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Proteomic and Biomarker Studies and Neurological Complications of Pediatric Sickle Cell Disease

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Abstract

Biomarker analysis and proteomic discovery in pediatric sickle cell disease has the potential to lead to important discoveries and improve care. The aim of this review article is to describe proteomic and biomarker articles involving neurological and developmental complications in this population. A systematic review was conducted to identify relevant research publications. Articles were selected for children under the age of 21 years with the most common subtypes of sickle cell disease. Included articles were focused on growth factors (platelet-derived growth factor), intra and extracellular brain proteins (glial fibrillary acidic protein, brain-derived neurotrophic factor), and inflammatory and coagulation markers (interleukin-1 β , L-selectin, thrombospondin-1, erythrocyte and platelet-derived microparticles). Positive findings include increases in plasma brain-derived neurotrophic factor and platelet-derived growth factor with elevated transcranial Dopplers velocities, increases in platelet-derived growth factor isoform AA with overt stroke, and increases in glial fibrillary acidic protein with acute brain injury. These promising potential neuro-biomarkers provide insight into pathophysiologic processes and clinical events, but their clinical utility is yet to be established. Additional proteomics research is needed, including broad-based proteomic discovery of plasma constituents and blood cell proteins, as well as urine and cerebrospinal fluid components, before, during and after neurological and developmental complications.

Keywords

biological markers; neurodevelopment; proteomics; sickle cell; stroke

BACKGROUND

Neurological complications in pediatric sickle cell disease (SCD) are associated with significant morbidity and mortality [1]. Ischemic stroke is quite common in this population, with an incidence rate of 0.44 per 100 patient-years in untreated children less than 20 years of age [2]. In addition to overt strokes, children with SCD often show magnetic resonance imaging (MRI) evidence of subclinical brain injury, without overt neurological abnormalities, referred to as silent cerebral infarctions (SCI) [3]. SCI are even more common than overt ischemic stroke, occurring in 37.1% of the SCD population before 14 years of age [4]. Although these children appear neurologically intact, SCI are associated with significant cognitive impairment, including attention deficits and poor school performance [3]. The deficits in attention and executive function associated with SCI are similar to other neurodevelopmental disorders, particularly attention deficit hyperactivity disorder (ADHD) and intellectual disability, which are also seen in children with SCD without obvious brain injury [5]. Current methods for detecting brain injury may be financially impractical, have limited sensitivity to detect subtle brain injury, and require the risk of sedation or anesthesia in younger children. Faster and more efficient methods are also needed to diagnose and predict neurological complications of SCD, so that appropriate prevention methods can be initiated earlier.

Common forms of ischemic brain injuries in pediatric SCD vary not only in presentation, but also in their locations and risk factors. Overt stroke typically occurs in large vessels, while SCI involve the watershed distributions of smaller vessels within the frontal lobes and deep white matter primarily [6]. Overlapping risk factors for overt stroke and SCI include anemia and hypertension. Specific risk factors for SCI include male gender and increasing age, while specific risk factors for overt ischemic stroke include prior transient ischemic attack or SCI, as well as frequent or recent acute chest syndrome and nocturnal hypoxemia [3, 6-9]. Increased cerebrovascular blood flow velocities correlate with risk for overt ischemic stroke, but do not appear to be associated with SCI [10]. These findings suggest that there are potential differences in the disease pathogenesis of common neurologic injuries within the pediatric SCD population. Identification of specific neuro-biomarkers that can distinguish between overt ischemic stroke and SCI risk using longitudinal measurements would allow precise diagnosis and earlier treatment of high-risk patients.

Current screening for ischemic stroke risk and SCI in children between 2 to 16 years of age is done with measurements of intracranial vessel blood flow velocities, using transcranial Doppler (TCD) ultrasound [11]. Elevated velocities typically indicate large intracranial vessel stenosis, which leads to increased stroke risk. Patients with elevated velocities undergo treatment with chronic transfusion therapy, decreasing their chance of stroke, but increasing their risk of infection and iron overload, as well as being a significant burden to families [12]. Measurement of specific validated neuro-biomarkers could be coupled with MRIs and TCDs to improve the sensitivity and specificity of stroke and SCI screening.

To date, there have been no review articles focusing solely on biomarker and proteomic studies involving the neurological complications of SCD, neither in the adult or pediatric populations. Recent review articles have focused on the expanding field of biomarkers and

proteomics discovery in SCD. Rees and Gipson discussed biomarkers in SCD involving red cell dehydration, rigidity, adhesion, inflammation, hemolysis, oxidative stress, reperfusion injury, hypercoagulability, nitric oxide metabolism and vasculopathy in SCD [13]. In addition, they reviewed findings regarding specific organ systems, including the spleen, kidney, bone, and heart [13]. Other biomarker review articles address specific adhesion molecules and their roles in SCD, particularly $\alpha 4\beta 1$ integrin [14, 15]. Additional proteomic reviews have focused on red cell membrane proteins in SCD [16, 17]. The proteomics research in these reviews discuss three major topics: sickle erythrocyte membranes proteins, proteins associated with various SCD clinical complications, such as vaso-occlusive crisis and acute chest syndrome, and the pharmacological effects of hydroxyurea on sickle erythrocyte membrane proteins.

As noted by Savage and Everett, pediatric biomarkers are badly needed to assess treatment effects, minimize invasive procedures, and validate adult studies in this young population [18]. The objective of this article is to provide a systematic review describing the existing research on proteomics and plasma, urine, and cerebrospinal fluid biomarkers associated with the neurological and developmental complications of pediatric SCD.

CRITERIA FOR CONSIDERING STUDIES FOR THIS REVIEW

We did a systematic search of the literature for studies meeting pre-determined criteria regarding biomarker and proteomic research in pediatric SCD. We included all types of studies in our review: randomized controlled trials, quasi randomized controlled trials, cohort studies, case control studies, and case reports to increase the comprehensive nature of the review. We excluded abstracts, as their data was considered unreliable and often not presented in enough detail to meet exclusion criteria. Review articles were not included in the study, but were extracted and reviewed for potential qualifying articles.

We included studies with children with specific subtypes of SCD (Hemoglobin SS, Hemoglobin S beta⁰ or beta⁺ thalassemia, Hemoglobin SC) from 0 to 21 years of age. These three subtypes are the most prevalent types of the disease, increasing the external validity of our study. Studies that included pediatric (less than 21 years of age) and adult (more than 21 years of age) subjects were excluded if the overall group mean or median age was at or above 14 years of age, more than 50% of subjects were adults, or separate analyses on the pediatric subset were not included or reported. These criteria were used to identify pediatric studies selectively, in order to target proteins and biomarkers pertaining to this high-risk population solely. There were no restrictions due to gender, ethnicity, or number of subjects.

We included studies that used proteomic techniques or measured biomarkers in plasma, urine, and cerebrospinal fluid. These markers are easy to measure and validate, with minimally invasive techniques. We defined biomarkers in accordance with the National Institutes of Health Biomarkers Definitions Working Group as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [19, pg. 91]. We excluded studies with measures of physiological correlates of disease such as

anemia. We excluded studies with technological, imaging, or genetic polymorphism biomarkers.

Neurodevelopmental complications of pediatric SCD were our primary outcomes. Specific neurological complications such as stroke, SCI, abnormal TCD velocities, seizures, headaches or developmental complications, such as intellectual disability and ADHD, were all considered. Seizures and headaches are quite common in the pediatric sickle cell disease population as are intellectual disability and ADHD [5, 20, 21].

SEARCH METHODS FOR IDENTIFICATION OF STUDIES

Two databases, PubMed and EMBASE, were searched without language or date restrictions for the following three concepts: biomarkers, SCD, and pediatrics. MeSH and Emtree headings and other keywords related to our concepts were used as well.

The initial PubMed search retrieved 145 articles using the keywords above. Terms were expanded to include “humans”, which increased the results to 465 articles, as well as the names of specific biomarkers (cystatin C, immunoglobulin E, ferritins), which increased the results to 1020 articles. These terms were identified using keywords from articles identified by the authors as relevant to the review paper. The final PubMed search on February 15th, 2014 found 1020 articles using the search terms “Biological Markers”[Mesh] OR “Biological Markers”[tw] OR “Proteomics”[Mesh] OR “Proteomics”[tw] OR “Genetics”[Mesh] OR “Cystatin C”[Mesh] OR “Immunoglobulin E”[Mesh] OR “Ferritins”[Mesh] AND “Anemia, Sickle Cell”[Mesh] OR “Anemia, Sickle Cell”[tw] OR “Sickle Cell Trait”[Mesh] OR “Sickle Cell Trait”[tw] OR “Hemoglobin SC Disease”[Mesh] OR “Hemoglobin SC Disease”[tw] AND “Pediatrics”[Mesh] OR “Pediatrics”[tw] OR “Child”[Mesh] OR “Child”[tw] OR “Humans”[Mesh]”. For EMBASE, a search on February 10th, 2014 found 55 total articles using the search terms ‘biological marker’/exp AND ‘sickle cell anemia’/exp AND ‘child’/exp. Six additional articles were added to the search after identification by the authors as relevant.

The 1081 total articles from these search results were combined in RefWorks Reference Management Software for de-duplication down to 1064 articles. Titles and abstracts were screened using inclusion and exclusion criteria noted above with 171 articles remaining. The full text versions of these remaining articles were pulled for final review and study inclusion. Articles were excluded for containing subjects outside the specified age range or not providing enough information regarding subjects’ ages (49), measuring factors not found in plasma, urine, or cerebrospinal fluid (43), including only non-neurological complications (43), representing review articles or abstracts (20), including subjects with disease other than SCD (4), and having a full text article not available in English (3). Through these criteria, the list was narrowed down to 16 articles for comprehensive review by all review authors. During group review, six articles were eliminated for measuring physiological correlates (anemia) as opposed to biomarkers and one article was eliminated due to measurement of brain natriuretic peptide, a cardiac biomarker, with no mention of neurologic complications. A total of 9 articles were included in the final review article, listed at the end of this paper. The bibliographies and articles citing the included articles were reviewed to identify

additional studies for inclusion through backward and forward citation searching. Forward citation searching for two articles was unable to be completed due to the articles not being available in a forward searching database [22, 23]. See Figure 1 for additional details regarding study selection.

DESCRIPTION OF STUDIES

Study Design and Participants

A description of all nine studies is included in Table 1. All of the included studies were published during or after 2008. Five studies did not explicitly state a study design in the manuscript. Identified study types included three cross-sectional studies and one case report. Several studies used blood samples from two pediatric SCD clinical trials, the Stroke Prevention Trial in sickle cell anemia (STOP) and the Silent Cerebral Infarct Multi-Center Clinical (SIT) Trial. STOP was a multi-center randomized trial to determine whether chronic transfusions could prevent cerebral infarcts in children with elevated TCD velocities who were considered to have high stroke risks (Clinical Trials.gov Identifier NCT00000592). SIT was a multi-center randomized trial to determine whether chronic transfusions could prevent SCI in children with SCD (Clinical Trials.gov Identifier NCT00072761). Samples from STOP were used in three studies and samples from SIT were used in two studies. One study used data from both the STOP study and a study exploring the effect of nutrition on inflammation in children with sickle cell anemia (NUTSCD).

All of the studies included children between the ages of 2 years to 18 years of age. A single study also used 28 adult subjects with overt brain injury from acute stroke, brain biopsy, or partial resection as a comparison group [24]. SCD types varied among the studies, with six studies of Hemoglobin SS and Hemoglobin S- β thalassemia, two studies with Hemoglobin SS only, and one study with Hemoglobin SS and Hemoglobin SC. Total participants ranged from 1 to 295 subjects per study. A total of four studies used data collected only in the United States; the other five studies took place in Egypt (2), England (1), and across multiple countries in North America and Europe (2).

Biomarker analyses and Proteomic discovery

All of the included studies measured candidate neuro-biomarkers identified by past research or through proteomic discovery. We defined candidate neuro-biomarkers in this review as plasma, urine, or cerebrospinal fluid laboratory markers associated with neurological (stroke, SCI, elevated TCD velocities, seizures, headache) or developmental (ADHD, intellectual disability) complications in children with sickle cell disease. Ideally, the marker would be involved in the proposed mechanistic pathway of the associated neurological and/or developmental disorder. This relationship should be reflected by negative or positive correlations with the marker and disease severity as well as significantly lower or higher levels of the marker in matched control groups. The mechanistic pathways of brain injury in sickle cell disease and the general population have some overlap, but the clear differences in disease epidemiology and pathogenesis suggest that markers need to be validated separately.

Similarly, the pathophysiology of ischemic stroke is likely different based on the epidemiology pattern of stroke in SCD. As described above, overt ischemic strokes have the

highest incidence in children ages 2 to 5 years, with a decline after ages 7 to 10 years [2]. Thereafter, the incidence of hemorrhagic stroke increases in the second decade of life, peaking during the third decade [2]. There is also a re-emergence of ischemic stroke in adults during the fourth and fifth decades of life [2]. In comparison, SCI occurs early in life, with the majority of events apparent before six years of age in girls and ten years of age in boys [25]. Taken together, these findings suggest that there may be important differences in the pathophysiology of stroke in children and adults, warranting separate consideration of these groups.

The included studies used measures associated with neurological or developmental complications in other populations that were not previously validated in the pediatric SCD population or measures that had only been associated with other non-neurodevelopmental complications in the adult or pediatric SCD populations. The majority of the candidate biomarkers in this study had been previously identified in studies of cerebral ischemia [24, 26-28], animal models of brain injury [24, 26, 28], vascular remodeling [27], and coagulation markers [22, 23], or showed previous associations with hemolysis [29], vaso-occlusive crises [30], abnormal TCD velocities [31] and stroke [30] in the SCD population. Proteomic discovery was used to find one candidate neuro-biomarker (glial fibrillary acidic protein [GFAP]) measured in two included studies, through testing of screening samples collected in the SIT study [24, 28].

All of the included studies measured samples collected during or after 1995. None of the included studies measured urine or cerebrospinal fluid markers. In the original search, no articles were identified involving cerebrospinal fluid markers, but 29 articles were identified involving urine markers in both adults and children; as such, these articles did not meet our other criteria for inclusion, as they were studied in the context of non-neurological complications, specifically renal complications, bone disorders, and hydroxyurea treatment effects. A total of nine studies used plasma samples, two used whole blood samples, and one used serum samples. Serum markers were also measured in two of the studies that used plasma samples. The most common markers include two isoforms (AA and AB/BB) of platelet-derived growth factor (PDGF), a regulator of cell growth, measured in 3 studies; brain-derived neurotrophic factor (BDNF), a regulator of nerve growth, measured in 2 studies; granulocyte monocyte-colony stimulating factor (GM-CSF), a bone marrow stimulator, measured in 2 studies; vascular endothelial growth factor (VEGF), an angiogenesis stimulator, measured in 2 studies; interleukin (IL)-1 β , IL-1RA, IL-4, IL-6, IL-10, IL-13, inflammatory cytokines, measured in 2 studies; interferon- γ (IFN- γ), measured in 2 studies; tumor necrosis factor α (TNF- α), measured in 2 studies; thrombin-antithrombin III (TAT-III), measured in 2 studies; D-dimers, measured in 2 studies; lactate dehydrogenase (LDH), a marker of hemolysis-related complications, measured in 2 studies; and GFAP, a central nervous system astrocyte cytoskeletal protein, measured in 2 studies. Only one study measured microparticles, small vesicles from platelets and erythrocytes [23].

The included studies also used a variety of techniques for protein analysis. Seven studies used immunoassays [22, 24, 26-28, 30-31]. Single protein biomarker analytes were assayed using either a commercially available immunoassay [30, 22] or a custom developed, highly sensitive, electro-chemiluminescent immunoassay employing the Meso Scale Discovery

system [24, 28]. Other studies used several different systems including Byk Sangtec Diagnostica, Behringwerke AG, Diagnostica stago, Diaclone SA, Roche Diagnostics, and Siemens Healthcare Diagnostics [22, 23]. Multiple analytes were assayed using the multiplex bead-based immunoassay systems by Biorad Bioplex [26, 27] and Millipore systems [27, 31]. Antibodies were also used in a flow-cytometry study to determine levels of platelet and erythrocyte microparticles [23]. Two studies used a biochemical assay to quantitate their biomarker [23, 29].

Outcomes

For the purposes of this review, we were interested in outcomes related to neurological and developmental complications in pediatric sickle cell disease. We included studies that reviewed differences in biomarker analyses and proteomic discovery between groups with and without these neurodevelopmental disorders. The included studies measured results with regards to four outcomes: overt stroke, SCI, abnormal TCD velocities, which are associated with an increased risk of stroke, and intellectual quotients (IQ), a partial measure of intellectual disability. No studies were identified looking at other neurological complications (seizures, headaches) or developmental complications (ADHD) of pediatric SCD.

Stroke—Stroke in SCD is quite common, occurring in 11% of untreated patients before 20 years of age (2). Biomarker and proteomic discovery in stroke in the general population has led to ways to predict, diagnose, and differentiate between different types of stroke [32–34]. Research, similar to our included SCD studies, has also identified coagulation, inflammatory, and microparticles biomarkers in childhood stroke in the general population [35, 36]. However, due to the potential mechanistic differences, candidate neuro-biomarkers discovered in the general adult and pediatric stroke population must still be validated in pediatric sickle cell disease.

The included studies focused on different types of strokes. In the STOP study, the endpoint of stroke was defined as a cerebral infarction or intracranial hemorrhage [37]. The occurrence of a stroke was determined by a blinded panel of neurologists who reviewed clinical and imaging data of each event to determine if a stroke had occurred. In the SIT study, the endpoint was new or enlarging SCI or overt stroke [38]. The occurrence of SCI or stroke was determined by three members of the neurology committee for the study, based on history, physical examination, and neuroimaging. Mourad et al. included subjects with clinically overt stroke and SCI determined by neuroimaging studies [22]. Tantawy et al defined stroke by a focal neurological deficit lasting longer than 24 hours or a deficit lasting less than 24 hours with acute infarction present on neuroimaging [23].

The included studies identified several proteins in sera or plasma that were associated with the development of overt stroke in children with SCD. IL-1 β is a non-specific pro-inflammatory cytokine that is associated with microglia, astrocytes, and neurons. Data from one study showed that overall stroke risk was decreased with higher levels of IL-1 β [26]; however, this finding was not replicated in a subsequent study by the same group in an expanded cohort [27]. Stroke risk was decreased with lower levels of BDNF, in addition to decreases in soluble vascular cell adhesion molecule-1 (s-VCAM-1), soluble intercellular

adhesion molecule-1 (sICAM-1), PDGF-AA, and tissue plasminogen activator inhibitor (tPAI-1), which are involved in nerve growth, inflammation, endothelial activation, and thrombogenesis [31]. A separate study showed that elevated levels of PDGF-AA correlated with stroke; however, association between the PDGF-AB/BB isoform and stroke was not found to be statistically significant [27]. Children with a history of stroke also had higher levels of fibrinopeptide A (FPA), TAT III, D-dimers, and serum platelet/endothelial cell adhesion molecule (PECAM-1) [22], as well as erythrocyte microparticles (ErMPs) and platelet-derived microparticles (PMPs) [23]. These markers, similar to those mentioned above, are associated with coagulation and thrombogenesis, highlighting the importance of these mechanisms in the stroke pathway for pediatric SCD. Moreover, elevated levels of PDGF-AA may suggest a relationship between arterial remodeling and stroke in the pediatric SCD population.

In addition, there were categories of circulating proteins that increased with development of acute stroke or SCI. A higher proportion of children with acute brain injury had elevated GFAP levels than children with and without a remote history of cerebral infarct [24]. GFAP is an intracellular intermediate filament protein expressed by supporting glial cells in the central nervous system that is known to be elevated in acute brain injury in the general population and may have a role in prediction and diagnosis of acute brain injury in the SCD population [39, 40, 41]. Of particular note is that children with SCD, with or without SCI, had significantly higher levels of GFAP than the general population of children, suggesting that children with SCD are subject to subclinical brain injury, perhaps on a chronic basis, that is not detectable by MRI. This finding is supported by subsequent imaging studies suggesting that acute silent cerebral ischemic events (ASCIEs), defined by positive diffusion weighted imaging (DWI) on MRI are 4x as common as SCI and may not be identified on subsequent T2 or DWI MRI scans [42]. Children with a history of SCI also had higher levels of thrombospondin-1 (TSP-1) and L-selectin (LSEL), biomarkers associated with other complications of pediatric SCD, than children without SCI [30, 43, 44]. Systolic blood pressure correlated positively with LSEL levels in children with SCI; this finding is consistent with high blood pressure as an established risk factor for ischemic brain injury [3, 30]. TSP-1 and LSEL are known biomarkers of overt stroke in SCD, and may have additional predictive value for SCI and other ischemic stroke risk factors as well [43, 44].

Abnormal transcranial Doppler velocities—As discussed above, elevated intracranial blood flow due to stenosis in the large arteries can be detected by TCD ultrasonography and is a significant clinical risk factor for development of stroke. In the STOP study, abnormal TCD values were defined as a time-averaged mean blood-flow velocity of greater than 200 cm/second by non-imaging TCD studies [37], the same protocol used by O'Driscoll et al [29]. Elevated TCD velocities are associated with an increased risk of stroke in children ages 2 to 16 years of age with sickle cell disease [11]. Elevated TCD velocities are also associated with deficits in syntactical language and increased risk of neurodevelopmental delay in children with sickle cell disease [45, 46].

In the included studies, correlations between certain proteins have been associated with abnormal TCD velocities. Children with SCD and abnormal TCD velocities had significantly higher levels of the neurotrophic factor BDNF, and growth factors PDGF-AA

and AB/BB, than children with SCD and normal TCD velocities [27]. In addition, PDGF-AA was directly associated with an increased likelihood of subsequent stroke in children with high TCD velocities [27]. These findings suggest that PDGF-AA may have predictive value in terms of determining stroke risk. In children with elevated TCD velocities, BDNF correlates positively with PDGF-AA and IL-10, an anti-inflammatory cytokine [27]. In this study, BDNF was proposed to reduce cell death from ischemia related to elevated TCD velocities; diminishing inflammation could aid this process, as shown by the positive correlation between BDNF and IL-10. In addition to PDGF-AA, which is associated with vascular remodeling [27], TCD velocities also correlate directly with LDH, a marker of hemolysis [29]. This finding may indicate that hemolysis is associated with risk factors for cerebrovascular disease in SCD.

Intellectual Disability—Intellectual disability, previously known as mental retardation, is defined by the Diagnostic and Statistical Manual of Mental Disorders (5th edition), as deficits in intellectual and adaptive functioning [47]. Intellectual ability is measured by psychological IQ testing, while adaptive function is an assessment of the skills of daily living in the social, communication, independence, and functional domains. Children with SCD with and without a history of stroke have been found to have cognitive and IQ deficits in comparison to sibling and age-matched controls [5]. None of the included studies measured intellectual disability in relation to candidate neuro-biomarkers as defined above. Savage et al. did find a negative correlation between GFAP levels and the performance IQ subtest of the Weschler Abbreviated Scale of Intelligence [24]. While this correlation does not meet the full criteria for intellectual disability, it is the only result linking a candidate neuro-biomarker to a developmental outcome found in our systematic review.

DISCUSSION

The included articles found for this review using a systematic approach describe the literature regarding plasma, urine, and cerebrospinal fluid biomarker analysis and proteomic discovery related to neurological complications in SCD. A summary of the candidate neurobiomarkers' functions and associations are listed in Table 2. Commonly studied potential neurobiomarkers include neurotrophic and vascular growth factors, as well as inflammatory, cell adhesion, coagulation, and intracellular glial cell protein markers. Several of these analytes (GFAP, TSP-1, LSEL) have been previously validated in neonatal and adult stroke or trauma populations [39-41, 48-49].

The literature on proteomic discovery and biomarkers of neurological injury, and stroke in particular, in the general population is extensive [50, 51, 52]. A focus of this work has been the use and discovery of brain specific proteins for organ specificity, which include: neuron-specific enolase (NSE) [53], heart-type fatty acid binding protein (H-FABP) [54, 55, 56], N-methyl D-aspartate (NMDA) receptor [57], visin-like protein 1 (VLP) [58], S100B [59, 60], myelin basic protein (MBP) [61], and GFAP [62]. GFAP, in particular, has shown value in detection of multiple mechanisms of acute brain injury (traumatic brain injury, cardiac arrest, and stroke) with further efficacy in stroke to differentiate hemorrhagic and ischemic stroke and estimate lesion volume [63, 64]. Remarkably, despite these findings and the high prevalence of stroke in adults with SCD, there is limited research regarding neuro-

biomarkers in adult sickle cell disease [65]; one SCD biomarker review article does not include a section regarding associated neurological complications [13]. Similarly, many of these analytes have not been studied in pediatric SCD. As discussed above, the spectrum of brain injury in pediatric SCD is immense, from large vessel infarction to subtle deficits in attention and executive function, to truly subclinical injury. While some of these neuro-biomarkers were less helpful in the general or adult sickle cell population, they may well be useful in pediatric studies, particularly to determine risk of neurological injury, detect otherwise subclinical and frequent injuries, and differentiate between different complications. These additional biomarkers deserve further evaluation in larger pediatric populations to determine their clinical value; similarly, more broad-based, non-biased investigations of biomarkers in patients with SCD are warranted and badly needed.

BDNF and PDGF are among the most widely studied potential neuro-biomarkers identified in this review. These markers are associated with cell survival, BDNF through protection from cerebral ischemia, and PDGF-AA through angiogenesis. Both markers are significantly associated with increased risk of stroke and elevated TCD velocity, which is itself a stroke risk factor in pediatric SCD across multiple studies. This evidence supports the future use of these biomarkers in appropriate prospective clinical trials, as they have been studied retrospectively in prospectively obtained samples from a major pediatric SCD clinical trial. It is also interesting that while PDGF-AA had significant associations with both abnormal TCD velocities and stroke in the two studies they were both measured in, the PDGF-AB/BB isoforms only had significant associations with TCD velocities in one study, and no associations with stroke in either study. LDH was also only associated with elevated TCD velocities as opposed to different types of stroke. With further study, BDNF and PDGF-AA could conceivably be used to both predict both risk of stroke and recovery or prognosis after stroke.

LSEL, TSP-1, and GFAP are other promising candidates, based on exploratory analyses using the SIT study samples. These preliminary studies associated LSEL and TSP-1 with the most common neurological complication in sickle cell disease, SCI. Silent clinical infarcts are extremely difficult to diagnose clinically, due to absence of acute focal neurological symptoms; however, they lead to neurodevelopmental disorders, such as ADHD and executive dysfunction [3]. In addition, currently SCI require MRI for diagnosis. In children less than six years of age with SCD, the most susceptible age group, MRI often cannot be accomplished without sedation, further increasing the difficulty of diagnosis and risk of complications. GFAP may be useful in diagnosing subclinical brain injury not detected on clinical grounds or by imaging studies. Establishing that plasma levels of LSEL, TSP-1, and GFAP, are capable of detecting silent ischemic brain injury would allow less need for neuroimaging requiring sedation or anesthesia in the pediatric population, as well as faster diagnosis and intervention. However, all three measures need further validation in the pediatric SCD population before clinical use can be considered.

Microparticles are another promising area of study with regards to associations with neurological complications in children. Current microparticle literature mainly focuses on pulmonary and cardiac complications within the SCD population [23, 66-68]. Although microparticle markers have been criticized in the past due to poor measurement validity,

PMPs and ErMPs may eventually be useful neurobiomarkers as well [69]. One limitation of our articles is that we included studies with only the most common types of SCD and used explicit age criteria to limit our review to only pediatric studies. Several biomarker and proteomic articles were excluded from the study for not including information regarding the age of participants or plasma sample donors; however, this exclusion does improve the validity of our review. While we recognize that anemia is an important risk factor for both overt and silent ischemic brain injury, it was not included in this review, due to its status as a physiological measure, as opposed to a biomarker [8].

Of note, no articles meeting our inclusion criteria were identified that used urine or cerebrospinal fluid measures. There are studies addressing these types of potential biomarkers, usually in relation to outcomes that are not neurologically or developmentally pertinent. Consideration of relevant neurological cerebrospinal fluid and urine analytes should be considered for broad-based proteomics studies of neurologic injury in SCD and future clinical trials; this is particularly true for urine, as it is minimally invasive to collect from most children.

Also, while several of the biomarker studies were based on previous proteomic discovery work, we were unable to find any articles related to a systematic discovery-based evaluation of proteomic biomarkers of neurological complications in pediatric SCD. This result is especially surprising, given the attention paid to red cell membrane proteins with regards to vaso-occlusive crisis and acute chest syndrome [70-75]. Red cell, white cell, and other cellular membrane proteins associated with neurological complications in pediatric SCD are also understudied areas in proteomic discovery.

We were also unable to find any articles regarding biomarker analysis or proteomic discovery in children with SCD and neurological and neurodevelopmental complications not directly related to stroke and SCI, such as seizures, headaches, intellectual disability, and ADHD. Performance IQ did have a weak negative correlation with GFAP levels; however, this is only one component of intellectual disability and additional adaptive function measures are needed. These neurological and developmental disorders are quite common in the general population, but may be difficult to characterize in pediatric SCD, due to varying disease presentations. All four disorders have been seen in children with SCD with and without a history of stroke. Further study and clarification of these clinical phenotypes is necessary before protein analyses can be undertaken.

It is worth noting that the studies with positive results mainly used plasma samples from large clinical trials, such as the STOP and SIT studies. Systematic collection of samples and a central repository for trials involving multiple centers is efficient and adds to the ease of data access and analysis. Clinical trials can and should be used for further studies of biomarker analyses and proteomic discovery, with appropriate sample collection. It is especially important that current findings be validated in prospective studies of this sort, either as primary studies, or ancillary to other studies in SCD.

In conclusion, the neuro-biomarkers in pediatric SCD have provided important contributions to the field thus far in a short timeline. Most of the findings at this time are exploratory, and

help more to understand pathophysiology than provide immediately useful clinical tests; however, several promising candidate have been established. Additional work is needed to validate these biomarkers, as well as those used in other developmental clinical disorders, such as ADHD, that may be useful for this population. To date, proteomics is particularly under-utilized with regards to associated neurological complications. Continued research in this area is needed to understand, predict, prevent, assess, follow, and treat the devastating neurological complications suffered by children with SCD.

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JFC has received an honorarium and travel expenses in the past and presently receives salary support through Johns Hopkins for providing consultative advice to Mast Pharmaceuticals (previously Adventrx Pharmaceuticals) regarding a proposed clinical trial of an agent for treating vaso-occlusive crisis in sickle cell disease. JFC and ADE are inventors with the Johns Hopkins discovered brain injury biomarkers licensed to ImmunArray, Inc. AE is a paid consultant to Immunarray, Inc. JFC and EBC have filed a provisional patent for a potential treatment for sickle cell disease.

Abbreviations

SCD	sickle cell disease
MRI	magnetic resonance imaging
SCI	silent cerebral infarctions
ADHD	attention deficit hyperactivity disorder
TCD	transcranial Doppler
STOP	Stroke Prevention Trial in sickle cell anemia
SIT Trial	Silent Cerebral Infarct Multi-Center Clinical Trial
NUTSCD	Nutrition on Inflammation in children with sickle cell anemia
GFAP	glial fibrillary acidic protein
PDGF	platelet-derived growth factor
BDNF	brain-derived neurotrophic factor
GM-CSF	granulocyte monocyte-colony stimulating factor
VEGF	vascular endothelial growth factor
IL	interleukin
IFN-γ	interferon- γ
TNF-α	tumor necrosis factor α
TAT-III	Thrombin-Antithrombin III
LDH	lactate dehydrogenase

IQ	intellectual quotient
s-VCAM	soluble vascular cell adhesion molecule
sICAM-1	soluble intercellular adhesion molecule-1
tPAI-1	tissue plasminogen activator inhibitor
FPA	fibrinopeptid A
PECAM-1	platelet/endothelial cell adhesion molecule
ErMPs	erythrocyte microparticles
PMPs	platelet-derived microparticles
ASCIEs	acute silent cerebral ischemic events
DWI	diffusion weighted imaging
TSP-1	thrombospondin-1
LSEL	L-selectin
NSE	neuron-specific enolase
H-FABP	heart-type fatty acid binding protein
NMDA	N-methyl D-aspartate
VLP	visin-like protein 1
MBP	myelin basic protein

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Figure 1.
Search Strategy of Biomarker and Proteomics Research in Pediatric Sickle Cell Disease
Neurological Complications

TABLE 1

Table of Included Studies

Authors, Year, Study Type	Participants/Samples	Biomarkers/Proteomics Measured	Results
Asare et al, 2010 Study type not identified	<p>Number: 39 children</p> <ul style="list-style-type: none"> 13 participants with elevated transcranial Doppler (TCD) velocities without stroke 13 participants with elevated TCD velocities with stroke 6 hemoglobin AA (HbAA) steady-state controls 7 HbSS controls <p>Age: 2-16 years</p> <p>Sickle Cell Disease (SCD) genotype: HbSS or HbS-β^0 thalassemia</p> <p>Study samples: Stroke Prevention in sickle cell anemia (STOP) * trial</p> <p>Sites: 14 STOP study centers in United States</p>	<p>Plasma samples collected upon recruitment from 1995 to 1997 and stored at -80°C</p> <p>Commercial multiplex calorimetric bead-based protein array system</p> <p>Inflammatory proteins assayed: (Bio-Rad Bioplex Beadlyte system, Hercules, CA): Interleukin (IL)-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, fibrocyte growth factor (FGF) basic, granulocyte-colony stimulating factor (G-CSF), granulocyte monocyte-colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), 10 kDa interferon-γ-induced protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory proteins-1α and β (MIP-1α and MIP-1β), platelet derived growth factor-BB (PDGF-BB), regulated upon activation normal T-cell expressed & secreted protein (RANTES), tumor necrosis factor α (TNF-α) and vascular endothelial growth factor (VEGF)</p> <p>No adjustments were made for multiple comparisons</p>	<p>1. Higher IL-1β concentration associated with a reduced risk of stroke in HbSS participants (OR 0.59, 95% CI: 0.36, 0.96, $P = 0.034$, AUC 0.852)</p>
Faulcon et al, 2013 Study type not identified	<p>Number: 116 children</p> <ul style="list-style-type: none"> 65 participants with silent cerebral infarct (SCI) 51 participants without SCI <p>Age: 5-14 years</p> <p>SCD genotype: HbSS or HbS-β^0 thalassemia</p> <p>Study samples: Convenience samples from Silent Cerebral Infarct Multi-Center Clinical Trial (SIT) **</p> <p>Sites: 25 SIT Trial sites in the United States, Canada, England, and France</p>	<p>Baseline plasma samples collected upon enrollment from February 2007 to May 2009</p> <p>Commercial immunoassays</p> <p>Plasma proteins assayed: thrombospondin 1 (TSP-1) and L-selectin (LSEL)</p>	<p>1. Participants with SCI had higher median TSP-1 concentrations than participants without SCI (8.4 vs 6.2 $\mu\text{g/mL}$, $p = 0.03$).</p> <p>2. Participants with SCI had higher median LSEL concentrations than participants without SCI (1.46 vs 1.35 $\mu\text{g/mL}$, $p = 0.03$).</p> <p>3. Systolic blood pressure correlated positively with LSEL in participants with silent cerebral infarct ($r = 0.28$, $p = 0.02$).</p>
Hyacinth et al, 2012 Cross-sectional, nested prospective study design	<p>Number: 39 children</p> <ul style="list-style-type: none"> 19 participants with abnormal TCD from STOP study 13 participants with normal TCD from Nutrition on Inflammation in children with sickle cell anemia (NUTSCD) study 7 healthy controls from the NUTSCD study <p>Age:</p> <ul style="list-style-type: none"> 2-16 years in STOP participants 6-12 years NUTSCD participants <p>SCD genotype:</p> <ul style="list-style-type: none"> STOP study - HbSS or HbS-β^0 thalassemia NUTSCD study- HbSS <p>STOP participants:</p> <p>Abnormal TCD = a time-averaged mean cerebral blood-flow velocity 200 cm/sec twice or 220 cm/sec once</p>	<p>Samples:</p> <ul style="list-style-type: none"> STOP -plasma samples collected at study entry from 1995 to 1997 and stored at -80°C NUTSCD-plasma samples collected starting in December 2009 stored at -80°C <p>Commercial multiplex, antibody immobilized bead-based protein array</p> <p>Pro-/anti-inflammatory cytokine, angiogenic and neurotropic markers assayed: (Millipore, Billerica, MA and Bio-Rad, Hercules, CA): IL-1β, IL-1ra, IL-4, IL-6, IL-10, IL-13, IFN-γ, transforming growth factor α (TGF-α), TNF-α, VEGF, GM-CSF, brain derived neurotrophic factor (BDNF), PDGF types AA and AB/BB</p>	<p>1. Participants with SCD and abnormal TCD velocities had higher mean plasma levels of BDNF (213.6 ± 91.4 pg/mL) than participants with normal TCDs (91.2 ± 97.7 pg/mL, $p = 0.004$) or controls (34.1 ± 20.3 pg/mL, $p < 0.001$).</p> <p>2. Participants with SCD and abnormal TCD velocities had higher mean plasma levels of PDGF-AA (346.7 ± 152.3 pg/mL) than participants with normal TCDs (111.5 ± 91.6 pg/mL, $p = 0.001$) or controls (33.7 ± 32.4 pg/mL, $p < 0.001$).</p> <p>3. Participants with SCD and abnormal TCD velocities had higher mean plasma levels of PDGF-AB/BB (790.6 ± 350.9 pg/mL) than controls (101.0 ± 72.3 pg/mL, $p < 0.001$).</p> <p>4. High TCD velocity correlated positively with plasma PDGF-AA levels among SCD HbSS participants ($r = 0.5$, $p = 0.032$).</p>

Authors, Year, Study Type	Participants/Samples	Biomarkers/Proteomics Measured	Results
	<ul style="list-style-type: none"> No history of stroke NUTSCD participants: • Normal TCD velocities • Not on hydroxyurea or chronic transfusion therapy for 4 months • Not on oral corticosteroids or non-steroidal anti-inflammatory drug (NSAID) therapy STOP trial Sites: 14 STOP * study centers in USA NUTSCD pilot trial Site: Morehouse School of Medicine in Atlanta, GA 		<p>5. In participants with SCD HbSS and high TCD velocity, plasma BDNF levels correlated positively with PDGF-AA ($r = 0.5$, $p = 0.038$) and IL-10 ($r = 0.5$, $p = 0.019$).</p> <p>6. Participants with SCD and high TCD velocities who later developed stroke had higher mean levels of PDGF-AA than participants who did not develop stroke (399.5 ± 143.4 pg/mL vs 209.6 ± 161.3 pg/mL, $p = 0.012$).</p> <p>7. Elevated BDNF and PDGF-AA plasma levels were significantly associated with increased odds of high TCD velocity in SCD HbSS participants (OR = 1.022/unit rise, $p = 0.004$ and OR = 1.023/unit rise, $p = 0.014$, respectively).</p> <p>8. Elevated PDGF-AA plasma levels were associated with a significant risk of developing stroke (OR = 1.014/unit rise, $p = 0.044$, AUC = 0.91).</p>
Hyacinth et al, 2014 Study type not identified	<p>Number: 80 children</p> <ul style="list-style-type: none"> • 40 participants on standard care • 40 participants on transfusion <p>Age: 2-16 years</p> <p>SCD genotype: HbSS or HbS-β^0 thalassemia</p> <p>Study samples: STOP study from Medical University of South Carolina (MUSC) only</p>	<p>Serum samples collected between 1995 – 1997 at baseline, study exit, and 1 year post trial</p> <p>Commercial multiplex, antibody immobilized bead-based protein array</p> <p>Analytes of the Human Neurodegenerative Panel kit assayed (Millipore, Billerica, MA): BDNF, soluble vascular cell adhesion molecule (sVCAM)-1, soluble intercellular adhesion molecule (sICAM)-1, myeloperoxidase (MPO), Cathepsin-D, PDGF-AA and PDGF-AB/BB, released upon activation normal T-cell expressed and secreted (RANTES, CCL5), tissue plasminogen activator inhibitor (tPAI)-1, and neural cell adhesion molecule (NCAM)-1</p>	<p>1. Participants with low baseline serum levels of BDNF ($P = 0.025$), sVCAM-1 ($P = 0.025$), PDGF-AA ($P = 0.01$), tPAI-1 ($P = 0.025$), and sICAM-1 ($P = 0.022$) had a significantly higher probability of stroke-free survival.</p> <p>2. Baseline variables differed between the transfusion and standard care arms – no known explanation.</p>
Mourad et al, 2008 Study type not identified	<p>Number: 20 children</p> <ul style="list-style-type: none"> • 10 children in steady state • 10 children with ongoing pain crisis <p>Neurological complications:</p> <ul style="list-style-type: none"> • 4 participants with stroke or SCI • 16 participants without stroke or SCI <p>SCD genotype: HbSS only</p> <p>Ages:</p> <ul style="list-style-type: none"> • steady state- 4-12 years • pain crisis- 5-11 years <p>Study samples collected: Tanta University Hospital, Tanta, Egypt</p>	<p>Plasma samples</p> <p>Quantitative sandwich enzyme immunoassay</p> <p>Analytes assayed: Fibrinogen A (FPA), Thrombin-Antithrombin III (TAT III), D-dimers, serum platelet/endothelial cell adhesion molecule (PECAM-1)</p>	<p>1. The stroke group had significantly elevated plasma concentrations of FPA (4.565, $p < 0.001$), TAT (3.537, $p < 0.01$), D-Dimer (4.029, $p < 0.001$), and serum PECAM-1 (3.336, $p < 0.01$) in comparison to the non-stroke group.</p>
O'Driscoll et al, 2008 Study type not identified	<p>Number: 115 children</p> <p>Age: 2.5-16.4 years</p> <p>SCD genotype:</p> <ul style="list-style-type: none"> • HbSS (n=97) • HbSC (n=18) <p>Study samples collected: King's College Hospital, London, UK</p>	<p>Blood samples from 2006 collected during clinical care</p> <p>Routine hematological and biochemical assays</p> <p>Protein assayed: total serum lactate dehydrogenase (LDH)</p>	<p>1. TCD velocities from the proximal extracranial internal carotid arteries ($r = 0.255$, $p = 0.008$), posterior cerebral arteries ($r = 0.336$, $p < 0.001$), anterior cerebral arteries ($r = 0.354$, $p < 0.001$), and middle cerebral arteries ($r = 0.402$, $p < 0.001$) correlate with total serum LDH levels in SCD participants.</p> <p>2. LDH was not an independent marker of hemolysis in multivariate analysis.</p>
Savage et al, 2011a	<p>Number:</p> <ul style="list-style-type: none"> • 295 children with SCD • 60 healthy controls 	<p>Baseline plasma samples collected in subjects at baseline between February 2007 - May 2009 and stored at -80°C</p>	<p>1. Participants with SCD had higher mean plasma GFAP concentrations than healthy pediatric controls (0.144 ± 0.368 ng/ml vs 0.072 ± 0.083 ng/ml, $p = 0.003$).</p>

Authors, Year, Study Type	Participants/Samples	Biomarkers/Proteomics Measured	Results
Cross sectional study	<ul style="list-style-type: none"> 28 adults with overt brain injury and no SCD Age: <ul style="list-style-type: none"> Children with SCD 5 - 14 years Healthy controls 5 - 16 years Adults SCD genotype: HbSS or HbS-β⁰ thalassemia Study samples: SIT * trial Sites: 25 SIT Trial sites in the United States, Canada, England, and France 	<p>Electrochemiluminescent immunoassay (MesoScale Discovery, Rockville, MD)</p> <p>Protein assayed: plasma glial fibrillary acidic protein (GFAP)</p>	<ol style="list-style-type: none"> Participants with SCD and acute cerebral infarct had a more elevated GFAP levels than healthy pediatric controls, as defined by the 95% cutoff (16%, $p = 0.04$). The proportion of participants with elevated GFAP was higher in participants with acute brain injury (defined by positive diffusion-weighted-imaging [DWI]) than SCD participants with cerebral infarct of undetermined age (but also DWI-negative) (50% vs 13.8%, $p = 0.05$) and SCD participants without cerebral infarct (50% vs 8.3%, $p = 0.01$). GFAP levels correlated negatively with performance IQ subtest of Wechsler Abbreviated Scale of Intelligence in participants with SCD ($r = -0.29$, $p = 0.04$).
Savage et al, 2011b Case Report	<p>Number:</p> <ul style="list-style-type: none"> 1 child with SCD 1 sibling control with SCD 60 healthy pediatric controls <p>Age:</p> <ul style="list-style-type: none"> case report child with SCD 12 years sibling control with SCD 8 years healthy controls 5-16 years <p>SCD genotype: HbSS</p> <p>Samples collected: Johns Hopkins Hospital, Baltimore, MD</p>	<p>Plasma samples collected from discard blood frozen and stored at -80°C</p> <p>Electrochemiluminescent immunoassay (MesoScale Discovery, Rockville, MD)</p> <p>Protein assayed: Plasma GFAP</p>	<ol style="list-style-type: none"> GFAP levels in the participant were 1.5 ng/ml at 32 hours prior to stroke, which is 6.6x higher than the normal 95% of pediatric controls (0.227ng/mL) and 17x higher than the sibling control (0.088ng/mL). Levels peaked at 2.83 ng/ml at the time of the stroke, and then declined to normal levels after the stroke.
Tantawy et al, 2013 Cross sectional study	<p>Number:</p> <ul style="list-style-type: none"> 50 children with SCD 40 healthy controls <p>Age:</p> <ul style="list-style-type: none"> children with SCD 2-18 years mean 11.1 ± 4.9 years controls 3-18 years mean 10.6 ± 3.4 years <p>SCD genotype: HbSS and HbS-β thalassemia</p> <p>Samples collected: Ain Shams University, Cairo, Egypt</p>	<p>Blood samples</p> <p>Standard three-color flow cytometry</p> <p>Hematological, coagulation and biochemical assays (Roche Diagnostics, Mannheim, Germany; Siemens Healthcare Diagnostics, Marburg, Germany)</p> <p>Microparticles assayed: erythrocyte-derived (ErMPs) and platelet-derived (PMPs)</p> <p>Proteins assayed: LDH, indirect bilirubin, C reactive protein, serum ferritin, D-dimers, TAT III, von Willebrand factor antigen</p>	<ol style="list-style-type: none"> Participants with a history of stroke had significantly higher PMPs levels than participants without a history of stroke (8.1 ± 1.2 vs 3.8 ± 0.61, $p < 0.001$). Participants with a history of stroke had significantly higher ErMPs levels than participants without a history of stroke (9.3 ± 0.83 vs 4.4 ± 0.53, $p < 0.001$).

* STOP Trial headquarters located at Medical University of South Carolina

** SIT Trial headquarters located at Washington University in St. Louis and Vanderbilt University – Biologic Repository and specimens for the SIT Trial located at Johns Hopkins University

Table 2

Candidate Neuro-biomarkers Of Pediatric Sickle Cell Disease

Candidate Neuro-biomarkers	Function	Association in Sickle Cell Disease	Level	Citation
Brain-derived neurotrophic factor (BDNF)	Nerve growth factor	Decreased risk of ischemic or hemorrhagic stroke Elevated transcranial Doppler velocities	Lower Higher	31 27
Cross-linked fibrin D fragments (D-Dimer)	Coagulation marker of fibrin degradation	History of overt stroke and silent cerebral infarction	Higher	22
Erythrocyte microparticle (ErMP)	Procoagulant factor Vascular injury biomarker	History of focal neurological deficit for greater than 24 hours or acute infarction	Higher	23
Fibrinopeptide A (FPA)	Coagulation marker of fibrin degradation	History of overt stroke and silent cerebral infarction	Higher	22
Glial fibrillary acidic protein (GFAP)	Astrocyte intermediate filament	History of acute cerebral infarction	Higher	24, 28
Interleukin- β (IL- β)	Inflammatory mediator	Decreased risk of ischemic or hemorrhagic stroke	Higher	26, 27*
Lactate dehydrogenase (LDH)	Intravascular hemolysis marker	Elevated transcranial Doppler velocities	Higher	29
L-selectin (LSEL)	Adhesion/homing receptor	History of silent cerebral infarction	Higher	30
Platelet-derived growth factor-AA (PDGF-AA)	Growth factor Endothelial activator	Increased risk of ischemic or hemorrhagic stroke Elevated transcranial Doppler velocities Decreased risk of ischemic or hemorrhagic stroke	Higher Higher Lower	27 27 31
Platelet-derived growth factor-AA/BB (PDGF-AA/BB)	Growth factor Endothelial activator	Elevated transcranial Doppler velocities	Higher	27
Platelet/endothelial cell adhesion molecule-1 (PECAM)	Immunoglobulin superfamily member	History of overt stroke and silent cerebral infarction	Higher	22
Platelet microparticle (PMP)	Procoagulant factor Vascular injury biomarker	History of focal neurological deficit for greater than 24 hours or acute infarction	Higher	23
Soluble intercellular adhesion molecule-1 (sICAM-1)	Inflammation marker	Decreased risk of ischemic or hemorrhagic stroke	Lower	31
Soluble vascular cell adhesion molecule-1 (sVCAM-1)	Inflammation marker	Decreased risk of ischemic or hemorrhagic stroke	Lower	31
Thrombin-antithrombin III complex (TAT)	Coagulation activation marker	History of overt stroke and silent cerebral infarction	Higher	22
Thrombospondin-1 (TSP-1)	Cell-cell and cell-matrix mediator	History of silent cerebral infarction	Higher	30
Tissue plasminogen activator inhibitor-1 (tPAI-1)	Tissue serine protease inhibitor	Decreased risk of ischemic or hemorrhagic stroke	Lower	31

* not statistically significant in subsequent study