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Original research

Proteomic changes in intracranial blood during human ischemic stroke

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ABSTRACT

Background Since 2015, mechanical thrombectomy has been the standard treatment for emergent large vessel occlusion ischemic stroke.

Objective To investigate, using the previously published Blood and Clot Thrombectomy Registry and Collaboration (BACTRAC) protocol (clinicaltrials.gov NCT03153683), how the protein expression of a patient's intracranial blood during ischemic stroke compares with the protein expression of their systemic arterial blood in order to better understand and treat stroke.

Methods Plasma samples from 25 subjects underwent proteomic analysis, where intracranial protein expression was compared with systemic protein levels. Data including sex, comorbidities, infarct volume, and infarct time were included for each subject.

Results A majority of important proteins had a lower expression in intracranial blood than in systemic arterial blood. Proteins with the most significant changes in expression were: endopeptidase at -0.26 (p<0.0001), phospholipid transfer protein (PLTP) at -0.26 (p=0.0005), uromodulin (UMOD) at -0.14 (p=0.002), ficolin-2 (FCN2) at -0.46 (p=0.005), C-C motif chemokine 19 (CCL19) at -0.51 (p<0.0001), C-C motif chemokine 20 (CCL20) at -0.40 (p<0.0001), fibroblast growth factor 21 at -0.37 (p=0.0002), and C-C motif chemokine (CCL23) at -0.43 (p=0.0003).

Conclusions Evaluation of proteomic changes in the intravascular space of a cerebral infarct in progress in human subjects suggested that changes in proteins such PLTP, fetuin-B (FETUB), and FCN2 may be involved in atherosclerotic changes, and chemokines such as CCL23 are known to play a role in the Th2 autoimmune response. These data provide a scientific springboard for identifying clinically relevant biomarkers for diagnosis/ prognosis, and targets for much needed neuroprotective/ neuroreparative pharmacotherapies.

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INTRODUCTION

Each year approximately $800\,000$ individuals have a stroke, of which 87% are ischemic.¹

Emergent large vessel occlusions (ELVOs) represent 30–40% of ischemic strokes and are the most severe acute cerebral blockages. Many randomized clinical trials have demonstrated the superiority of mechanical thrombectomy over medical management alone; as a result, thrombectomy has become standard treatment for ELVO.^{2–5} At the University of Kentucky, we initiated the Blood and Clot Thrombectomy Registry and Collaboration (BACTRAC) tissue bank to evaluate molecular mechanisms of stroke in the human condition (clinicaltrials.gov NCT03153683).⁶ The BACTRAC protocol isolates intracranial blood within the artery immediately downstream of the clot, in the clot itself, and in systemic blood proximal to the clot. Since the establishment of BACTRAC, efforts have been focused on understanding acid–base balance, electrolyte chemistry, and transcriptomics at the site of the occlusion.⁷⁸ Here, we report novel proteomic data on blood distal to the thrombus, providing insight into the peri-infarct vascular microenvironment in human stroke.

METHODS

Sample acquisition and Olink analysis

BACTRAC is a continually enrolling tissue registry. Samples included for analyses presented here were obtained between May 11, 2017 and December 31, 2019. Other than pregnant or imprisoned individuals, all thrombectomy patients were considered for enrollment. The main exclusions occurred due to inability to obtain consent within the 72 hours window. Tissue samples were obtained and processed in accordance with the published BACTRAC protocol.⁶ During standard mechanical thrombectomy, a microcatheter is navigated distal to the thrombus to allow for a 1 cc aspiration of intracranial blood with a micro-syringe. Additionally, during thrombectomy, suction through the guide or distal access catheter allows systemic arterial blood samples to be obtained from the carotid system. These samples are obtained on the first pass of thrombectomy to reduce any risk of cross-contamination of specimens. Plasma was immediately isolated and frozen at -80° C. Aliquots $(40\,\mu\text{L})$ of plasma were randomized and placed into a 96-well plate (Starstedt, Nümbrecht, Germany), covered with Micro-Amp clear adhesion film (Thermo-Fisher Scientific), and shipped overnight on dry ice to Olink Proteomics (Olink Proteomics, Boston, Massachusetts, USA). Olink recommends that samples be randomized within the plate, so intracranial and systemic samples were randomly placed throughout the plate rather than in sequential order. Additionally, Olink recommends that previous samples from a prior run should be analyzed with each new sample set. Thus a previous sample was sent with this cohort of 25 new subjects. We requested the standardized cardiometabolic and



inflammatory panels on $1\,\mu L$ aliquots of plasma using a proximity extension assay. For this study, we compared the intracranial blood distal to the thrombus, using each subject's systemic arterial blood as an internal comparative control.

Protein analysis

Olink Proteomics provides a Normalized Protein eXpression (NPX), a unit that is in log2 scale which allows for individual protein analysis across a sample set. The intracranial versus systemic fold change in NPX was calculated by subtracting the systemic NPX (NPX) from the intracranial NPX (NPX). In order to determine which proteins had the most significant changes, a series of 184 paired t-tests were performed, one for each of the 92 cardiometabolic and 92 inflammatory proteins. Within each panel, proteins were then ranked based on the associated p values. Benjamini and Hochberg's linear step up procedure was used to control the false discovery rate at 0.05.9 Additionally, linear regression was used to determine the impact of potential confounding factors on the degree to which protein expression differs in the intracranial versus systemic blood. For the top changing proteins, the original fold change was controlled for using baseline National Institutes of Health Stroke Scale (NIHSS) values. The significance of baseline NIHSS scores and the percent change in the original compared with adjusted fold change values were used to determine the impact of these potential confounders. All data analysis was performed using SAS software version 9.4 (SAS Institute Inc., Cary, North Carolina, USA).

RESULTS

Patient characteristics

Subject demographics are shown in table 1. Twenty-five adult subjects were included in the study with a median age of 64 (24–91), of whom 15 (60%) were female. Ten subjects had a normal body mass index, 12 were overweight, and three were obese. Of note, 7 (28%) were current smokers, and 3 (12%) were previous smokers. According to the NIHSS score on admission, 1 (4%) of the patients had a minor stroke (NIHSS score 1–4), 9 (36%) were considered to have a moderate stroke (NIHSS score 5–15), and 8 (32%) were considered to have a moderate, 10 (40%) were considered to have a minor stroke, 11 (44%) were considered to have a moderate stroke a severe stroke (NIHSS score 21). The mean last known normal to thrombectomy completion time was 513 ± 246 min and the mean infarct volume was $58 172\pm82284$ mm³.

Proteomics

The cardiometabolic panel analyzed 92 protein biomarkers, including proteins involved in cellular metabolic processes, cell adhesion, immune response, and complement activation. The inflammatory panel was an immune assay of 92 inflammation-related protein biomarkers. Figures 1 and 2 demonstrate log2 proteomic fold change (NPX_i–NPX_s) and p values for cardiometabolic and inflammatory proteins, respectively. After controlling for the false discovery rate across all 184 proteins, 42 cardiometabolic and 53 inflammatory proteins had significantly different expressions in intracranial versus systemic blood. All these 95 proteins exhibited lower expression in the intracranial blood. For the cardiometabolic panel, the top fivefold changes were: prolyl endopeptidase (FAP) at -0.26 (p=0.0005), uromodulin (UMOD) at -0.14 (p=0.002), fetuin-B (FETUB) at

 Table 1
 Demographics and characteristics for thrombectomy subjects

	Value (%)	
Age (median; range)	64 (24–91)	
Sex		
Female	15 (60)	
Male	10 (40)	
BMI		
<24.9	10 (40)	
25–29.9	12 (48)	
30–39.9	3 (12)	
>40	0 (0)	
Comorbidities		
Hypertension	16 (64)	
Diabetes mellitus 2	4 (16)	
Hyperlipidemia	4 (16)	
Previous stroke	6 (24)	
Previous MI	1 (4)	
Smoking status		
Never	15 (60)	
Currently	7 (28)	
Previously (>6 months)	3 (12)	
NIHSS score on admission		
Minor stroke (1–4)	1 (4)	
Moderate stroke (5–15)	9 (36)	
Moderate/severe (16–20)	8 (32)	
Severe stroke (≥21)	7 (28)	
NIHSS score at discharge*		
Minor stroke (1–4)	10 (40)	
Moderate stroke (5–15)	12 (44)	
Moderate/severe (16–20)	0 (0)	
Severe stroke (≥21)	2 (4)	
TICI score		
2a = <50% perfusion	0 (0)	
2b = >50% perfusion	10 (40)	
3 = full perfusion	15 (60)	
LKN to thrombectomy		
Completion time (min)*	513±246	
Infarct volume (mm ³)†	58 172±82 284	
CTA collateral score†		
0	4 (16)	
1	16 (64)	
2	3 (12)	

Values are median with range, mean±SD, or (%).

*Data for one patient were missing (n=24).

†Data for two patients were were missing (n=23).

BMI, body mass index; CTA, CT angiography; LKN, last known normal; MI, myocardial infarction; NIHSS, National Institutes of Health Stroke Scale; TICI, Thrombolysis in Cerebral Infarction.

-0.31 (p=0.002), and ficolin-2 (FCN2) at -0.46 (p=0.005). For the inflammatory panel, the top fivefold changes were: C-C motif chemokine 19 (CCL19) at -0.51 (p<0.0001), C-C motif chemokine 20 (CCL20) at -0.40 (p<0.0001), fibroblast



Figure 1 Cardiometabolic panel volcano plot illustrating proteomic log2 fold changes in Normalized Protein eXpression (NPX) in intracranial blood compared with systemic blood. Labeled proteins include prolyl endopeptidase (FAP), phospholipid transfer protein (PLTP), fetuin-B (FETUB), uromodulin (UMOD), ficolin-2 (FCN2), and superoxide dismutase 1 (SOD1). Proteins with negative fold change are located to the left of the vertical line and indicate higher expression in systemic blood. Proteins located above the horizontal line are significant (p<0.05).

growth factor 21 (FGF21) at -0.37 (p=0.0002), transforming growth factor alfa (TGF- α) at -0.28 (p=0.0002), and C-C motif chemokine (CCL23) at -0.43 (p=0.0003). Although not statistically significant after controlling for the false discovery rate, the two proteins whose fold change in expression had the greatest increase in the intracranial blood were axin-1 (AXIN1) at 0.54 (p=0.035) and superoxide dismutase 1 (SOD1) at 0.33 (p=0.055) from the inflammatory and cardiometabolic panels, respectively. A table of all 95 significant proteins can be found in online supplementary table 1.

Table 2 presents results from the series of linear regression analyses predicting the fold changes from the 12 proteins labeled in the volcano plots controlling for baseline NIHSS values. All adjusted fold change values were significant. For the six cardiometabolic proteins, controlling for stroke severity



Figure 2 Inflammatory panel volcano plot illustrating proteomic log2 fold changes in NormalizedProtein eXpression (NPX) in intracranial blood compared with systemic blood. Labeled proteins include C-C motif chemokine 19 (CCL19), C-C motif chemokine 20 (CCL20), fibroblast growth factor 21 (FGF21), transforming growth factor alfa (TGF- α), C-C motif chemokine 23 (CCL23), and axin-1 (AXIN1). Proteins with negative fold change are located to the left of the vertical line and indicate higher expression in systemic blood. Proteins located above the horizontal line are significant (p<0.05). Changes in AXIN1 were found to be non-significant after controlling for the false discovery rate.

resulted in adjusted fold change values that were nearly identical to the originals (largest percent change=0%; smallest p value for NIHSS score predicting fold change=0.3955). For the top five inflammatory proteins with higher expression in the systemic blood, controlling for baseline NIHSS score resulted in an average adjustment of -15.06%. However, baseline NIHSS score was only significantly related to the fold change in TGF- α (p=0.025). Higher levels of baseline NIHSS values were associated with larger discrepancies in protein expression in intracranial blood compared with systemic blood.

DISCUSSION

In this study, we report significant proteomic changes occurring at the site of infarct in ischemic stroke in the human condition. Data provided here offer insight into biomolecular and cell signaling responses that occur hours after vessel occlusion. Of the cardiometabolic proteins, SOD1 is an antioxidant that is known to protect from reactive oxygen species after cerebral ischemia and reperfusion. Further, in mouse models, it has been shown that overexpression of SOD1 prevents the early release of mitochondrial cytochrome c after focal ischemia and reperfusion.¹⁰ These protective effects prove to be particularly interesting in these data, as SOD1 showed a trend (p=0.055) of higher expression in the intracranial blood and may be contributing to a neuroprotective response. The protein PLTP is present in human platelets and has been shown to play a role in the initiation of thrombin generation and platelet aggregation participating in hypercoagulation.¹¹ PLTP has also been shown to have hyperlipidemic properties and that increased expression can increase the risk of cardiovascular disease in humans.^{12 13} Additionally, FCN2 has been shown to be deposited in human carotid plaques and may play a role in complement activation and atherosclerosis.¹⁴ The protein FETUB has been reported to be elevated in patients with acute myocardial infarction in comparison with patients with stable angina.¹⁵ This study suggests that FETUB may play a role in acute myocardial infarction modulation, lipid deposition, and plaque-stabilizing factors in regards to ischemia. Future studies will investigate the roles of PLTP, FCN2, and FETUB in stroke predisposition and outcome, stratified based on factors such as coagulopathies, anticoagulation/antiplatelet medications, and dyslipidemia. Studies have reported that neuropeptides such as FAP play a role in central nervous system neurotransmission and neuromodulation. Specifically, administration of an FAP inhibitor may work to ameliorate memory impairment due to middle cerebral artery occlusion by restoring the decreased thyrotropin-releasing hormone activity in rodents.¹⁶ However, the literature is underdeveloped with regard to the relationship of FAP with acute ischemic stroke in humans and may provide an opportunity for these data to establish a novel relationship between FAP and ELVO stroke. Additionally, UMOD is known to be expressed in the thick ascending loop of the kidneys, and no clear relationship with ischemic stroke has been reported to our knowledge.

Of the inflammatory proteins, it has been reported that FGF21 may play a neuroprotective role against injury in cerebral microvascular endothelial cells during hypoxic stress.¹⁷ As this protein was found to have significantly lower expression levels in the intracranial samples, it may indicate a diminished or slower local neuroprotective response or extravasation of FGF21 into brain parenchyma. Future studies will investigate how FGF21 might relate to infarct time and infarct volume. In this panel, we also report a complex set of chemokine signaling occurring at the time of infarct. CCL23 is known to be a chemotactic agent, probably involved in inflammation and atherosclerosis.¹⁸ CCL23

Protein panel	Original fold change		Adjusted fold change			NIHSS score at admission significance	
	NPX _i -NPX _s	P value	NPX _i -NPX _s	% Change*	P value	Slope estimate	P value
Cardiometabolic							
FAP	-0.2604	<0.0001	-0.2604	0.00%	0.0001	-0.0072	0.3955
PLTP	-0.2647	0.0005	-0.2647	0.00%	0.0006	0.0012	0.9018
FETUB	-0.3136	0.0023	-0.3136	0.00%	0.0028	-0.0034	0.8104
UMOD	-0.1437	0.0020	-0.1437	0.00%	0.0024	-0.0030	0.6362
FCN2	-0.4628	0.0045	-0.4628	0.00%	0.0053	0.0076	0.7375
SOD1	0.3290	0.0548	0.3290	0.00%	0.0601	-0.0043	0.8631
Inflammatory							
CCL19	-0.5111	< 0.0001	-0.4358	-14.73%	<0.0001	-0.0186	0.1261
CCL20	-0.4034	<0.0001	-0.3483	-13.66%	<0.0001	-0.0137	0.1839
FGF21	-0.3687	0.0002	-0.3114	-15.54%	<0.0001	-0.0122	0.2035
TGFα	-0.2839	0.0002	-0.2422	-14.69%	<0.0001	-0.0173	0.0250
CCL23	-0.4255	0.0003	-0.3546	-16.66%	< 0.0001	-0.0155	0.1730
AXIN1	0.5389	0.0349	0.5611	4.12%	0.0382	0.0130	0.7345

*(Original fold change – adjusted fold change)/original fold change.

AXIN1, axin-1; CCL, C-C motif chemokine; FAP, prolyl endopeptidase; FCN2, ficolin-2; FETUB, fetuin-B; FGF21, fibroblast growth factor 21; PLTP, phospholipid transfer protein; SOD1, superoxide dismutase 1; TGF α , transforming growth factor alfa; UMOD, uromodulin.

blood levels can discriminate brain damaging diseases and may be a biomarker of stroke prognosis and predictor of patient outcome at hospital discharge.¹⁹ The same study found that patients with acute cerebral injury present with higher baseline levels of circulating CCL23. This response may be more systemic rather than local, as our study demonstrates lower expression levels of CCL23 at the site of infarct than systemically. Future studies using age-matched controls will help establish timeline and locality of this chemokine response. The interaction of CCL19 and its receptor CCR7 may play an important role in arteriogenesis in ischemia, as well as the migration and homing of T-lymphocytes.²⁰ Reduced intracranial levels of CCL23 and CCL19 may be due to chemokine binding, chemokine activation timeline, or extravasation out of the intraluminal space.

Linear regression analysis showed that a higher baseline NIHSS score was associated with a greater difference between TGF- α expression in the systemic blood than in intracranial blood samples. It has been shown that in mice, treatment with TGF- α can reduce infarct size, and that TGF- α may play a role in angiogenesis, neurogenesis, and neuroprotection after stroke.²¹

Here, we report that stroke severity, based on baseline NIHSS score, relates to the discrepancy of expression levels between intracranial and systemic arterial blood samples. This finding may be due to diminished peri-infarct levels of TGF- α , leading to a dampened neuroprotective effect. Future studies will further investigate the role TGF- α plays in stroke severity, infarct volume, and functional recovery.

Previously, we reported that genes related to a Th2 autoimmune response are elevated in intracranial blood from subjects during thrombectomy.⁸ Changes in CCL23 support the gene data as CCL23 is induced by interleukin 4, a classic Th2 cytokine.²² Proteins are drug targets for ischemic stroke.²³ Th2 drug targets already exist and have been developed to alter pathways for conditions such as asthma.²⁴ Understanding these responses occurring at the site of infarct in ischemic stroke will open doors for drug development and particularly, in the area of repurposing FDA approved agents for use as an adjuvant for the thrombectomy procedure.

Interestingly, all significant proteins reported here were found to have a lower level of expression in the intracranial blood than in systemic blood. This finding may be due to a combination of factors. It may indicate proteomic extravasation into brain parenchyma as many of these proteins are inflammatory, reactive, and migratory. It may be a result of the protein expression timeline and how that relates to infarct time for each subject with stroke. Other possible explanations include cellular binding of proteins preventing extraction from plasma, low levels of collateral blood supply precluding protein influx into the intraluminal sample space, or a robust systemic response blunting our primary measure, change in NPX. Lastly, this finding may be related to the cohort of patients with varying stroke severity, baseline characteristics, and stroke etiology. As our tissue registry continues to grow, we will be able to stratify these data for future analyses to investigate potential confounders and mechanisms that may influence intracranial expression levels in relation to systemic expression levels.

A recent study investigated leukocyte invasion into the site of infarct by isolating blood distal to the clot as well as carotid and femoral control samples.²⁵ They reported that the total number of leukocytes, primarily neutrophils, were significantly increased in the ischemic area compared with the control samples. Additionally, they reported increased levels of CXCL11 under occlusion conditions. As their study primarily investigated leukocytes, our study provides a novel proteomic view of the intracranial space during this inflammatory response. As our cohort grows, we plan to investigate how the proteomic data presented here relate to sex, comorbidities, pharmacotherapies, infarct time, infarct volume, stroke severity, and functional recovery. Future planned studies will include data points such as 3-month modified Rankin Scale scores, 3-month Montreal Cognitive Assessment, and 3-month/6-month neurocognitive batteries. We expect these future studies will elucidate relationships between individual proteins or patterns of proteins and their role in stroke predisposition, severity, and recovery. We hope these data will provide an impetus towards uncovering biomarkers or potential biosignatures used in prognostication and treatment.

Basic science

Our study has some notable limitations. Inherent in studies of human disease are possibilities for heterogeneity that can limit conclusions. Variations in proteases, ischemia timeline, patient comorbidities, reperfusion injury, and basal levels of systemic proteins could confound our results. However, these would tend to reduce the significance of recognized changes. As such, our data may represent a muted expression of true protein changes. Future planned studies to expand the cohort, both at our institution and at multicenters will help to reduce this variance.

Technical specifics of protein detection represent another limitation. A subset of our data were listed by Olink as below the limit of detection (LOD). We followed Olink guidelines, and chose to include actual data below LOD because of our multiplate study, where LOD is a conservative measurement. This is a technical limitation in the detection assays, but it underscores the need in future studies to use multiple methodologies to study individual protein changes. This is the value of translational multidisciplinary collaborations. Using these human data to select an array of protein changes, we will, in future studies, take the significantly altered protein changes, and study them using in vivo and in vitro models.

Another potential limitation is the sample size. Though the sample size was small (n=25), we still managed to find significant changes in 95 proteins after controlling for the false discovery rate. Across all proteins, the median achieved power was relatively low at 52% (IQR 23%–65%), but was adequate for the top 10 proteins at 72%. Although these findings are by no means definitive, they help to identify proteins that warrant future research. Finally, the nature of our study cohort limits the generalizability of the findings. In central Kentucky, our population with stroke is primarily Caucasian, with significant notable co-morbidities, which may be different in other populations.²⁶ This does not negate our results, but emphasizes the importance of our planned future multi-institutional collaborations.

CONCLUSIONS

We evaluated inflammatory and cardiometabolic proteomic changes in the intravascular space of a cerebral infarct in progress in human subjects by comparing intracranial protein expression with systemic expression. These data are preliminary, but they provide insight into acute changes in protein activities in the brain during ischemia. Our banking process provides a vital road map to others for future research. Finally, these changes may provide a scientific springboard for identifying clinically relevant biomarkers for diagnosis/prognosis, and targets for much needed neuroprotective/neuroreparative pharmacotherapies.

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Basic science

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