%20panel%201%20human%20insert.pdf. Accessed Aug. 6, 2022.
‡Normal and elevated IL-6 based on reference range: median 1.89 pg/mL, upper 95th percentile 4.45 pg/mL. Todd J., Simpson P., Estis J., Torres V., Wub A. H. Reference range and short- and long-term biological variation of interleukin (IL)-6, IL-17A and tissue necrosis factor-alpha using high sensitivity assays. Cytokine. Dec 2013;64(3):660-665.

Table S4. Comparison of stroke-onset and 24-hour plasma neuroinflammatory factors in stroke patients with ECL

Blood-based markers	Normal Range†	Ultra-early (on MSU)	24-hour (acute hospital care)	Δ (MSU to 24h)
		N=8	N=6	
Minutes from stroke symptom onset		46 (36-133)	1435 (1215-1596)	
Pro-inflammatory cytokines				
IL-17A (pg/mL)	0.091 (0.030-0.90)	0.36 (0.12-1.42)	0.35 (0.19-2.42)	+0.07 (-0.19 to + +1.33)
Normal		8 (100)	5 (83)	
Elevated		0 (0)	1 (17)	
IL-1β (pg/mL)	0.070 (ND-5.90)	0.11 (0.05-0.29)	0.21 (0.05-0.56)	+0.11 (-0.12 to +0.44)
Normal		8 (100)	6 (100)	
Elevated		0 (0)	0 (0)	
IL-6 (pg/mL)	1.60 (0.330-5.80)	7.27 (1.76-36.76)	39.74 (5.64-94.05)	+12.96 (+3.88 to +84.79)
Normal‡		1 (12)	0 (0)	
Elevated‡		7 (88)	6 (100)	
TNFα (pg/mL)	0.160 (0.110-5.50)	0.47 (0.34-0.74)	0.76 (0.35-1.03)	+0.21 (0.0 to +0.39)
Normal		8 (100)	6 (100)	
Elevated		0 (0)	0 (0)	
IFN-γ (pg/mL)	0.170 (0.038-1.70)	0.51 (0.10-41.15)	0.18 (0.10-56.10)	-0.05 (-0.83 to +14.95)
Normal		8 (100)	6 (100)	
Elevated		0 (0)	0 (0)	

Data are N (%), median (range). MSU, mobile stroke unit, ECL, electrochemiluminescence assay; IL-17A, interleukin 17A; IL-1β, interleukin 1β; IL-6, interleukin 6; TNFα, tumor necrosis factor alpha; IFN-γ, interferon gamma; ND, none detected.
†Mesoscale Discovery S-PLEX Proinflammatory Panel 1 human insert. https://www.mesoscale.com/~/media/files/product%20inserts/s-plex%20proinflammatory%20panel%201%20human%20insert.pdf. Accessed Aug. 6, 2022.
‡Normal and elevated IL-6 based on reference range: median 1.89 pg/mL, upper 95th percentile 4.45 pg/mL. Todd J., Simpson P., Estis J., Torres V., Wub A. H. Reference range and short- and long-term biological variation of interleukin (IL)-6, IL-17A and tissue necrosis factor-alpha using high sensitivity assays. Cytokine. Dec 2013;64(3):660-665.

Table S5. Comparison of plasma neuroinflammatory factors and stroke proteins in stroke patients and controls with ELISA

Blood-based markers	Healthy Controls	Ultra-early (MSU)	24-hour (acute hospital care)	Δ (MSU to 24h)
Plasma pro-neuroinflammatory factors	N=5	N=7	N=5	N=5
Minutes from stroke symptom onset		48 (38-133)	1,473 (1,215-1,596)	
IL-6 (pg/mL)	2.30 (0.93-6.22)	7.36 (2.03-41.05)	41.03 (4.31-54.75)	+10.97 (+2.28 to +47.39)
Normal†	3 (60)	2 (29)	1 (20)	
Elevated†	2 (40)	5 (71)	4 (80)	
Δ during first 24h				
Increase				5 (100)
Decrease				0 (0)
Plasma stroke proteins				
NSE (pg/mL)	N/A	9,244 (6,610-22,067)	4,552 (2,709-6,728)	-4,953 (-1,964 to -18,014)
Normal		N/A	N/A	
Elevated		N/A	N/A	
NfL (pg/mL)	24.66 (9.46-26.37)	31.94 (1.94-130.82)	17.82 (2.36-112.52)	+0.42 (-18.30 to +13.17)
Normal*		3 (43)	3 (60)	
Elevated*		4 (57)	2 (40)	
UCH-L1 (pg/mL)	18.89 (11.88-61.61)	44.19 (20.54-85.98)	25.84 (10.99-164.19)	-8.39 (-17.06 to +120.00)
Normal*		0 (0)	2 (40)	
Elevated*		7 (100)	3 (60)	
GFAP (pg/mL)	187.87 (91.19-359.53)	184.90 (52.77-1,526.74)	312.74 (53.03-3,537.32)	+221.90 (+0.26 to +3,303.05)
Normal*		4 (57)	1 (20)	
Elevated*		3 (43)	4 (80)	

Data are N (%), median (range). ELISA, enzyme-linked immunoassay; MSU, mobile stroke unit; IL-6, interleukin 6; NSE, neuron specific enolase; NfL, neurofilament light chain; UCH-L1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein.

† Normal and elevated IL-6 based on reference range: median 1.89 pg/mL, upper 95th percentile 4.45 pg/mL. Todd J., Simpson P., Estis J., Torres V., Wub A. H. Reference range and short- and long-term biological variation of interleukin (IL)-6, IL-17A and tissue necrosis factor-alpha using high sensitivity assays. Cytokine. Dec 2013;64(3):660-665.

*Compared with median plasma concentration in healthy controls.

Table S6. Comparison of plasma neuroinflammatory factors and stroke proteins in stroke patients and controls with SIMOA

Blood-based markers	Healthy Controls	Ultra-early (MSU)	24-hour (acute hospital care)	Δ (MSU to 24h)
Plasma proteins	N=21	N=6	N=5	N=5
Time from stroke symptom onset (mins)		76 (38-133)	1,473 (1,215-1,596)	
NfL (pg/mL)	4.09 (1.34-8.84)	26.63 (1.94-130.82)	17.82 (2.36-112.52)	+0.42 (-18.30 to +13.17)
Normal†		1 (17)	1 (20)	
Elevated‡		5 (83)	4 (80)	
Δ first 24h				3 (60)
Increase				2 (40)
Decrease				
UCH-L1 (pg/mL)	17.38 (2.98-32.91)	41.27 (20.54-85.98)	25.84 (10.99-164.19)	-8.39 (-17.06 to +120.00)
Normal		0 (0)	2 (40)	
Elevated		6 (100)	3 (60)	
Δ first 24h				
Increase				2 (40)
Decrease				3 (60)
GFAP (pg/mL)	80.37 (56.43-132.86)	195.22 (52.77-1,526.74)	312.70 (53.00-3,537.30)	+221.86 (+0.23 to +3,303.03)
Normal		2 (33)	1 (20)	
Elevated		4 (67)	4 (80)	
Δ first 24h				
Increase				5 (100)
Decrease				0 (0)

Data are N (%), median (range). SIMOA, single molecule array; MSU, mobile stroke unit; NfL, neurofilament light chain; UCH-L1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein.

† Compared with median plasma concentration in healthy controls.

Table S7. Extracellular vesicle-derived neuroinflammatory factors in confirmed stroke patients

	Healthy Controls	Ultra-early (MSU)	24-hour (acute hospital care)	Δ (MSU to 24h)
EV-based neuroinflammatory factors	(Pooled HC)	N=5	N=5	N=5
Time from stroke symptom onset (mins)		83 (38-133)	1,473 (1,215-1,596)	
CRP (pixel density)	8,959	12,875 (10,758-26,823)	16,473 (12,042-33,008)	+3,598 (+268 to +6,185)
Normal†		0 (0)	0 (0)	
Elevated†		5 (100)	5 (100)	
Δ first 24h				5 (100)
Increase				0 (0)
Decrease				
MMP-9 (pixel density)	1,765	4,708 (1,542-8,134)	6,846 (1,087-16,473)	+983 (-3,621 to +8,339)
Normal		0 (0)	1 (20)	
Elevated		5 (100)	4 (80)	
Δ first 24h				
Increase				2 (40)
Decrease				3 (60)
CXCL4 (pixel density)	702	9,734 (6,771-14,781)	11,684 (5,450-14,932)	+151 (-1,321 to +1,950)
Normal		0 (0)	0 (0)	
Elevated		5 (100)	5 (100)	
Δ first 24h				
Increase				3 (60)
Decrease				2 (40)
PECAM1 (pixel density)	1,804	4,756 (2,739-5,309)	3,582 (2,171-5,948)	-568 (-2,206 to +1,192)
Normal		0 (0)	0 (0)	
Elevated		5 (100)	5 (100)	
Δ first 24h				
Increase				2 (40)
Decrease				3 (60)
IL-6 (pixel density)	510	1,047 (510-1,859)	942 (570-2,973)	+60 (-105 to +1,926)
Normal		1 (20)	0 (0)	
Elevated		4 (80)	5 (100)	
Δ first 24h				
Increase				3 (60)
Decrease				2 (40)
OPN (pixel density)	2,451	5,557 (3,409-12,432)	6,758 (3,874-12,575)	+987 (+143 to +1.646)
Normal		0 (0)	0 (0)	
Elevated		5 (100)	5 (100)	
Δ first 24h				
Increase				5 (100)
Decrease				0 (0)

Data are N (%), median (range). MSU, mobile stroke unit; EV, extracellular vesicle; CRP, C-reactive protein; MMP-9, matrix metalloproteinase-9; CXCL4, chemokine (C-X-C motif) ligand 4; PECAM1, platelet and endothelial cell adhesion molecule 1; IL-6, interleukin-6; OPN, osteopontin.

Table S8. Acute leukocyte levels in MSU patients after stroke onset

	Normal Range†	All Patients (N=15)	Confirmed Stroke (N=8)	Stroke Mimic* (N=7)
Clinical leukocyte values (CBC)				
Minutes from stroke symptom onset		133 (79-865)	116 (84-242)	452 (79-865)
WBC (10^9/L)	4-11.1	9.3 (4.6-21.1)	10.6 (4.6-12.5)	8.6 (6.5-21.1)
Normal		10/14 (71)	4/7 (57)	6 (86)
Elevated		4/14 (29)	3/7 (43)	1 (14)
Neutrophils (10^9/L)	1.8-6.6	5.8 (3-17.6)	7.5 (3-11.1)	5.4 (4.7-17.6)
Normal		8/13 (61)	3/6 (50)	5 (71)
Elevated		5/13 (38)	3/6 (50)	2 (29)
Lymphocytes (10^9/L)	1.0-4.8	1.6 (0.7-3.0)	1.5 (0.7-2.4)	1.6 (1.0-3.0)
Normal		13/13 (100)	6/6 (100)	7 (100)
Elevated		0 (0)	0 (0)	0 (0)
Monocytes (10^9/L)	0.2-0.9	0.7 (0.4-1.6)	0.5 (0.4-1.0)	0.8 (0.4-1.6)
Normal		11 (73)	5/6 (83)	6 (86)
Elevated		2 (13)	1/6 (17)	1 (14)
Eosinophils (10^9/L)	0.0-0.4	0.0 (0.0-0.3)	0.5 (0.0-0.3)	0.0 (0.0-0.3)
Normal		13/13 (100)	6/6 (100)	7 (100)
Elevated		0 (0)	0 (0)	0 (0)

Data are N (%), median (range). CBC, complete blood count; MSU, mobile stroke unit.
*Includes one patient with suspected TIA, and one patient with a possible ischemic stroke aborted by tPA.
†University of Colorado Hospital clinical reference ranges.

Table S9. Summary table of normal reference ranges for study biomarker comparisons

Biomarker test utilized for study subject plasma	Biomarker	Units	Normal reference values [median, (range)]	Normal range source	N evaluated for normal range
SIMOA	NfL	pg/mL	4.09 (1.34-8.84)	Study healthy adult controls	21
	UCH-L1		17.38 (2.98-32.91)		
	GFAP		80.37 (56.43-132.86)		
ELISA	NfL	pg/mL	24.66 (9.46-26.37)	Study healthy adult controls	5
	UCH-L1		18.89 (11.88-61.61)		
	GFAP		187.87 (91.19-359.53)		
ECL	IL-17A	pg/mL	0.091 (0.030-0.90)	MSD S-Plex reference[1]	17
	IL-1β		0.070 (ND-5.90)		
	TNFα		0.160 (0.110-5.50)		
	IFN-γ		0.170 (0.038-1.70)		
	IL-6		1.60 (0.330-5.80)		
			1.89 (4.45)†	Todd et a, *Cytokine*[2]	125
			≤1.8	Mayo Clinic Laboratories[3]	N/A
EV*	CRP	pixel density	8,959	Pooled sample healthy adults**	Pooled
	MMP-9		1,765		
	CXCL4		702		
	PECAM1		1,804		
	IL-6		510		
	OPN		2,451		

SIMOA, single molecule array; ELISA, enzyme-linked immunoassay; ECL, electrochemiluminescence assay; EV, extracellular vesicle; NfL, neurofilament light chain; UCH-L1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein; IL-17A, interleukin 17A; IL-1β, interleukin 1β; TNFα, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL-6, interleukin 6; CRP, C-reactive protein; MMP-9, matrix metalloproteinase-9; CXCL4, chemokine (C-X-C motif) ligand 4; PECAM1, platelet and endothelial cell adhesion molecule 1; OPN, osteopontin.

*EVs isolated from study subject plasma. Biomarkers quantified with membrane-based sandwich immunoassay (Proteome Profiler Human XL Cytokine Array).
**Pooled plasma-derived serum sample, Innovative Research, MI, USA, ISERAB-100 mL.
†Normal reference range reported as mean (upper 95th percentile).

1. Mesoscale Discovery S-PLEX Proinflammatory Panel 1 human insert. https://www.mesoscale.com/~/media/files/product%20inserts/s-plex%20proinflammatory%20panel%201%20human%20insert.pdf. Accessed Aug. 6, 2022.
2. Todd J., Simpson P., Estis J., Torres V., Wub A. H. Reference range and short- and long-term biological variation of interleukin (IL)-6, IL-17A and tissue necrosis factor-alpha using high sensitivity assays. Cytokine. Dec 2013;64(3):660-665.
3. Mayo Clinic Laboratories Plasma Interleukin-6 Clinical Information. https://www.mayocliniclabs.com/test-catalog/Overview/63020#Clinical-and-Interpretive. Accessed June 8, 2022.

Table S10. Association of blood sample processing times with biomarker concentrations

	Blood sample handling times confirmed stroke patients					
	Time from MSU blood draw to 4 °C		Time from 4 °C to -80 °C		Time from MSU blood draw to -80 °C	
Time in transition (minutes)						
All stroke patients (N=8)	23 (12-37)		1,501 (339-4,058)		1,521 (351-4,093)	
ELISA (N=7)	21 (12-37)		1,471 (339-4,058)		1,490 (351-4,093)	
SIMOA (N=6)	20 (12-37)		1,388 (339-1,553)		1,406 (351-1,590)	
ECL (N=8)	23 (12-37)		1,501 (339-4,058)		1,521 (351-4,093)	
EV (N=5)	21 (16-37)		1,471 (1,113-1,553)		1,490 (1,148-1,590)	
Immunoassay and Inflammatory Factor	Spearman's Correlation coefficient	P value	Spearman's Correlation coefficient	P value	Spearman's Correlation coefficient	P value
ELISA (N=7)						
IL-6	-0.108	0.818	-0.500	0.253	-0.500	0.253
NSE	0.314	0.544	0.371	0.468	0.371	0.468
NfL	-0.270	0.558	0.143	0.760	0.143	0.760
UCH-L1	-0.450	0.310	-0.214	0.645	-0.214	0.645
GFAP	-0.396	0.379	-0.607	0.148	-0.607	0.148
SIMOA (N=6)						
NfL	-0.486	0.329	-0.257	0.623	-0.257	0.623
UCH-L1	-0.657	0.156	-0.600	0.208	-0.600	0.208
GFAP	-0.468	0.329	-0.829	0.042	-0.829	0.042
ECL (N=8)						
IL-17A	-0.407	0.317	-0.595	0.120	-0.524	0.310
IL-1β	0.132	0.756	-0.119	0.779	-0.095	0.823
IL-6	- 0.072	0.866	-0.595	0.120	-0.571	0.139
TNFα	0.347	0.399	0.024	0.955	0.095	0.823
IFN-γ	0.275	0.509	-0.357	0.385	-0.238	0.570
Extracellular Vesicles (N=5)						
CRP	-0.100	0.873	-0.200	0.747	-0.200	0.747
MMP-9	-0.100	0.873	-0.700	0.188	-0.700	0.188
CXCL4	-0.100	0.873	-0.200	0.747	-0.200	0.747
PECAM1	-0.100	0.873	0.700	0.188	0.700	0.188
IL-6	0.051	0.935	-0.051	0.935	-0.051	0.935
OPN	-0.300	0.624	0.000	1.000	0.000	1.000

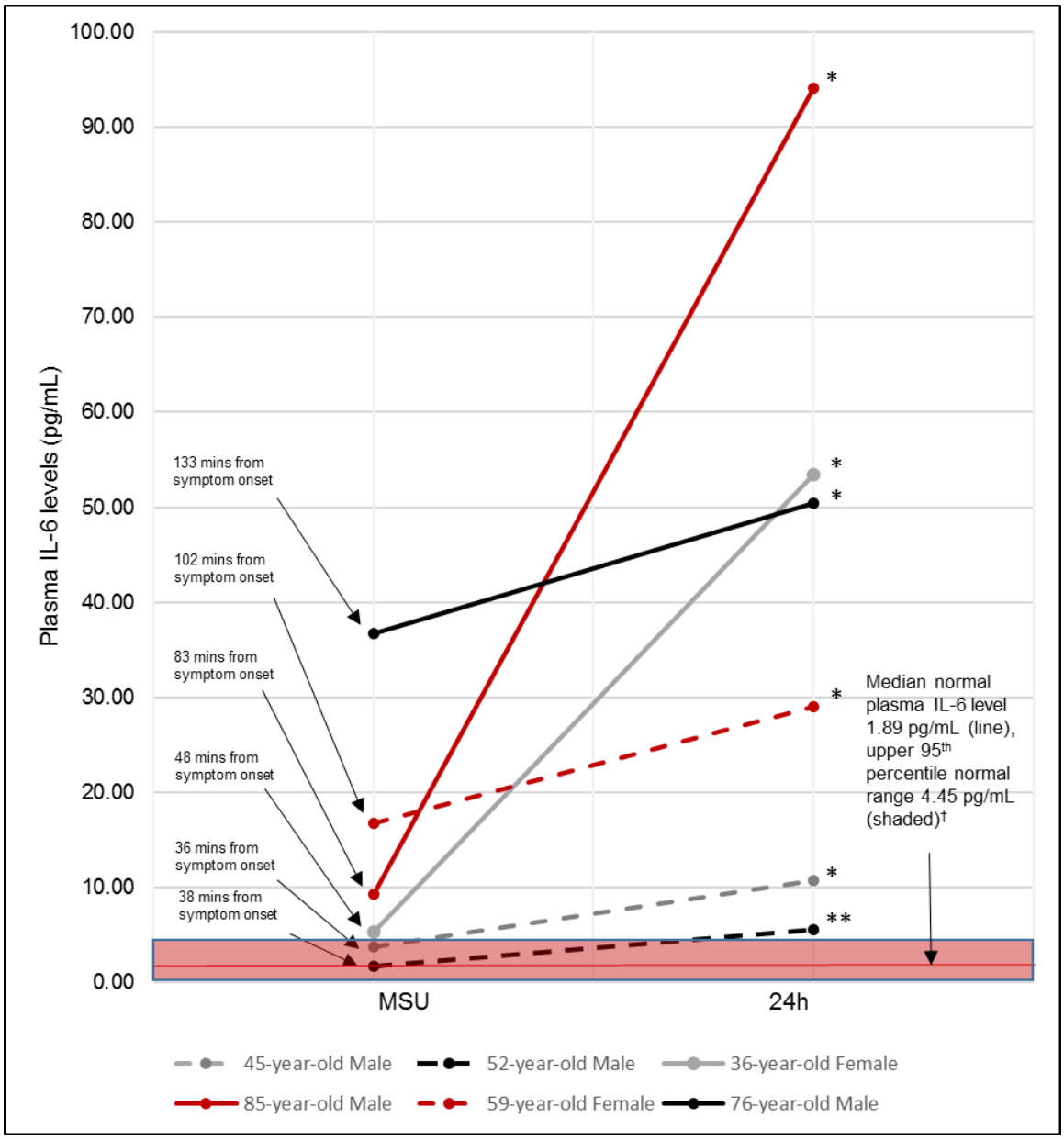
Data are median (range). ELISA, enzyme-linked immunosorbent assay; SIMOA, single molecule array; ECL, electrochemiluminescence immunoassay; EV, extracellular vesicle; MSU, mobile stroke unit; IL-6, interleukin 6; NSE, neuron-specific enolase; NfL, neurofilament light chain; UCH-L1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein; IL-17A, interleukin 17A; IL-1β, interleukin 1β; CRP, C-reactive protein; MMP-9, matrix metalloproteinase 9; CXCL4, C-X-C motif ligand 4; PECAM-1, platelet endothelial cell adhesion molecule-1; OPN, osteopontin.

Table S11. Acute hospitalization outcomes in patients treated on mobile stroke unit

Outcome Characteristics	All Patients (N=15)	Confirmed Stroke (N=8)	Stroke Mimic* (N=7)
Clinical and functional disability status			
NIHSS score at hospital discharge	4 (0-6)†	4.5 (1-9)	0 (0-0)‡
0-4. Minor	7 (64)	4 (50)	3 (100)
5-15. Moderate	3 (27)	3 (37)	0 (0)
16-20. Moderate to severe	1 (9)	1 (12)	0 (0)
21-42. Severe	0 (0)	0 (0)	0 (0)
Δ NIHSS score (MSU to hospital discharge)	-6 (-1 to -7)	-5.5 (-0.25 to -9.25)	-6 (-1 to -7)
Improved	9 (82)	6 (75)	3 (100)
Unchanged	1 (9)	1 (12)	0 (0)
Worsened	1 (9)	1 (12)	0 (0)
Modified Rankin Scale score at hospital discharge			
0. No symptoms	4 (27)	2 (25)	2 (29)
1. No significant disability	1 (7)	0 (0)	1 (14)
2. Slight disability	2 (13)	2 (25)	0 (0)
3. Moderate disability	2 (13)	2 (25)	0 (0)
4. Moderately severe disability	5 (33)	1 (12)	4 (57)
5. Severe Disability	1 (7)	1 (12)	0 (0)
6. Death	0 (0)	0 (0)	0 (0)
Discharge Disposition			
Home	8 (53)	3 (37)	5 (71)
Inpatient Stroke rehabilitation	3 (20)	3 (37)	0 (0)
Skilled nursing facility	2 (13)	1 (12)	1 (14)
Hospice care	2 (13)	1 (12)	1 (14)

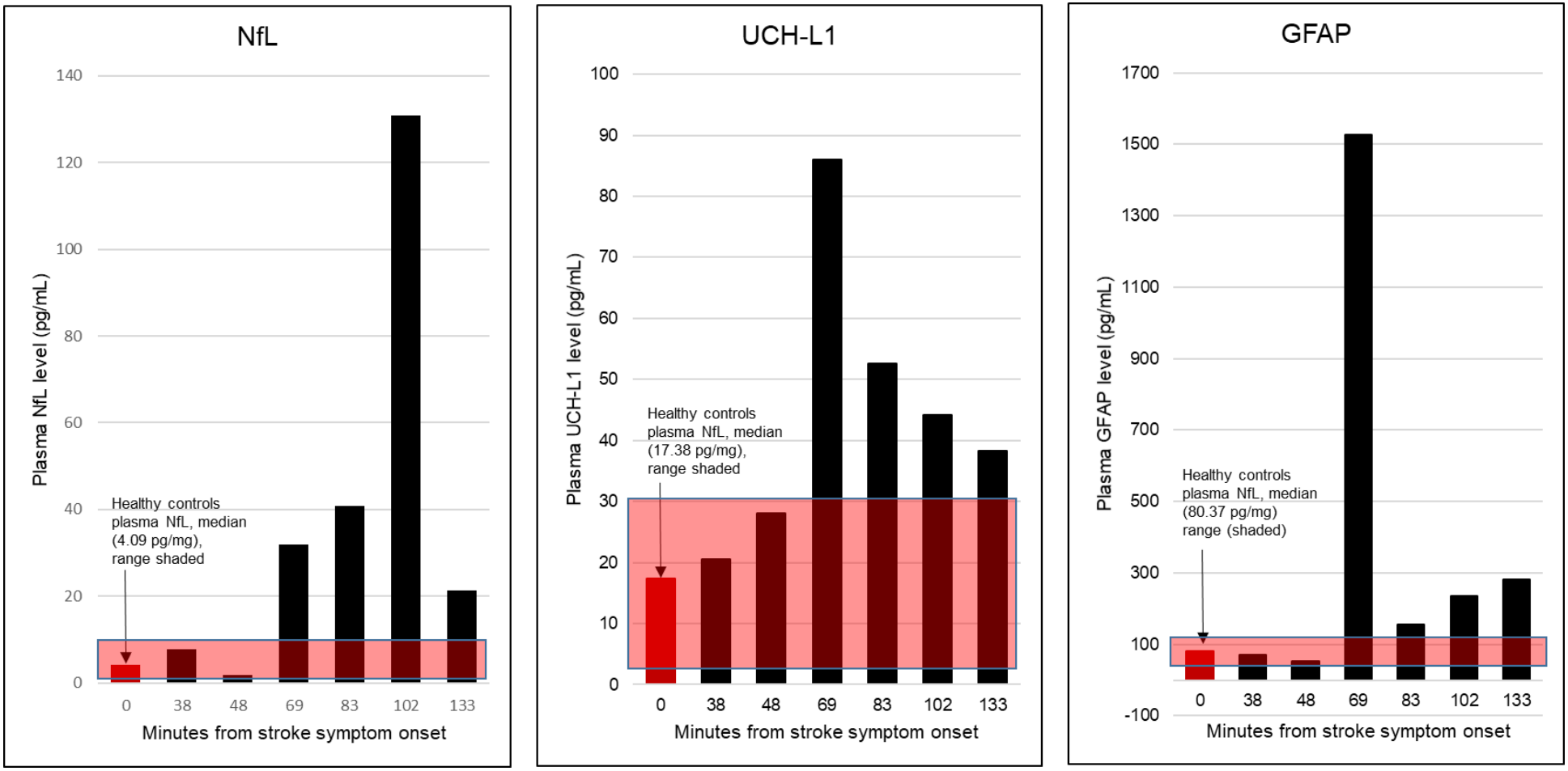
Data are N (%), median (IQR). NIHSS, National Institutes of Health Stroke Scale; MSU, mobile stroke unit.
*Includes one patient with suspected TIA, and one patient with a possible ischemic stroke aborted by tPA.
†Discharge NIHSS score available for 11 of 15 patients treated on mobile stroke unit.
‡Discharge NIHSS score available for 3 of 7 stroke mimic patients.

Figure S1. Stroke-onset and 24-hour plasma IL-6 levels, in confirmed stroke patients



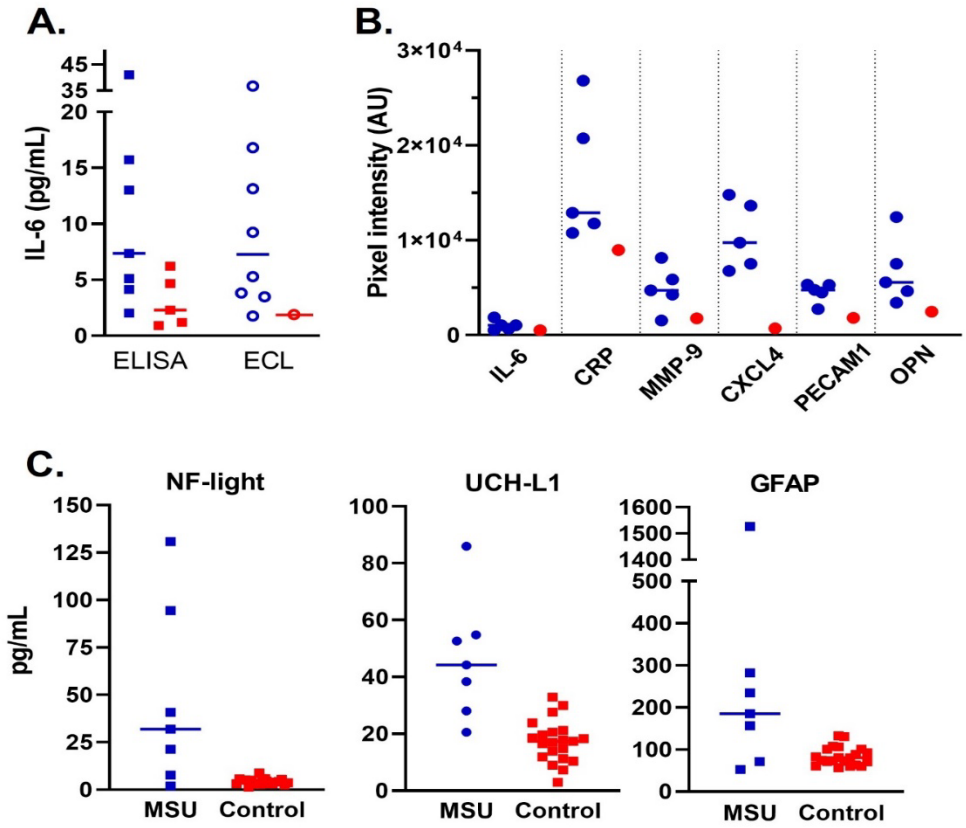
MSU, mobile stroke unit; IL-6, interleukin 6.
*Ischemic stroke, middle cerebral artery (MCA) territory.
**Thalamic intracerebral hemorrhage (ICH).
†Todd J., Simpson P., Estis J., Torres V., Wub A. H. Reference range and short- and long-term biological variation of interleukin (IL)-6, IL-17A and tissue necrosis factor-alpha using high sensitivity assays. *Cytokine.* Dec 2013;64(3):660-665.

Figure S2. Stroke-onset plasma protein and enzyme levels, in confirmed stroke patients*



NfL, neurofilament light chain; UCH-L1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein.
*Plasma biomarker levels measured with single molecule array (SIMOA).

Figure S3. Ultra-early plasma neuroinflammatory factor levels in acute stroke patients[†*]



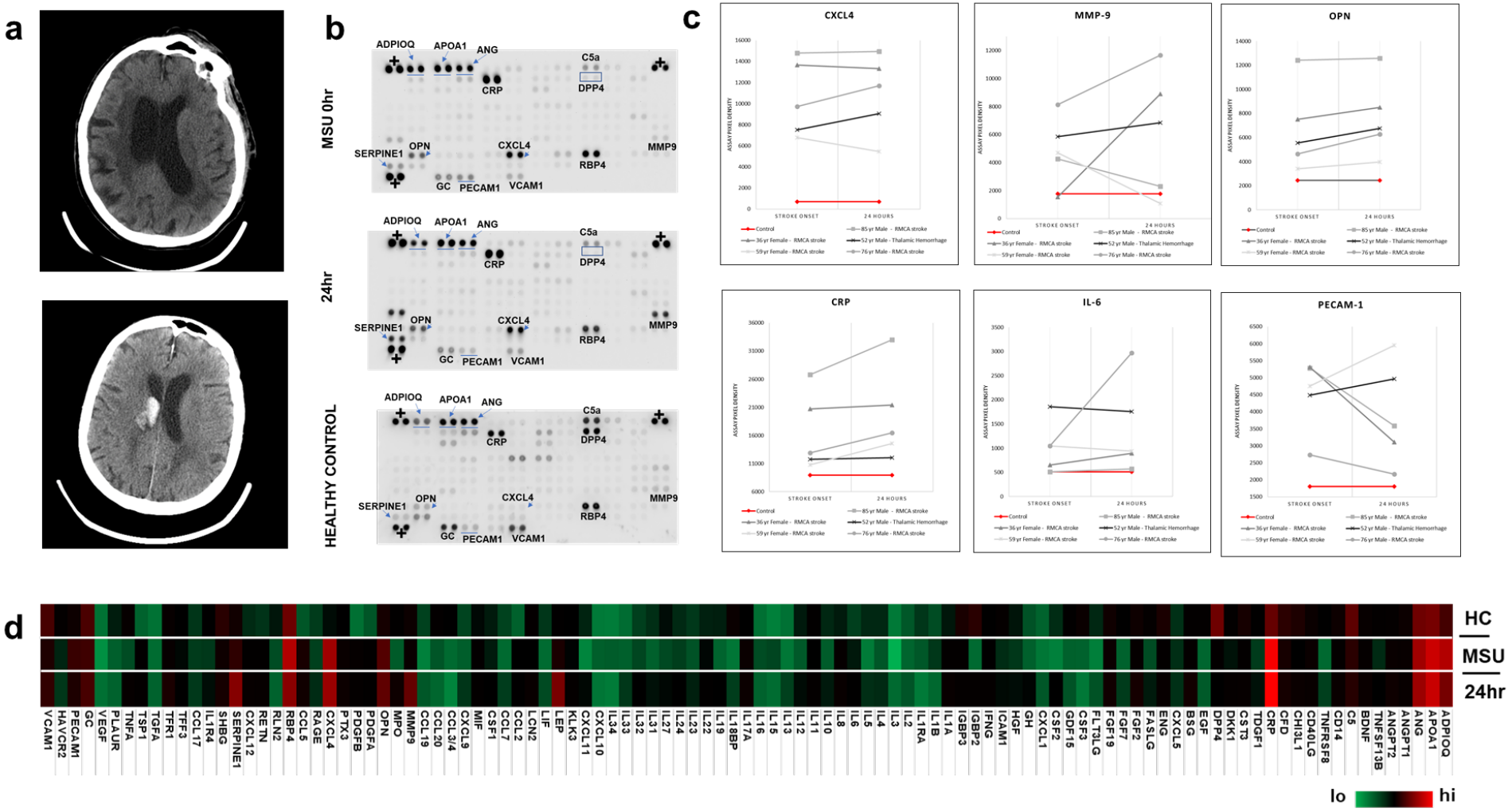
ECL, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; SIMOA, single molecule array; MSU, mobile stroke unit; IL-6, interleukin 6; NfL, neurofilament light chain; UCH-L1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein; CRP, C-reactive protein; MMP-9, matrix metalloproteinase 9; CXCL4, C-X-C motif ligand 4; PECAM-1, platelet endothelial cell adhesion molecule-1; OPN, osteopontin.

[†]All biomarkers are derived from plasma of blood collected from patients on the MSU immediately after stroke symptom onset.

*Values are presented as median and full range. Blue represents acute stroke patient; red denotes control subject or reference value.

A) IL-6 concentrations quantified for by ELISA and ECL. For ECL, the reference is 1.89 pg/mL (Todd J., Simpson P., Estis J., Torres V., Wub A. H. Reference range and short- and long-term biological variation of interleukin (IL)-6, IL-17A and tissue necrosis factor-alpha using high sensitivity assays. Cytokine. Dec 2013;64(3):660-665.) **B)** Biomarkers derived from extracellular vesicles via heat-shock protein pulldown from plasma. **C)** Biomarker concentrations quantified by SIMOA.

Figure S4. Extracellular Vesicle-derived biomarkers levels at stroke onset and 24 hours in confirmed stroke patients



Neuroinflammatory factor concentrations derived from a subset of extracellular vesicles (EVs) in circulating blood of confirmed stroke patients treated on the mobile stroke unit (MSU). A) computed tomography (CT) brain imaging for acute ischemic stroke (upper) and intracerebral hemorrhage (lower) patients included in EV analysis. B) Proteome Profiler Human XL Cytokine Array (R&D, Minneapolis, MN #ARY022B), for one patient, including stroke-onset blood drawn on MSU, 24-hour blood drawn during acute hospitalization, and pooled blood for healthy adult controls. C) comparison of stroke onset and 24-hour spot pixel density concentrations of neuroinflammatory factors. D) heatmap of neuroinflammatory factors measured from EVs isolated from MSU, 24-hour and healthy control plasma.

Figure S5. Extracellular vesicle-derived neuroinflammatory factors

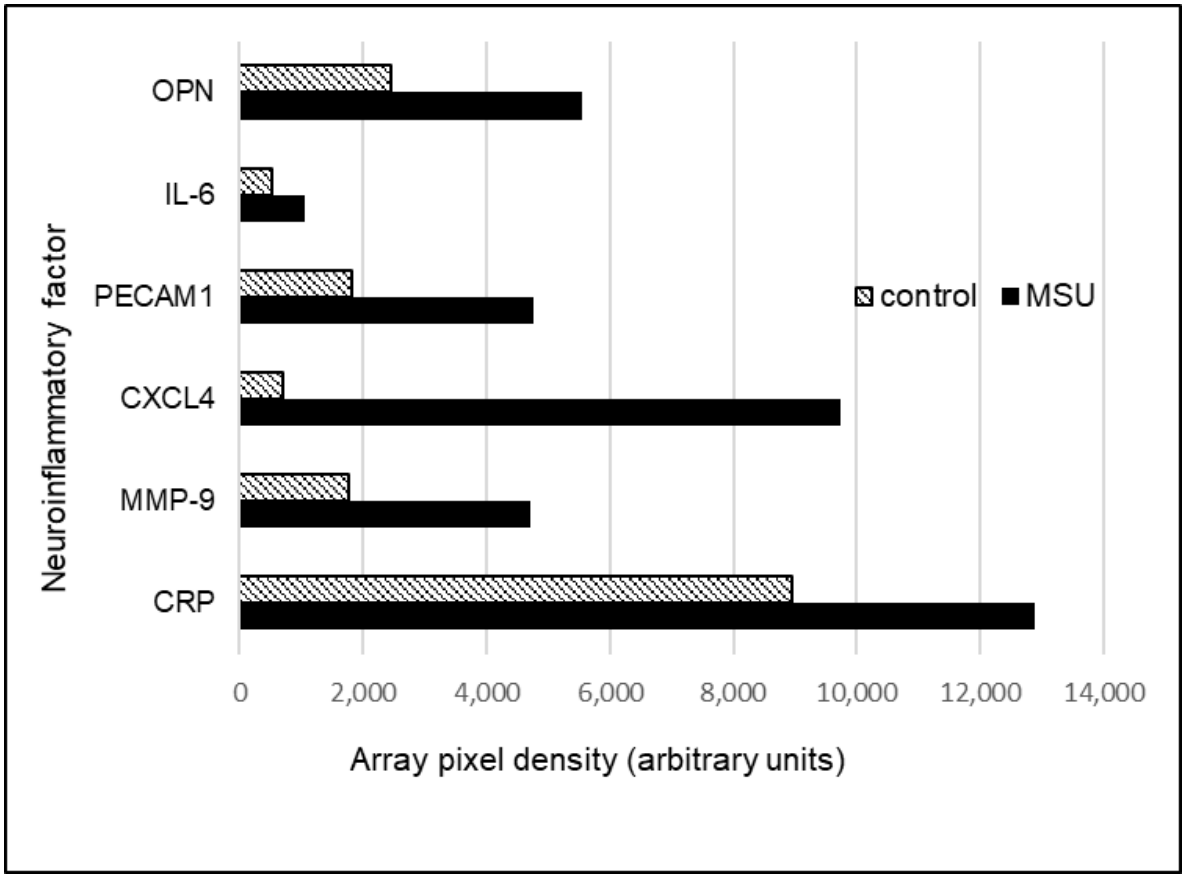
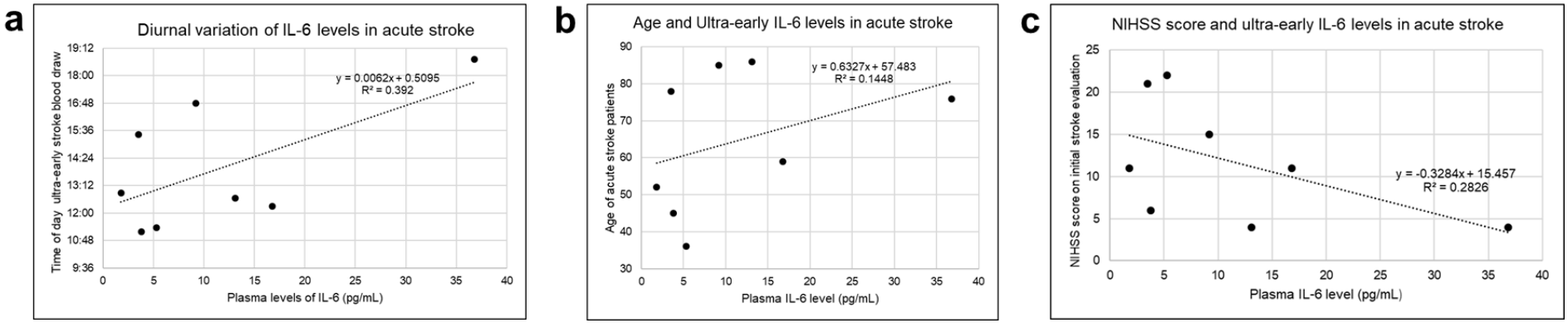


Figure S6. Comparison of stroke-onset plasma IL-6 concentration and time of day, patient age and NIHSS score.



Comparison of plasma Il-6 levels at the time of stroke onset with possible mitigating factors. A) Comparison of plasma IL-6 with time of day blood collected. B) Comparison of plasma IL-6 levels and patient age. C) Comparison of plasma IL-6 levels and initial National Institutes of Health Stroke Scale (NIHSS) score on the mobile stroke unit (MSU).