Proteomic discovery in sickle cell disease: Elevated neurogranin levels in children with sickle cell disease

Eboni I. Lance¹,², Lisa M. Faulcon³, Zongming Fu⁴, Jun Yang⁵, Donna Whyte-Stewart⁴, John J. Strouse⁴,⁶, Emily Barron-Casella⁴, Kimberly Jones⁴, Jennifer E. Van Eyk⁷,⁸, James F. Casella⁴, Allen D. Everett⁵

¹Department of Neurodevelopmental Medicine, Kennedy Krieger Institute, Baltimore, Maryland, USA
²Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
³Food and Drug Administration, Silver Spring, Maryland, USA
⁴Division of Pediatric Hematology, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
⁵Division of Pediatric Cardiology, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
⁶Division of Hematology, Department of Medicine, Duke University School of Medicine, Durham, North Carolina
⁷Division of Cardiology, Department of Internal Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
⁸The Smidt Heart Institute, Cedars-Sinai Medical Center, Los Angeles, California, USA

Abstract

Purpose: Sickle cell disease (SCD) is an inherited hemoglobinopathy that causes stroke and silent cerebral infarct (SCI). Our aim was to identify markers of brain injury in SCD.

Experimental Design: Plasma proteomes were analyzed using a sequential separation approach of hemoglobin (Hb) and top abundant plasma protein depletion, followed by reverse phase separation of intact proteins, trypsin digestion, and tandem mass spectrometry. We compared plasma proteomes of children with SCD with and without SCI in the Silent Cerebral Infarct Multi-Center Clinical Trial (SIT Trial) to age-matched, healthy non-SCD controls.
Results: From the SCD group, 1172 proteins were identified. Twenty-five percent (289/1172) were solely in the SCI group. Twenty-five proteins with enriched expression in the human brain were identified in the SCD group. Neurogranin (NRGN) was the most abundant brain-enriched protein in plasma of children with SCD. Using a NRGN sandwich immunoassay and SIT Trial samples, median NRGN levels were higher at study entry in children with SCD (0.28 ng/mL, N = 100) compared to control participants (0.12 ng/mL, N = 25, p < 0.0004).

Conclusions and Clinical Relevance: NRGN levels are elevated in children with SCD. NRGN and other brain-enriched plasma proteins identified in plasma of children with SCD may provide biochemical evidence of neurological injury.

Keywords
neurogranin; sickle cell disease; silent cerebral infarction; stroke

1 | INTRODUCTION

Sickle cell disease (SCD) is an inherited disorder of red blood cells that can have widespread systemic effects, including hemorrhagic or ischemic stroke and silent cerebral infarcts (SCI) [1,2]. SCI is defined as ischemic lesions at least 3 mm in diameter visible on T2-weighted magnetic resonance imaging (MRI) that are not associated with a focal neurologic deficit in the same vascular distribution [3–6]. SCI is associated with decreased neurocognitive function [7,8], and increased risk for new or enlarging SCI or stroke [9,10]. Surveillance MRI for SCI is costly and not done routinely at many institutions. The ability to detect children with SCD who are at risk for SCI remains limited, as no laboratory test for SCI exists. As a result, many individuals with SCD and SCI either remain undiagnosed or are diagnosed after they have SCI-related neurocognitive impairments [11]. With recent trials showing successful treatments for decreasing SCI recurrence and stroke, additional methods are needed for diagnosis, prognostication, and assessment of treatment response of SCI and other neurological complications in SCD [12–16].

Proteomic techniques have the potential to identify proteins associated with brain injury in children with SCD [17]. We hypothesized that children with SCD would have circulating brain-derived proteins in plasma that could be used as surrogate markers for subclinical brain injury and provide insight into the pathophysiology of the disease and that these markers may be elevated in children with SCD and SCI in comparison to children with SCD without SCI, or children with SCD when compared to control participants. In this study, LC–MS based proteomics was used to discover potential brain injury associated proteins, and then develop an immunoassay for detection of one of the brain-enriched proteins in plasma from children with SCD. For verification, neurogranin (NRGN), a neuron-specific signaling protein and one of the brain proteins identified in plasma samples from participants with SCD screened for the SIT Trial, was explored further using longitudinal plasma samples collected from participants in the SIT Trial.
2 | MATERIALS AND METHODS

2.1 | Study population

Plasma used in the discovery and verification SCD cohorts were obtained from participants enrolled in the IRB approved Silent Cerebral Infarct Multi-Center Clinical Trial (SIT) (ClinicalTrials.gov identifier NCT00072761), a multi-center, international clinical study, and stored in a biologic repository, as well as samples from participants with SCI and SCD who declined randomization in the SIT Trial, SCI negative participants with SCD who did not qualify for randomization for the SIT Trial, and healthy control participants without SCD [5]. Control participants were matched by group characteristics, specifically age, gender, and race. Children with SCD were screened for the SIT Trial with blood samples and MRI obtained at enrollment, followed by randomization of patients with SCI to either monthly transfusion or standard of care (observation). The plasma samples used for the proteomics discovery analyses were screening samples from two groups: children enrolled in the SIT Trial with SCD \((n = 15)\) and healthy age-matched children with no SCD \((n = 6)\), including three children who had sickle cell trait. Samples from children with SCD were divided into two groups: those with SCI \((n = 7)\) and those without SCI \((n = 8)\) matched for age, hemoglobin (Hb) and white blood cell counts (WBC).

For verification of the MS-identified protein NRGN, stored plasma samples obtained at various timepoints during the SIT Trial were tested for NRGN levels by immunoassay. The majority of these samples were from an ancillary study to collect longitudinal samples from SIT Trial participants that started after the SIT Trial had begun, at a subset of sites that agreed to participate. Groups of subjects in the treatment \((n = 68)\), observation \((n = 72)\), declined randomization \((n = 11)\), and SCI negative \((n = 43)\) groups had high quality proteomic samples collected longitudinally at 0, 6, 12, 24, and 36 months after enrollment. Eighty-nine participants (43 observation, 46 treatment) exiting during the ancillary study had proteomic grade exit samples; 30 participants had standard entry plasma samples that were analyzed. There was no overlap between the discovery group and the verification group samples. Samples that were not collected or handled according to protocol were not included in this study; all other samples were included. An additional 25 cross-sectional plasma samples from healthy, age, gender, and race comparable non-SCD pediatric controls unrelated to the SIT Trial, without evidence of acute/chronic illness (except for asthma, attentiondeficit-hyperactivity disorder (ADHD), mood disorders, bipolar disorder, sleep disorders, allergies, iron deficiency, thyroglossal duct cyst, and esotropia) were obtained from the Harriet Lane Pediatrics Clinic at the Johns Hopkins Hospital through a separate IRB-approved study. Figure 1 shows a flowchart of the study participants.

2.2 | Sample preparation and hemoglobin depletion

SIT Trial screening samples collected at study entry were shipped and stored at room temperature (storage time median 2 days, range 1 to 6 days), aliquoted and frozen at \(-80^\circ C\). Longitudinal proteomic-grade samples and healthy control participant samples were frozen at \(80^\circ C\) on site within a 4 h window after phlebotomy, and processed per the SIT Trial protocol [18]. Obvious hemolysis was observed in the SIT screening discovery SCD samples. To enrich for low abundance proteins, Hb was depleted from SCD plasma samples.
using nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen) [19]. Non-SCD discovery plasma samples had no observable hemolysis and were not subjected to this depletion step. In a separate study, NRGN levels were found to be stable after sitting at room temperature for 4 days [20].

2.3 | **Plasma abundant protein depletion and fractionation**

Using the ProteomeLab IgY-12 LC10 column kit (Beckman Coulter, Inc., Fullerton, CA) and the manufacturer’s protocol, samples underwent immunoaffinity depletion of the top 14 abundant plasma proteins [21]. Subsequently, 400 μg of the depleted protein samples were separated by reversed phase HPLC using PS-HPRP 2D (4.6 × 33 mm) columns (Beckman-Coulter, Inc.), also on a PF 2D LC platform (Beckman Coulter, Inc., Fullerton, CA). Solvent A was composed of 0.1% TFA in water and solvent B was 0.08% TFA in acetonitrile. The AB gradient was run from 5 to 15% B in 1 min, 15 to 25% in 2 min, 25 to 31% in 2 min, 31 to 41% in 10 min, 41 to 47% in 6 min, 47 to 67% in 4 min, finally up to 100% B in 3 min, held for 1 min, and back to 5% in 1 min at a flow rate of 1 mL/min. The resulting 39 reversed phase (RP)-HPLC fractions were collected in 1 mL 96-well plates. The fractionated proteins were neutralized, vacuum-dried, digested with sequencing-grade modified trypsin (Promega, Madison, WI) and desalted according to Sheng et al. [21].

2.4 | **MS analysis for protein identification**

Tandem (LC-MS/MS) experiments were performed on a linear trap quadrupole (LTQ)-Orbitrap ELITE mass spectrometer (ThermoFisher, San Jose, CA) equipped with an on-line nano-HPLC (Agilent Technologies, 1200 Series, Wilmington, DE), as previously described [19]. The MS raw data were analyzed using Proteomics Alternative Splicing Screening (PASS) (Integrated Analysis, Bethesda, MD) with X!Tandem searches (www.thegpm.org; version 2008.12.01) of the non-redundant International Protein Index (IPI) peptide database (human, 3.19). Peptide identifications were accepted if they could be established at greater than 95% probability and contained at least 2 unique identified spectra per peptide [22], with probability based Mowse scores greater than 35 ($p < 0.05$) and charge of +2. To remove protein name redundancy, the dataset was filtered based on 90% amino acid sequence homology using cluster database at high identity with tolerance (CD-HIT) [23]. All single peptide proteins had their MS spectrum manually validated. All isoforms were identified based on observed peptide to an amino acid sequence that is unique to the specific isoform.

2.5 | **Brain-enriched protein database**

To develop a brain-enriched protein list to query our plasma MS dataset, publicly available data sources for oligonucleotide microarray (http://www.genecards.org/index.shtml), expressed sequence tags (EST) (https://ncbiinsights.ncbi.nlm.nih.gov/2019/07/30/the-unigene-web-pages-are-now-retired) and serial analysis of gene expression (SAGE) databases (https://mitelmandatabase.isb-cgc.org) were used to identify proteins that are specific or enriched in the brain. When data were available, the Human Protein Atlas (http://www.proteinatlas.org/) was also used to confirm enriched brain protein expression.

Proteins were given a brain-enriched score based on their relative expression of transcripts in human brain, and 27 other normal human tissues as assessed by available microarray
Scoring criteria included: microarray data showing greater than ten-fold increase in expression over baseline, EST and SAGE data showing presence of the protein in less than two other tissues. Proteins received either a score of 1 or 0 for each category, with a maximum score of 3 when all three brain enrichment categories were met. A composite list of brain proteins meeting these criteria was used to filter the MS data to identify brain proteins in children with and without SCD and SCI.

2.6 | Ingenuity pathway analysis

Ingenuity Pathway Analysis (IPA) program (http://www.ingenuity.com) was used to analyze the pathway network of the proteins with abundance changes that were identified through MS. The protein accession numbers and corresponding expression values were uploaded as an Excel spreadsheet file into the Ingenuity software, which algorithmically generate networks between proteins with differential expression using the Ingenuity Knowledge Base. Each network is assigned a score, used to rank networks according to their relevance to the proteins in the dataset. A score > 2 is considered as a valid network. Identified networks were analyzed to rank significant biological functions. Biological functions were categorized into diseases/disorders, molecular/cellular functions, and physiological system development/function. Canonical pathways were grouped in metabolic and signaling pathways. Right-tailed Fisher’s exact tests were used to calculate p values to determine the probability of network assignment due to chance.

2.7 | Neurogranin (NRGN) ELISA

A human NRGN ELISA that our group developed was used as previously described [24], an electro-chemiluminescent sandwich immunoassay for NRGN based on the MesoScale Discovery platform (MesoScale Discovery, Gaithersburg, MD). A purified, mouse monoclonal anti-NRGN was used as the capture antibody, and an unlabeled polyclonal rabbit anti-NRGN was used for detection, and identified by a MesoScale Discovery Sulfo-TAG-labelled goat anti-rabbit antibody (R32AB). The standard curve (from 40–0.055 ng/mL) was constructed by serial dilutions of purified recombinant hNRGN in 1 X PBS containing 1% bovine serum albumin (SeraCare Life Sciences, Milford, MA).

2.8 | Statistical analyses

For the verification group, NRGN immunoassay concentration levels were analyzed in duplicate using parametric and non-parametric statistical tests to compare groups. We compared longitudinal changes in plasma concentration differences for NRGN using a multi-level mixed effects linear regression model. Spearman test was used to analyze the correlations between NRGN concentrations and other variables. Sample assays were repeated with appropriate controls if values had a coefficient of variation (CV%) greater than 20%. The average lower limit of quantification for the assay was 0.039 ng/mL and the average lower limit of detection for the assay was 0.012 ng/mL. Values of NRGN that were below the lower limit of quantification of the assay, but above the lower limit of detection of the assay, were recorded as half of the value of the lower limit of quantification for the assay. We also did a sensitivity analysis to look at the impact of processing time duration on NRGN values. All samples processed over a period of 4 days or greater were withheld...
while statistical analyses were repeated. A p value less than 0.05 was considered statistically
significant. Statistical analyses were conducted using Stata version 11.0 (StataCorp., College
Station, Texas).

3 | RESULTS

3.1 | Baseline characteristics of children with SCD and controls

Characteristics and differences between the discovery groups are presented in Tables 1 and 2. The SCI positive group had a total of 28 hospitalizations/emergency department visits for SCD related issues (pain crises, acute chest syndrome, asthma/respiratory symptoms) vs. 40 visits for the SCI negative group. The SCI positive group had a total of 57 lesions (mean 8.1, range 2 to 14), with an average total lesion volume of 7.4 (range 5.2 to 10.8). Data on lesion size was not available for one participant. None of the participants were on hydroxyurea at the time of screening for the SIT Trial.

3.2 | Characterization of the plasma proteome of children with SCD

In all, 819 fractions were quantified using LC-MS yielding 672460 spectra. Using X!
Tandem searches of the IPI Proteomics and Uniprot databases, we identified a total of 1172 unambiguous proteins in the plasma proteome of children with SCD (Figure 2 and Table S1). Excluding the proteins found in the control group, the SCI group uniquely contained 25% (289/1172), the SCI negative group uniquely contained 29% (335/1172) of these proteins, and 13% (148/1172) of proteins were common to both groups. Of the proteins identified, 239 proteins were found only in healthy controls, and not in SCD participants (Figure 2 and Table S2).

There were 23 proteins detected in at least two individuals of the SCI positive and SCI negative groups with spectral counts greater than two-fold difference between the two groups (Table 3). Inflammatory pathway proteins were commonly elevated in the SCI negative group, including L-selectin, a homing receptor for leukocytes to endothelial cells [25] and S100A11, a ligand for the receptor for advanced glycation end products (RAGE) receptor [26]. Complement proteins were also increased in the SCI negative group, including complement proteins C1q subcomponent subunits A and B [27], C4b-binding protein beta chain [28] and C8 gamma chain [29]. Platelet basic protein (CXCL7), a platelet-derived chemokine that functions to activate and attract neutrophils [30], was abundantly elevated in the SCI negative group. Elevated levels of teneurin-3 (TEMN3), involved in connectivity and axon guidance [31], were seen in the SCI negative group (6.5 fold) and cell death regulator Aven (AVEN), an apoptosis and caspase activation inhibitor [31], in the SCI positive group (2.5 fold).

Analysis using IPA revealed that the proteins identified in SCD plasma demonstrated overrepresentation of a number of biological pathways. Neurological disease was ranked among the top 5 diseases in the SCI positive group, but not in the SCI negative group. Additional IPA analysis revealed that proteins identified in the SCI group pathways are involved in more specific disease processes that have already been implicated in SCD, namely ischemia-reperfusion injury [32,33], endothelial dysfunction [34] and neuronal...
injury and death [35]. Specific protein pathways linked by IPA in this study include: (1) tauopathy (microtubule-associated protein tau [MAPT] and glial fibrillary acidic protein [GFAP]), (2) axon loss (MAPT) and (3) cerebral amyloid angiopathy (cystatin-C [CST3] and vimentin [VIM]).

### 3.3 Identification of brain proteins

An iterative process was used to identify circulating brain proteins from our discovery cohort. A review of publicly available oligonucleotide microarray, EST and SAGE databases identified 524 genes with increased messenger ribonucleic acid (mRNA) expression in brain (Table S3). Our MS protein identification data were filtered against this list of expressed brain proteins to produce a composite list of brain proteins found in plasma from children with SCD, but not found in plasma from age, gender and race-matched healthy control children. Using this methodology, we identified a total of 25 brain-specific proteins in plasma from children with SCD (Table 4). When we filtered the MS protein identification data for age-matched non-SCD controls against the list of expressed brain proteins listed in Table S3, we identified two brain-specific proteins: low density lipoprotein receptor-related protein 4 (LRP4; accession # O75096) and rabphilin (RPH3A accession# Q9Y2J0). These proteins were not found in plasma from children with SCD.

These brain-specific proteins are derived from both neuronal and astrocyte/glial cells across the brain. The proteins identified encompassed all cell compartments, but were predominately membrane-bound (11/25, 44%) and identified at relatively low levels (1 peptide, 18/25, 72%), as would be expected for a brain protein in plasma. The significance of low level detection in SCD has been demonstrated for GFAP, which was identified at the 2 peptide level [18,35]. The most abundant (spectral count = 15) brain protein identified in the plasma of children with SCD was NRGN, a small (7.6 kilodalton, 78 amino acids) calcium-dependent neuronal signaling protein not previously identified in plasma from patients with SCD [36]. Therefore, we developed an ELISA for NRGN for verification as described in Yang et al. [24].

### 3.4 Plasma NRGN levels in the verification cohort

We used the NRGN ELISA to verify the discovery proteomic data with plasma samples from the SIT Trial Biologic Repository from children with SCD and SCI (n = 152) and no SCI (n = 43), as well as from healthy children (n = 25). Table 5 shows the characteristics of the entire group of participants.

Using initial study visit samples (earliest available sample from either the participant’s screening or baseline visit), there was a significant difference in median NRGN levels between the SCD (n = 101) and pediatric healthy control groups (n = 25), (0.28 vs. 0.12 ng/mL, 25–75%IQR: 0.11–0.83 vs. 0.09–0.15 ng/mL, p < 0.0004) (Figure 3). Using the initial study visit samples from the SIT Trial, there was no significant difference in median NRGN levels between the SCI negative (n = 34) and SCI positive groups (n = 67) (0.54 vs 0.2 ng/mL, 25–75%IQR: 0.1–1.01 vs. 0.11–0.66 ng/mL, p = 0.27, 0.21 in sensitivity analysis). Given the expected significant difference in age between the SCI positive and SCI negative groups (Table 5), and as age is a known risk factor for SCI, we compared the mean...
age at the initial visit between the SCD and pediatric healthy control group and did not find a significant difference between the groups (111.4 vs. 126.5 months, 95% CI: 104.2–118.7 vs. 110.9 – 142.1, p = 0.07).

As shown in the supplemental analyses, there was no association of NRGN with age, neuropsychological measures of executive function or change over time in SCI or non-SCI groups.

4 | DISCUSSION

Biomarkers of subclinical brain injury in SCD are needed to diagnose and monitor therapy and disease progression, as well as aid in the development of molecular targeted therapies. Proteomics provides an opportunity to discover these biochemical markers in complex mixtures, such as plasma. Proteomic techniques have been used for biomarker discovery of brain proteins in a number of disease states, including brain cancer [37,38], Alzheimer’s disease [39,40], traumatic brain injury (TBI) [41,42], and stroke. [43,44]. However, very few studies have used plasma proteomics for clinical biomarker discovery in SCD [45]. We used a proteomic-based approach to test the hypothesis that children with SCD with and without SCI have brain proteins circulating in their plasma proteome that are associated with subclinical brain injury. We also explored the hypothesis that difference would be seen between children with SCD and normal control participants. We then verified our experimental identification of one circulating brain protein, NRGN, using longitudinal samples from children with SCD and SCI, children with SCD and without SCI, and healthy control participants.

Limited information is available regarding circulating biomarkers for neurologic and other complications of SCD. Using a targeted candidate approach, we have previously reported associations between the vascular stress proteins thrombospondin and L-selectin with SCI in SCD as well as neuronally secreted brain-derived neurotrophic factor (BDNF) in SCD participants in comparison to control participants [46,47]. As described in this study, we pursued a non-biased approach to identify circulating proteins that could differentiate SCD patients at risk of SCI. There were 239 unique proteins identified in the SCI discovery group. Similarly, Tewari et al. found elevated levels of 13 proteins in SCD pediatric participants with SCI in comparison to SCI negative participants with SCD, including one protein, fibrinogen gamma chain, which we also found to be elevated in our SCD group per spectral counts [48]. Kakhniashvili et al. used two-dimensional fluorescence difference gel electrophoresis (2D DIGE) and tandem MS (LC-MS/MS) to evaluate quantitative changes in the red blood cell (RBC) membrane proteome and described elevations of proteins involved in repair in SCD after oxidative stress [49]. Others have used SELDI-TOF and MALDI-TOF MS to evaluate biomarkers of pulmonary hypertension [50] and acute painful episodes [51] in SCD.

In our proteomics study, use of complementary and overlapping mRNA/protein databases (SAGE, EST, microarray and Human Protein Atlas) identified 524 expressed genes enriched in the brain. This brain-enriched gene list may implicate neuroaxonal injury in the pathophysiology of subclinical brain injury in children with SCD. Potential
mechanisms for this neuroaxonal damage include mitochondrial injury and defects in calcium homeostasis [52] (copine-6), damaging changes in sodium channel function [53] (amiloride-sensitive cation channel 4), glutamate receptor activation [54] (glutamate receptor, metabotropic 4 variant), invading proteolytic enzymes [55] (β-Ala-His dipeptidase/carnosinase), neuronal damage (NRGN) [56], as well as impaired neurite outgrowth [57] (SLIT and Neurotrophic Tyrosine Kinase Receptor [NTRK]-like protein 3), neuronal differentiation [58,59] (transcription factor SRY-Box Transcription Factor 3 [SOX]–3 and neuronal membrane glycoprotein M6-a).

The results from IPA suggest that children with SCD are at risk for neuronal injury and cell death, through tauopathy and axonal loss. These analyses suggest that the presence in plasma of GFAP, a known biomarker of stroke and traumatic brain injury, could be due to brain injury in children with SCD and SCI. GFAP is elevated in participants with SCD when compared to healthy controls and associated with ischemic brain injury, and inversely correlated with performance IQ [18,35]. Similarly, MAPT, an axonal cytoskeletal protein that has been implicated in several neurodegenerative disorders [60] and TBI [61], was detected in the plasma of SCD children (average spectral count = 2). Abnormal phosphorylation of MAPT can lead to the formation of neurotoxic insoluble tau aggregates, which results in loss of neurons [62]. The identification of MAPT suggests a potential role for axonal loss in the pathophysiology of SCI in children with SCD. Furthermore, CST3, a basic protein that inhibits cysteine proteases implicated in cerebral amyloid angiopathy and neuroprotective in TBI [63,64], was identified in both SCI positive and SCI negative groups (average SC = 7.2). CST3 has been used as an indicator of renal glomerular dysfunction in participants with SCD [65,66], but has not been studied in subclinical brain injury in SCD.

We measured our most abundant MS discovery protein, NRGN, with a new ELISA in a cohort of children with SCD from the SIT Trial and a group non-SCD control children of similar age, gender and race. NRGN levels were significantly different between non-SCD controls and SCD participants at enrollment. When studied longitudinally in SIT participants, NRGN levels were not significantly different between the SCI observation and SCI transfusion treatment groups and did not significantly change over time.

The significance of elevated levels of NRGN, a neuron-specific signaling protein, in the blood of children with SCD is presently unknown; however, circulating levels of NRGN likely reflect cellular injury, especially necrosis. Elevated levels of NRGN have been found in the serum of individuals with TBI [24] and plasma NRGN levels correlate with infarct volume in adult acute ischemic stroke patients [67]. Most studies have investigated NRGN genetic polymorphisms in adults with schizophrenia [68–70] and in cerebrospinal fluid (CSF) in Alzheimer’s disease [71,72], relating NRGN to learning and memory impairment [73–75]. Another study noted that NRGN levels decreased in cognitively intact older adults using two samples collected between 3 and 11 year intervals [76]. NRGN is of particular interest in regards to SCD, as a calcium-sensitive, calmodulin-binding, neuron-specific signaling protein, which has been implicated in synaptic development and remodeling [73], thyroid hormone signaling [77], stroke [67] and learning [78]. Its role in cognition is also demonstrated in NRGN knockout mice, which have structurally normal brains, but considerable learning deficits [79]. While we did not see differences in NRGN levels.
between participants with SCD with and without SCI, this could be due to the timing of the blood draws or the sensitivity of the NRGN assay may not be able to discriminate the SCI– and SCI+ groups.

The conclusions of this evaluation are limited by several factors. For example, the SIT Trial samples were not designed to measure time-dependent correlations with acute brain injury. Also, children with the highest risk of stroke (elevated transcranial Dopper [TCD] velocity) were excluded from the trial, which precludes us from determining a causal relationship between TCD velocities and NRGN or other lead proteins identified. In addition, a small number of samples were used for the initial proteomic discovery analysis and a limited number of control samples were available for the verification assays, though matched for group characteristics. The amount of mass spectrometry time required for the study design precluded doing larger sample sizes, however, the initial step is intended only for identification of candidate proteins, and the study design compensates for weaknesses of small sample size during discovery with larger subsequent validation cohorts. Furthermore, we have previously shown that the use of nickel beads for Hb depletion makes relative concentration determinations of some proteins challenging [19]. Hb depletion using Ni-NTA beads was only done in the SCD group, as excess plasma Hb was not present in the control group; therefore, our list of brain proteins in plasma from children with SCD may not be exhaustive for proteins involved in the pathophysiology of subclinical brain injury, and ratios of spectral counts after depletion may have been affected by plasma hemoglobin levels. This may have contributed to discrepant results between the discovery cohort and the verification results and the L-selectin levels between the current study and our prior results (L-selectin levels were higher in the plasma of individuals with SCI compared to those with no SCI in the prior study [47], whereas spectral counts were lower in patients within SCI in the current study). These differences may also reflect the limitation of quantitation of protein levels using spectral counts; however, these factors should not have affected the results of the verification assays in the current study [19].

In summary, we have developed and verified a proteomic workflow for brain biomarker discovery in children with SCD. We are the first to report significant elevations of NRGN in children with SCD as compared to non-SCD controls. While this study focused largely on NGRN, the ultimate value of this study may be in the numerous other brain proteins potentially involved in brain injury in SCD that deserve additional investigation. Collectively, these findings support further proteomic discovery research in children with SCD, which may provide new biomarkers for determining extent of disease, following the course of injury and response to therapy, predicting brain injury and establishing potential targets for therapeutic drug discovery.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the Monoclonal Antibody Core Facility (MACF) at Johns Hopkins University, Department of Neuroscience. This work was supported by grants from the National Institutes of Health National
The current affiliation for LMF is the Food and Drug Administration, Silver Spring, MD. Although the author is a FDA/CTP employee, this work was not done as part his/her official duties. This publication reflects the views of the author and should not be construed to reflect the FDA/CTP’s views or policies. The current affiliation for JJS is Division of Hematology, Duke University School of Medicine, Durham, NC. The current affiliation of J.V.E. is Biostatistics and Informatics Core, Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, USA.

CONFLICT OF INTEREST

J.F.C. has received an honorarium and travel expenses and received salary support in the past through Johns Hopkins for providing consultative advice to Mast Pharmaceuticals (previously Adventrx Pharmaceuticals) regarding a clinical trial of an agent for treating vaso-occlusive crisis in sickle cell disease. Under a license agreement between ImmunArray Ltd. and the Johns Hopkins University, J.F.C., J.V.E. and A.D.E. are entitled to royalties on an invention described in this article. In addition, A.D.E. and J.V.E. were paid consultants to Immunarray, Ltd. and E.B.C. is the spouse of J.F.C.. These arrangements have been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies. The other authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The SIT Trial data used in this manuscript are available on request from the corresponding author. The MS data are not publicly available due to patient consent issues.

Abbreviations:

| Abbreviation | Description                        |
|--------------|------------------------------------|
| SCD          | sickle cell disease                |
| SCI          | silent cerebral infarcts           |
| MRI          | magnetic resonance imaging         |
| NRGN         | neurogranin                        |
| SIT Trial    | Silent Cerebral Infarct Multi-Center Clinical Trial |
| Hb           | haemoglobin                        |
| WBC          | white blood cell counts            |
| ADHD         | attention-deficit-hyperactivity disorder |
| Ni-NTA       | nickel-nitrilotriacetic acid        |
| RP           | reversed phase                     |
| LTQ          | linear trap quadrupole             |
| PASS         | Proteomics Alternative Splicing Screening |
| IPI          | international protein index        |
| CD-HIT       | cluster database at high identity with tolerance |
| EST          | expressed sequence tags            |

Proteomics Clin Appl. Author manuscript; available in PMC 2022 September 01.
| Acronym | Description |
|---------|-------------|
| SAGE    | serial analysis of gene expression |
| NCBI    | National Center for Biotechnology Information |
| IPA     | Ingenuity Pathway Analysis |
| CV      | coefficient of variation |
| RAGE    | receptor for advanced glycation end products |
| CXCL7   | platelet basic protein |
| TEMN3   | teneurin-3 |
| AVEN    | aven |
| MAPT    | microtubule-associated protein tau |
| GFAP    | glial fibrillary acidic protein |
| CST3    | cystatin-C |
| VIM     | vimentin |
| mRNA    | Messenger ribonucleic acid |
| LRP4    | low density lipoprotein receptor-related protein 4 |
| RPH3A   | rabphilin |
| BRIEF   | behavior rating inventory of executive function |
| IQ      | intelligence quotient |
| WASI    | Wechsler Abbreviated Scale of Intelligence |
| TBI     | traumatic brain injury |
| BDNF    | brain-derived neurotrophic factor |
| RBC     | red blood cell |
| NTRK    | Neurotrophic Tyrosine Kinase Receptor |
| SOX     | SRY-Box Transcription Factor |
| CSF     | cerebrospinal fluid |
| TCD     | transcranial Doppler |

REFERENCES

1. Rees DC, Williams TN, & Gladwin MT (2010). Sickle-cell disease. Lancet, 376, 2018–2031. 10.1016/S0140-6736(10)61029-X [PubMed: 21131035]
2. Debaun MR, & Kirkham FJ (2016). Central nervous system complications and management in sickle cell disease. Blood, 127, 829–838. 10.1182/blood-2015-09-618579 [PubMed: 26758917]
3. Moser FG, Miller ST, Bello JA, Pegelow CH, Zimmerman RA, Wang WC, Ohene-Frempong K, Schwartz A, Vichinsky EP, Gallagher D, & Kinney TR (1996). The spectrum of brain MR abnormalities in sickle-cell disease: A report from the Cooperative Study of Sickle Cell Disease. Ajnr American Journal of Neuroradiology, 17, 965–972. [PubMed: 8733975]

4. Vendt BA, Mckinstry RC, Ball WS, Kraut MA, Prior FW, Barton B, Casella JF, & Debaun MR (2009). Silent cerebral infract transfusion (SIT) trial imaging core: Application of novel imaging information technology for rapid and central review of MRI of the brain. Journal of Digital Imaging, 22, 326–343. 10.1007/s10278-008-9114-3 [PubMed: 18398653]

5. Casella JF, King AA, Barton B, White DA, Noetzel MJ, Ichord RN, Terrill C, Hirtz D, Mckinstry RC, Strouse JJ, Howard TH, Coates TD, Minniti CP, Campbell AD, Vendt BA, Lehmah H, & Debaun MR (2010). Design of the silent cerebral infract transfusion (SIT) trial. Pediatric Hematology and Oncology, 27, 69–89. 10.3109/088800109033630637 [PubMed: 20201689]

6. Glauser TA, Siegel MJ, Lee BCP, & Debaun MR (1995). Accuracy of neurologic examination and history in detecting evidence of MRI-diagnosed cerebral infarctions in children with sickle cell hemoglobinopathy. Journal of Child Neurology, 10, 88–92. 10.1177/088307389501000203 [PubMed: 7782614]

7. Schatz J, Brown RT, Pascual JM, Hsu L, & Debaun MR (2001). Poor school and cognitive functioning with silent cerebral infarcts and sickle cell disease. Neurology, 56, 1109–1111. 10.1212/WNL.56.8.1109 [PubMed: 11320190]

8. Armstrong FD, Thompson RJ Jr, Wang W, Zimmerman R, Pegelow CH, Miller S, Moser F, Bello J, Hurtig A, & Vass K (1996). Cognitive functioning and brain magnetic resonance imaging in children with sickle cell disease. neuropsychology committee of the cooperative study of sickle cell disease. Pediatrics, 97, 864–870. 10.1177/088307389501000203 [PubMed: 7782614]

9. Pegelow CH (2002). Longitudinal changes in brain magnetic resonance imaging findings in children with sickle cell disease. Blood, 99, 3014–3018. 10.1182/blood.V99.8.3014 [PubMed: 11929794]

10. Miller ST, Macklin EA, Pegelow CH, Kinney TR, Sleeper LA, Bello JA, Dewitt LD, Gallagher DM, Guzini L, Moser FG, Ohene-Frempong K, Sanchez N, Vichinsky EP, Wang WC, Wethers DL, Younkin DP, Zimmerman RA, Debaun MR; Cooperative Study of Sickle Cell Disease (2001). Silent infarction as a risk factor for overt stroke in children with sickle cell anemia: A report from the cooperative study of sickle cell disease. Journal of Pediatrics, 139, 385–390.

11. Debaun MR, Armstrong FD, Mckinstry RC, Ware RE, Vichinsky E, & Kirkham FJ (2012). Silent cerebral infarcts: A review on a prevalent and progressive cause of neurologic injury in sickle cell anemia. Blood, 119, 4587–4596. 10.1182/blood-2011-02-272682 [PubMed: 22354000]

12. Adams RJ, Mckie VC, Brambilla D, Carl E, Gallagher D, Nichols FT, Roach S, Abboud M, Berman B, Driscoll C, Files B, Hsu L, Hurlet A, Miller S, Olivieri N, Pegelow C, Scher C, Vichinsky E, Wang W, …, Waclawiw MA (1998). Stroke prevention trial in sickle cell anemia. Controlled Clinical Trials, 19, 110–129. 10.1016/S0197-2456(97)00099-8 [PubMed: 9492971]

13. Adams R, & Brambilla D (2005). Optimizing Primary Stroke Prevention in Sickle Cell Anemia (STOP 2) Trial Investigators. Discontinuing prophylactic transfusions used to prevent stroke in sickle cell disease. New England Journal of Medicine, 353, 2769–2778.

14. Ware RE, Helms RW, Switch Investigators. (2012). Stroke with transfusions changing to hydroxyurea (SWITCH). Blood, 119, 3925–3932. 10.1182/blood-2011-11-392340 [PubMed: 22318199]

15. Debaun MR, Gordon M, Mckinstry RC, Noetzel MJ, White DA, Sarnaik SA, Meier ER, Howard TH, Majumdar S, Inusa BPD, Telfer PT, Kirby-Allen M, McCavit TL, Kamdem A, Airewele G, Woods GM, Berman B, Paneptino JA, Fuh BR, …, Casella JF (2014). Controlled trial of transfusions for silent cerebral infarcts in sickle cell anemia. New England Journal of Medicine, 371, 699–710. 10.1056/NEJMoa1401731

16. Ware RE, Davis BR, Schultz WH, Brown RC, Aygun B, Sarnaik S, Odame I, Fuh B, George A, Owen W, Luchtman-Jones L, Rogers ZR, Hilliard L, Gauger C, Piccone C, Lee MT, Kwiatkowski JL, Jackson S, Miller ST, …, Adams RJ (2016). Hydroxyurea versus chronic transfusion for maintenance of transcranial Doppler flow velocities in children with sickle cell anaemia–TCD With Transfusions Changing to Hydroxyurea (TWITCH): A multicentre, open-label, phase 3, non-inferiority trial. Lancet, 387, 661–670. 10.1016/S0140-6736(15)01041-7 [PubMed: 26670617]
17. Lance EI, Casella JF, Everett AD, & Barron-Casella E (2014). Proteomic and biomarker studies and neurological complications of pediatric sickle cell disease. PROTEOMICS – Clinical Applications, 8, 813–827. 10.1002/prca.201400069 [PubMed: 25290359]

18. Savage WJ, Barron-Casella E, Fu Z, Dulloor P, Williams L, Crain BJ, White DA, Jennings JM, Van Eyk JE, Debaun MR, Everett A, & Casella JF (2011). Plasma glial fibrillary acidic protein levels in children with sickle cell disease. American Journal of Hematology, 86, 427–429. 10.1002/ajh.21995 [PubMed: 21523806]

19. Williams LM, Fu Z, Dulloor P, Yen T, Barron-Casella E, Savage W, Van Eyk JE, Casella JF, & Everett A (2010). Hemoglobin depletion from plasma: Considerations for proteomic discovery in sickle cell disease and other hemolytic processes. PROTEOMICS – Clinical Applications, 4, 926–930. 10.1002/prca.201000054 [PubMed: 21179892]

20. Gutierrez S, Everett A, Bembea M, & Schwartz J (2014). Impact of delayed plasma storage on brain injury biomarker stability [abstract 533]. Critical Care Medicine, 42, A1488. 10.1097/01.ccm.0000458030.91776.65

21. Sheng S, Skalnikova H, Meng A, Tra J, Fu Q, Everett A, & Van Eyk J (2011). Intact protein separation by one- and two-dimensional liquid chromatography for the comparative proteomic separation of partitioned serum or plasma. Methods in Molecular Biology, 728, 29–46. [PubMed: 21468939]

22. Choi H, Fermin D, & Nesvizhskii AI (2008). Significance analysis of spectral count data in label-free shotgun proteomics. Molecular and Cellular Proteomics, 7, 2373–2385. [PubMed: 18644780]

23. Li W, & Godzik A (2006). Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics, 22, 1658–1659. 10.1093/bioinformatics/btl158 [PubMed: 16731699]

24. Yang J, Korley FK, Dai M, & Everett AD (2015). Serum neurogranin measurement as a biomarker of acute traumatic brain injury. Clinical Biochemistry, 48, 843–848. 10.1016/j.clinbiochem.2015.05.015 [PubMed: 26025774]

25. Biancone L, Cantaluppi V, Duó D, Deregibus MC, Torres C, & Camussi G (2004). Role of L-selectin in the vascular homing of peripheral blood-derived endothelial progenitor cells. Journal of Immunology, 173, 5268–5274. 10.4049/jimmunol.173.8.5268

26. Donato R (2007). RAGE: A single receptor for several ligands and different cellular responses: The case of certain S100 proteins. Current Molecular Medicine, 7, 711–724. 10.2174/156652407783220688 [PubMed: 18331229]

27. Stephan AH, Barres BA, & Stevens B (2012). The complement system: An unexpected role in synaptic pruning during development and disease. Annual Review of Neuroscience, 35, 369–389. 10.1146/annurev-neuro-061010-113810

28. Ermert D, & Blom AM (2016). C4b-binding protein: The good, the bad and the deadly. Nobel functions of an old friend. Immunology Letters, 169, 82–92. 10.1016/j.imlet.2015.11.014 [PubMed: 26658464]

29. Parker CL, & Sodetz JM (2002). Role of the human C8 subunits in complement-mediated bacterial killing: Evidence that C8Î± is not essential. Molecular Immunology, 39, 453–458. 10.1016/S0161-5890(02)00121-9 [PubMed: 12413696]

30. Brown AJ, Sepuru KM, Sawant KV, & Rajaratnam K (2017). Platelet-derived chemokine CXCL7 dimer preferentially exists in the glycosaminoglycan-bound form: Implications for neutrophil-platelet crosstalk. Frontiers in immunology, 8, 1248. 10.3389/fimmu.2017.01248 [PubMed: 29038657]

31. Antinucci P, Nikolaou N, Meyer MP, & Hindges R (2013). Teneurin-3 specifies morphological and functional connectivity of retinal ganglion cells in the vertebrate visual system. Cell Reports, 5, 582–592. 10.1016/j.celrep.2013.09.045 [PubMed: 24183672]

32. Chau BN, Cheng EHY, Kerr DA, & Hardwick JM (2000). Aven, a Novel Inhibitor of Caspase Activation, Binds Bcl-xxl and Apaf-1. Molecular Cell, 6, 31–40. [PubMed: 10949025]

33. Kato GJ, Hebbel RP, Steinberg MH, & Gladwin MT (2009). Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. American Journal of Hematology, 84, 618–625. 10.1002/ajh.21475 [PubMed: 19610078]
34. Kaul DK, & Hebbel RP (2000). Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. Journal of Clinical Investigation, 106, 411–420. 10.1172/JCI9225

35. Kato GJ, Martyr S, Blackwelder WC, Nichols JS, Coles WA, Hunter LA, Brennan M-L, Hazen SL, & Gladwin MT (2005). Levels of soluble endothelium-derived adhesion molecules in patients with sickle cell disease are associated with pulmonary hypertension, organ dysfunction, and mortality. British Journal of Haematology, 130, 943–953. 10.1111/j.1365-2414.2005.05701.x [PubMed: 16156864]

36. Savage WJ, Everett AD, & Casella JF (2011). Plasma glial fibrillary acidic protein levels in a child with sickle cell disease and stroke. Acta Haematologica, 125, 103–106. 10.1159/000321791 [PubMed: 21099215]

37. Prichard L, Deloulme JC, & Storm DR (1999). Interactions between neurogranin and calmodulin in vivo. Journal of Biological Chemistry, 274, 7689–7694. 10.1074/jbc.274.12.7689

38. Zhang R, Tremblay T-L, Mcdermid A, Thibault P, & Stamirovic D (2003). Identification of differentially expressed proteins in human glioblastoma cell lines and tumors. Glia, 42, 194–208. 10.1002/glia.10222 [PubMed: 12655603]

39. Odreman F, Vindigni M, Gonzales ML, Niccolini B, Candiano G, Zanotti B, Skrap M, Pizzolitto S, Stanta G, & Vindigni A (2005). Proteomic studies on low- and high-grade human brain astrocytomas. Journal of Proteome Research, 4, 698–708. 10.1021/pr0498180 [PubMed: 15952716]

40. Korolainen MA, Nyman TA, Aittokallio T, & Pirttilä T (2010). An update on clinical proteomics in Alzheimer’s research. Journal of Neurochemistry, 112, 1386–1414. 10.1111/j.1471-4159.2009.06558.x [PubMed: 20050976]

41. Di Domenico F, Sultana R, Barone E, Perluigi M, Cini C, Mancuso C, Cai J, Pierce WM, & Butterfield DA (2011). Quantitative proteomics analysis of phosphorylated proteins in the hippocampus of Alzheimer’s disease subjects. Journal of Proteomics, 74, 1091–1103. 10.1016/j.jprot.2011.03.033 [PubMed: 21515431]

42. Wang KK, Ottens AK, Liu MC, Lewis SB, Meegan C, Oli MW, Tortella FC, & Hayes RL (2005). Proteomic identification of biomarkers of traumatic brain injury. Expert Review of Proteomics, 2, 603–614. 10.1586/14789450.2.4.603 [PubMed: 16097892]

43. Zupanc GKH (2007). Proteomics of traumatic brain injury and regeneration. PROTEOMICS – Clinical Applications, 1, 1362–1372. 10.1002/prca.200700420 [PubMed: 21136636]

44. Sironi L, Tremoli E, Miller I, Guerrini U, Calvio AM, Eberini I, Gemenier M, Asdente M, Paolelli R, & Gianazza E (2001). Acute-phase proteins before cerebral ischemia in stroke-prone rats. Stroke; A Journal of Cerebral Circulation, 32, 753–760. 10.1161/01.STR.32.3.753

45. Ning M, Sarracino DA, Khon AT, Guo S, Lee S-R, Krastins B, Buonanno FS, Vizcaíno JA, Orchard S, Mcmullin D, Wang X, & Lo EH (2011). Proteomic temporal profile of human brain endothelium after oxidative stress. Stroke; A Journal of Cerebral Circulation, 42, 37–43. 10.1161/STROKEAHA.110.585703

46. Yuditskaya S, Suffredini AF, & Kato GJ (2010). The proteome of sickle cell disease: Insights from exploratory proteomic profiling. Expert Rev Proteomics, 7, 833–848. 10.1586/14789450.7.4.833 [PubMed: 21142886]

47. Faulcon LM, Fu Z, Duloor P, Barron-Casella E, Savage W, Jennings JM, Van Eyk JE, Debaun M, Casella JF, & Everett A (2013). Thrombospondin-1 and L-selectin are associated with silent cerebral infarct in children with sickle cell anemia. British Journal of Haematology, 162, 421–424. 10.1111/bjh.12374 [PubMed: 23672305]

48. Lance EI, Barron-Casella E, Everett AD, Casella JF (2020). Brain-derived neurotrophic factor levels in pediatric sickle cell disease. Pediatric Blood & Cancer, 67, e28076. 10.1002/pbc.28076 [PubMed: 31736231]

49. Tewari S, Renney G, Brewin J, Gardner K, Kirkham F, Inusa B, Menzel S, Thein SL, Ward M, & Rees DC (2018). Proteomic analysis of plasma from children with sickle cell anemia and silent cerebral infarction. Haematologica, 103, 1136–1142. 10.3324/haematol.2018.187815 [PubMed: 29545349]
50. Kakhniashvili DG, Griko NB, Bulla LA, & Goodman SR (2005). The proteomics of sickle cell disease: Profiling of erythrocyte membrane proteins by 2D-DIGE and tandem mass spectrometry. Experimental Biology and Medicine (Maywood, N.J.), 230, 787–792. 10.1177/153537020523001102

51. Yuditskaya S, Tumblin A, Hoehn GT, Wang G, Drake SK, Xu X, Ying S, Chi AH, Remaley AT, Shen R-F, Munson PJ, Suffredini AF, Kato GJ (2009). Proteomic identification of altered apolipoprotein patterns in pulmonary hypertension and vasculopathy of sickle cell disease. Blood, 113, 1122–1128. 10.1182/blood-2008-03-142604 [PubMed: 19023114]

52. Tumblin A, Tailor A, Hoehn GT, Mack AK, Mendelsohn L, Freeman L, Xu X, Remaley AT, Munson PJ, Suffredini AF, & Kato GJ (2010). Apolipoprotein A-I and serum amyloid A plasma levels are biomarkers of acute painful episodes in patients with sickle cell disease. Haematologica, 95, 1467–1472. 10.3324/haematol.2009.018044 [PubMed: 20378559]

53. Perestenko PV, Pooler AM, Noorbakhshnia M, Gray A, Bauccio C, & Jeffrey Mcilhinney RA (2010). Copines-1, −2, −3, −6 and −7 show different calcium-dependent intracellular membrane translocation and targeting. The Febs Journal, 277, 5174–5189. 10.1111/j.1742-4658.2010.07935.x [PubMed: 21087455]

54. Xiong Z-G (2007). Acid sensing ion channels - novel therapeutic targets for ischemic brain injury. Frontiers in Bioscience, 12, 1376–1386. 10.2741/2154 [PubMed: 17127388]

55. Gong QZ, Phillips LL, & Lyeth BG (1999). Metabotropic glutamate receptor protein alterations after traumatic brain injury in rats. Journal of Neurotrauma, 16, 893–902. 10.1089/neu.1999.16.893 [PubMed: 10547098]

56. Wassif WS, Sherwood RA, Amir A, Idowu B, Summers B, Leigh N, & Peters TJ (1994). Serum carnosinase activities in central nervous system disorders. Clinica Chimica Acta, 225, 57–64. 10.1016/0009-8981(94)90027-2

57. Watson JB, Sutcliffe JG, & Fisher RS (1992). Localization of the protein kinase C phosphorylation/calmodulin-binding substrate RC3 in dendritic spines of neostriatal neurons. PNAS, 89, 8581–8585. [PubMed: 1528865]

58. Aruga J, & Mikoshiba K (2003). Identification and characterization of slitrk, a novel neuronal transmembrane protein family controlling neurite outgrowth. Molecular and Cellular Neuroscience, 24, 117–129. 10.1016/S1044-7313(03)00129-5 [PubMed: 14550773]

59. Stevanovic M (2003). Modulation of SOX2 and SOX3 gene expression during differentiation of human neuronal precursor cell line NTERA2. Molecular Biology Reports, 30, 127–132. 10.1023/A:1023961009869 [PubMed: 12841584]

60. Michibata H, Okuno T, Konishi N, Kyono K, Wakimoto K, Aoki K, Kondo Y, Takata K, Kitamura Y, & Taniguchi T (2009). Human GPM6A is associated with differentiation and neuronal migration of neurons derived from human embryonic stem cells. Stem Cells and Development, 18, 629–639. 10.1089/scd.2008.0215 [PubMed: 19298174]

61. Rademakers R, Cruts M, & Van Broeckhoven C (2004). The role of tau (MAPT) in frontotemporal dementia and related tauopathies. Human Mutation, 24, 277–295. 10.1002/humu.20086 [PubMed: 15365985]

62. Liu T, Qian W-J, Griskenko MA, Xiao W, Moldawer LL, Kaushal A, Monroe ME, Varnum SM, Moore RJ, Purvine SO, Maier RV, Davis RW, Tompkins RG, Campbell D, & Smith RD; Inflammation and the Host Response to Injury Large Scale Collaborative Research Program. (2006). High dynamic range characterization of the trauma patient plasma proteome. Molecular and Cellular Proteomics, 5, 1899–1913. 10.1074/mcp.M600068-MCP200 [PubMed: 16684767]

63. Kimura T, Fukuda T, Sahara N, Yamashita S, Murayama M, Mizoroki T, Yoshiike Y, Lee B, Sotiropoulos I, Maeda S, & Takashima A (2010). Aggregation of Detergent-insoluble Tau Is Involved in Neuronal Loss but Not in Synaptic Loss*. Journal of Biological Chemistry, 285, 38692–38699. 10.1074/jbc.M110.136630

64. Alvarez O, Zilleruelo G, Wright D, Montane B, & Lopez-Mitnik G (2006). Serum cystatin C levels in children with sickle cell disease. Pediatric Nephrology, 21, 533–537. 10.1007/s00467-006-0033-6 [PubMed: 16491413]

65. Voskaridou E, Terpos E, Michail S, Hantzi E, Anagnostopoulos A, Margeli A, Simirloglou D, Loukopoulos D, & Papassotiriou I (2006). Early markers of renal dysfunction in patients

Proteomics Clin Appl. Author manuscript; available in PMC 2022 September 01.
with sickle cell/beta-thalassemia. Kidney International, 69, 2037–2042. 10.1038/sj.ki.5000248 [PubMed: 16501491]

66. Martinez-Vargas M, Gonzalez-Rivera R, Soto-Nuñez M, Cisneros-Martinez M, Huerta-Saquero A, Morales-Gomez J, Molina-Guarneros J, & Navarro L (2006). Recovery after a traumatic brain injury depends on diurnal variations. Neuroscience Letters, 400, 21–24. 10.1016/j.neulet.2006.02.010 [PubMed: 16519999]

67. Tizon B, Sahoo S, Yu H, Gauthier S, Kumar AR, Mohan P, Figliola R, Cisneros-Martinez M, Huerta-Saquero A, Uchiyama Y, Bandypadhyay U, Cuervo AM, Nixon RA, & Levy E (2010). Induction of autophagy by cystatin C: A mechanism that protects murine primary cortical neurons and neuronal cell lines. Plos One, 5, e9819. 10.1371/journal.pone.0009819 [PubMed: 20352108]

68. De Vos A, Bjerke M, Brouns R, De Roeck N, Jacobs D, Van Den Abbeele L, Guldolf K, Zetterberg H, Blennow K, Engelborghs S, & Vanmechelen E (2017). Neurogranin and tau in cerebrospinal fluid and plasma of patients with acute ischemic stroke. BMC Neurology [Electronic Resource], 17, 170. 10.1186/s12883-017-0945-8

69. Broadbelt K, Ramprasaud A, & Jones LB (2006). Evidence of altered neurogranin immunoreactivity in areas 9 and 32 of schizophrenic prefrontal cortex. Schizophrenia Research, 87, 6–14. 10.1016/j.schres.2006.04.028 [PubMed: 16797925]

70. Shen Y-C, Tsai H-M, Cheng M-C, Hsu S-H, Chen S-F, & Chen C-H (2012). Genetic and functional analysis of the gene encoding neurogranin in schizophrenia. Schizophrenia Research, 137, 7–13. 10.1016/j.schres.2012.01.011 [PubMed: 22306195]

71. Smith RL, Knight D, Williams H, Dwyer S, Richards A, Kirov G, O’donovan MC, & Owen MJ (2011). Analysis of neurogranin (NRGN) in schizophrenia. American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics, 156B, 532–535. 10.1002/ajmg.b.31191

72. Thorsell A, Bjerke M, Brouns R, De Roeck N, Jacobs D, Van Den Abbeele L, Guldolf K, Zetterberg H, Blennow K (2010). Neurogranin in cerebrospinal fluid as a marker of synaptic degeneration in Alzheimer's disease. Brain Research, 1362, 13–22. 10.1016/j.brainres.2010.09.073 [PubMed: 20875798]

73. Reddy PH, Mani G, Park BS, Jacques J, Murdoch G, Whetsell W, Kaye J, & Manczak M (2005). Differential loss of synaptic proteins in Alzheimer’s disease: Implications for synaptic dysfunction. Journal of Alzheimer's Disease, 7, 103–117. 10.3233/JAD-2005-7203

74. Pak JH, Huang FL, Li J, Balschun D, Reymann KG, Chiang C, Westphal H, & Huang K-P (2000). Involvement of neurogranin in the modulation of calcium/calmodulin-dependent protein kinase II, synaptic plasticity, and spatial learning: A study with knockout mice. PNAS, 97, 11232–11237. 10.1073/pnas.210184697 [PubMed: 11016969]

75. Huang K-P (2004). Neurogranin/RC3 enhances long-term potentiation and learning by promoting calcium-mediated signaling. Journal of Neuroscience, 24, 10660–10669. 10.1523/JNEUROSCI.2213-04.2004 [PubMed: 15564582]

76. Krug A, Krach S, Jansen A, Nieratschker V, Witt SH, Shah NJ, Nöthen MM, Rietschel M, & Kircher T (2013). The effect of neurogranin on neural correlates of episodic memory encoding and retrieval. Schizophrenia Bulletin, 39, 141–150. 10.1093/schbul/sbr076 [PubMed: 21799211]

77. Abner EL, Jicha GA, Shaw LM, Trojanowski JQ, & Goetzl EJ (2016). Plasma neuronal exosomal levels of Alzheimer’s disease biomarkers in normal aging. Annals of Clinical and Translational Neurology, 3, 399–403. 10.1002/acn3.309 [PubMed: 27231710]

78. De Arrieta CM, Morte B, Coloma A, & Bernal J (1999). The human RC3 gene homolog, NRGN contains a thyroid hormone-responsive element located in the first intron. Endocrinology, 140, 335–343. 10.1210/endo.140.1.6461 [PubMed: 9886843]

79. Huang FL, Huang K-P, Wu J, & Boucheron C (2006). Environmental enrichment enhances neurogranin expression and hippocampal learning and memory but fails to rescue the impairments of neurogranin null mutant mice. Journal of Neuroscience, 26, 6230–6237. 10.1523/JNEUROSCI.1182-06.2006 [PubMed: 16763030]

80. Miyakawa T, Yared E, Pak JH, Huang FL, Huang K-P, & Crawley JN (2001). Neurogranin null mutant mice display performance deficits on spatial learning tasks with anxiety related components. Hippocampus, 11, 763–775. 10.1002/hipo.1092 [PubMed: 11811671]
81. Kawadler JM, Clayden JD, Clark CA, & Kirkham FJ (2016). Intelligence quotient in paediatric sickle cell disease: A systematic review and meta-analysis. Developmental Medicine and Child Neurology, 58, 672–679. 10.1111/dmcn.13113 [PubMed: 27038278]
Statement of Clinical Relevance

Limited proteomic discovery work has been done involving sickle cell disease and neurological complications. This study identified multiple new proteins of interest with regards to silent cerebral infarction in pediatric sickle cell disease. An ELISA assay was used to measure the levels of one protein, neurogranin, a neuronal protein. Neurogranin levels were elevated in children with sickle cell disease in comparison to healthy control participants.
FIGURE 1.
Flow chart of participants samples selected for verification analysis
FIGURE 2.
Venn diagram of the number of proteins identified and the overlap in the normal, non-silent cerebral infarction and silent cerebral infarction groups. SCI – Silent Cerebral Infarction.
FIGURE 3.
Median neurogranin levels by sickle cell disease status. Box plot of neurogranin levels from a group of children with sickle cell disease \((n = 104)\) and a group of healthy pediatric control participants \((n = 25)\). Line in middle of box represents median neurogranin level. Horizontal line at top of box represents 75th percentile value. Horizontal line at bottom of box represents 25th percentile value. Line at top of whisker represents upper adjacent value. Line at bottom of whisker represents lower adjacent value. Outliers were removed from the graph. SCD – Sickle Cell Disease. NRGN – Neurogranin.
TABLE 1
Clinical characteristics of children with sickle cell disease and healthy, non-sickle cell disease control participants, used for proteomic discovery analysis

| Clinical characteristics                  | SCD [n= 15] | Control group [n= 6] |
|------------------------------------------|-------------|---------------------|
| % Sickle cell trait [n] *                | 0 [15]      | 50 [3]              |
| % Male [n]                               | 67 [10]     | 50 [3]              |
| Mean age (SD)                            | 9.4 (2.7)   | 11.5 (2.1)          |
| Median reticulocyte % (IQR) *            | 10.3 (8.2–12.2) | 7.5 (5.5–12)      |
| Median hemoglobin g/dL (IQR) *           | 8.9 (7.6–9.1) | 12.05 (11.8–12.4) |
| Median hematocrit % (IQR) *              | 24 (22–25)  | 36.45 (34.8–37.8)  |
| Median WBC x10⁹/L (IQR) *               | 12.8 (10–17.3) | 4.9 (4.3–5.5)      |
| Median platelet x10⁹/L (IQR) *           | 445 (368–570) | 327.5 (319–332)    |

Abbreviations: SCD, sickle cell disease; SD, standard deviation; IQR, interquartile range; WBC, white blood cell count.

Results represent mean ± standard deviation (SD).

* p < 0.05 between groups by Student’s t-test.
| Clinical characteristics | SCI positive [n= 7] | SCI negative [n= 8] |
|--------------------------|---------------------|--------------------|
| % Male [n]               | 71 [5]              | 63 [5]             |
| Mean age (SD)            | 9.8 (2.4)           | 9 (3.1)            |
| Median reticulocyte % (IQR) | 10.7 (9.4–13.1)    | 9.6 (7–12)         |
| Median hemoglobin g/dL (IQR) | 8.4 (7.4–9.1)      | 8.9 (8.2–9)        |
| Median hematocrit % (IQR) | 24 (21–25)          | 24.5 (22.5–25)     |
| WBC x10⁹/L (IQR)         | 13 (9.27–23.4)      | 12.8 (11–14)       |
| Platelet x10⁹/L (IQR)    | 418 (335–456)       | 516 (437–597)      |

Abbreviations: IQR, interquartile range; SCI, silent cerebral infarction; SD, standard deviation; WBC, white blood cell count.

Results represent median (25% Interquartile range [IQR] – 75% IQR) unless otherwise specified.

*p < 0.05 between groups by Student’s t test.
Table 3: Protein differences (>2 < 0.5-fold spectral counts) identified in SCI positive and SCI negative SCD groups

| Accession # | Protein name                              | Total samples (SCI negative) | Total samples (SCI positive) | Avg SC/protein (SCI negative) | Avg SC/protein (SCI positive) | SC Ratio (SCI negative/SCI positive) |
|-------------|-------------------------------------------|-----------------------------|-------------------------------|--------------------------------|-------------------------------|--------------------------------------|
| Q9P273      | Teneurin-3                                | 7.0                         | 3.0                           | 51.9                           | 8.0                           | 6.5                                  |
| P31949      | Protein S100-A11                          | 4.0                         | 2.0                           | 41.5                           | 7.5                           | 5.5                                  |
| P15401      | Histone H1.5                              | 2.0                         | 2.0                           | 21.0                           | 4.5                           | 4.7                                  |
| Q9UJ43      | L-selectin                                | 8.0                         | 2.0                           | 17.8                           | 4.0                           | 4.4                                  |
| P50552      | Vasodilator-stimulated phosphoprotein      | 5.0                         | 3.0                           | 15.8                           | 4.0                           | 4.0                                  |
| P18286      | Vinculin                                  | 7.0                         | 3.0                           | 34.3                           | 10.3                          | 3.3                                  |
| P10599      | Thioredoxin                               | 7.0                         | 2.0                           | 44.1                           | 13.5                          | 3.3                                  |
| P20851      | C4b-binding protein beta chain            | 8.0                         | 5.0                           | 22.1                           | 7.8                           | 2.8                                  |
| P02775      | Platelet basic protein                    | 8.0                         | 7.0                           | 174.7                          | 65.0                          | 2.7                                  |
| P02745      | Complement C1q subcomponent A             | 5.0                         | 2.0                           | 19.4                           | 7.5                           | 2.6                                  |
| Q9UK55      | Protein Z-dependent protease inhibitor     | 6.0                         | 4.0                           | 19.5                           | 8.3                           | 2.4                                  |
| Q92954      | Proteoglycan 4                            | 8.0                         | 6.0                           | 26.2                           | 11.7                          | 2.2                                  |
| P02746      | Complement C1q subcomponent B             | 8.0                         | 7.0                           | 105.4                          | 47.0                          | 2.2                                  |
| Q96F45      | Carboxypeptidase B2                       | 7.0                         | 4.0                           | 14.3                           | 6.5                           | 2.2                                  |
| P07360      | Complement component C8 gamma chain       | 8.0                         | 7.0                           | 53.2                           | 25.3                          | 2.1                                  |
| P05233      | Keratin, type I cytoskeletal 14           | 7.0                         | 5.0                           | 43.6                           | 20.8                          | 2.1                                  |
| P03670      | Vimentin                                  | 5.0                         | 4.0                           | 12.6                           | 6.3                           | 2.0                                  |
| Q96PD5      | N-acetylmuramoyl-L-alanine amidase         | 8.0                         | 7.0                           | 305.0                          | 153.0                         | 2.0                                  |
| Q6B823      | Histone H4                                 | 3.0                         | 2.0                           | 5.0                            | 10.0                          | 0.5                                  |
| Q96F45      | Zinc finger protein 503                   | 3.0                         | 2.0                           | 2.0                            | 4.0                           | 0.5                                  |
| P02679      | Fibrinogen gamma chain                    | 8.0                         | 7.0                           | 92.1                           | 185.9                         | 0.5                                  |
| Q9NQS1      | Cell death regulator Aven                 | 3.0                         | 2.0                           | 4.0                            | 10.0                          | 0.4                                  |
| P02461      | Collagen alpha-1(III) chain               | 3.0                         | 2.0                           | 3.7                            | 9.5                           | 0.4                                  |

Abbreviations: SCI, silent cerebral infarction; SCD, sickle cell disease; SC, spectral counts; Samples, total number of samples where protein is identified.
| Accession # | Protein name                                      | Protein function                                                                 | Cellular component          | Average SC per Protein | Log(e)  |
|------------|--------------------------------------------------|----------------------------------------------------------------------------------|-----------------------------|------------------------|---------|
| Q92686     | Neurogranin                                      | Acts as a “third messenger” substrate of protein kinase C-mediated molecular cascades during synaptic development and remodeling. Binds to calmodulin in the absence of calcium. | Membrane; Cytoplasm         | 15                     | −6.40   |
| Q96KN2     | Beta-Ala-His dipeptidase                          | Preferential hydrolysis of the beta-Ala-His dipeptide (carnosine)                 | Secreted                    | 7.25                   | −25.08  |
| Q99884     | Sodium-dependent proline transporter             | Terminates the action of proline by its high affinity sodium-dependent reuptake into presynaptic terminals. | Membrane                    | 6                      | −2     |
| P01213     | Beta-neoendorphin-dynorphin                      | Pain perception and responses to stress                                           | Secreted                    | 2                      | −9.9   |
| P56975     | Pro-neuregulin-3, membrane-bound isoform         | Direct ligand for the ERBB4 tyrosine kinase receptor                             | Membrane                    | 2                      | −1.10  |
| P14136     | Gliad fibrillary acidic protein                  | Distinguishes astrocytes from other glial cells during the development of the CNS | Cytoplasm                   | 2                      | −6.33  |
| Q94933     | SLIT and NTRK-like protein 3                     | Suppresses neurite outgrowth                                                      | Membrane                    | 2                      | −5.3   |
| P08908     | 5-hydroxytryptamine receptor 1A                  | Serotonin receptor                                                              | Membrane                    | 1                      | −1.1   |
| A7E2E4     | Dipeptidyl-peptidase 6                          | May modulate the cell surface expression and the activity of the potassium channel KCND2 | Membrane                    | 1                      | −1.1   |
| Q96FT7     | Amiloride-sensitive cation channel 4             | Cation channel with high affinity for sodium                                     | Membrane                    | 1                      | −1.2   |
| B3KXG7     | Protein tyrosine phosphatase, non-receptor type 5 | Hydrolyase receptor                                                             | Endoplasmic reticulum membrane | 1          | −1.5   |
| Q9P218     | Collagen alpha-1(XX)                            | Collagen protein                                                               | Secreted                    | 1                      | −1.5   |
| O95741     | Copine-6                                         | Membrane trafficking and in synaptic plasticity                                  | Mitochondrion                | 1                      | −1.2   |
| Q9U147     | Catenin alpha-3                                  | Formation of stretch-resistant cell-cell adhesion complexes                      | Cytoplasm                    | 1                      | −1.3   |
| P51674     | Neuronal membrane glycoprotein M6-a              | Neuronal differentiation, including differentiation and migration of neuronal stem cells | Membrane                    | 1                      | −1.4   |
| Q9UQM7     | Calcium/calmodulin-dependent protein kinase type II subunit alpha | Long-term potentiation and neurotransmitter release | Membrane                    | 1                      | −1.2   |
| Q96NJ5     | Kelch-like protein 32                            | Unknown                                                                       | Unknown                      | 1                      | −2     |
| Q8N967; Q6ZR1 | Leucine-rich repeat and transmembrane domain-     | Unknown                                                                       | Membrane                     | 1                      | −1.4   |
| Q96NK8     | Neurogenic differentiation factor 6              | Trans-acting factor involved in the development and maintenance of the mammalian nervous system | Nucleus                      | 1                      | −1.5   |
| Q8N987     | N-terminal EF-hand calcium-binding protein 1     | Calcium ion binding                                                            | Cytoplasm                    | 1                      | −1.6   |
| Q135I6     | Oligodendrocyte transcription factor 2           | Oligodendrocyte and motor neuron specification in the spinal cord; development of somatic motor neurons in the hindbrain | Nucleus; Cytoplasm         | 1                      | −1.1   |
| Accession # | Protein name                             | Protein function                                                                 | Cellular component | Average SC per Protein | Log(e) |
|------------|------------------------------------------|----------------------------------------------------------------------------------|--------------------|------------------------|---------|
| Q59GK5     | Glutamate receptor, metabotropic 4 variant | Receptor                                                                        | Membrane           | 1                      | -1.4    |
| Q16650     | T-box brain protein 1                    | Transcriptional regulator of brain development                                   | Nucleus            | 1                      | -1.8    |
| Q504Y0     | Zinc transporter ZIP12                   | Zinc-influx transporter                                                          | Membrane           | 1                      | -1      |
| P41225     | Transcription factor SOX-3               | Required for formation of hypothalamo-pituitary axis; counteracts the activity of proneural proteins and suppresses neuronal differentiation | Nucleus            | 1                      | -1.30   |

Abbreviations: SCD, sickle cell disease; SC, spectral counts; CNS, central nervous system; Log(e), natural logarithm
TABLE 5
Clinical characteristics of children with SCD and healthy, non-SCD controls, from verification analysis

|                      | All SCD subjects | Treatment group | Observation group | SCI positive | SCI negative | Control group |
|----------------------|------------------|-----------------|------------------|--------------|--------------|---------------|
| **Total n**          | 194              | 68              | 72               | 151          | 43           | 25            |
| **SCD type**         |                  |                 |                  |              |              |               |
| HbSS                 | 175              | 62              | 64               | 136          | 39           | Unknown       |
| HbS-βthal            | 11               | 3               | 4                | 8            | 3            |               |
| **Male (%)**         | 107 (55)         | 41 (60)         | 39 (54)          | 88 (58)      | 19 (44)      | 9 (36)        |
| **Mean BMI in kg/m² (SD)** | 16.86 (2.76)   | 16.72 (2.17)    | 17.15 (3.22)     | 16.9 (2.71)  | 16.65 (3.14) |               |
| **Mean age at initial study visit in months (SD)** | 111.4 (36.8)    | 117.4 (34)      | 121.1 (41.1)     | 118.9 (37.4) | 96.7 (31.3)  | 126.5 (37.9) |
| **Race (%)**         |                  |                 |                  |              |              |               |
| Black                | 177 (91)         | 62 (91)         | 66 (92)          | 139 (92)     | 38 (88)      | 25 (100)      |
| Asian                | 1 (1)            | 6 (9)           | 1 (1)            | 1 (1)        | 1 (2)        |               |
| Pacific Islander     | 1 (1)            | 5 (7)           | 11 (7)           | 4 (9)        |              |               |
| White                | 15 (8)           |                 |                  |              |              |               |
| **Ethnicity**        |                  |                 |                  |              |              | Unknown       |
| Hispanic             | 3                | 1               | 1                | 2            | 1            |               |
| Not Hispanic         | 189              | 67              | 71               | 148          | 41           |               |
| Unknown              | 2                |                 |                  | 1            | 1            |               |
| **History of (%)**   |                  |                 |                  |              |              |               |
| Asthma               | 47 (24)          | 17 (25)         | 20 (28)          | 38 (25)      | 9 (21)       | 10 (40)       |
| ADHD                 |                  |                 |                  |              |              | 4 (16)        |
| Sleep disorder       |                  |                 |                  |              |              | 2 (8)         |
| **TCD status (%)**   |                  |                 |                  |              |              |               |
| Normal               | 144 (74)         | 52 (76.5)       | 57 (79)          | 118 (78)     | 26 (60)      | Not applicable|
| Conditional          | 26 (13)          | 13 (19)         | 11 (15.5)        | 24 (16)      | 2 (5)        |               |
| High                 | 5 (3)            | 2 (3)           | 1 (1.5)          | 4 (3)        | 1 (2)        |               |
| Missing              | 19 (10)          | 1 (1.5)         | 3 (4)            | 5 (3)        | 14 (33)      |               |
| **Median steady state hemoglobin in g/dL (25%−75%IQR)** | 7.8             | 7.8             | 7.9              | 7.8          | 7.7          | Unknown       |
|                      | (7.4–8.7)        | (7.2–8.4)       | (7.5–9.1)        | (7.4–8.8)    | (7.5–8.7)    |               |
| Study Measure | All SCD subjects | Treatment group | Observation group | SCI positive | SCI negative | Control group |
|---------------|-----------------|-----------------|------------------|--------------|-------------|---------------|
| Median % Steady State Reticulocytes | 11.2 (8.6–16.2) | 12.5 (9.6–16.8) | 10 (7.6–13.6) | 11.2 (8.2–16.3) | 11.6 (10.6–14.6) | Unknown |
| Median platelet count per mm$^3$ | 435000 (380000–526000) | 413000 (376000–513000) | 446500 (371500–520000) | 433000 (376000–520000) | 475000 (391500–578500) | Unknown |
| Median % Hgb F | 7.2 (4.2–12.2) | 6.3 (4–11.7) | 6.5 (4.8–11.8) | 6.5 (4–12.2) | 8.9 (5–14.4) | Not Applicable |
| Median steady state WBC per mm$^3$ | 12445 (9950–14400) | 12250 (9265–14400) | 12100 (9550–14180) | 12445 (9550–14400) | 12450 (10550–14550) | Unknown |

Abbreviations: SCD, sickle cell disease; SD, standard deviation; WBC, white blood cell count; TCD, transcranial Doppler

\[a\] \( p < 0.004.\)

\[b\] \( p < 0.02.\)