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Authors

Samarron, Sandra L
Miller, Joshua W
Cheung, Anthony T
et al.

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Homocysteine is Associated with Severity of Microvasculopathy in Sickle Cell Disease Patients

Sandra L. Samarron, PhD[1], **Joshua W. Miller, PhD**[2], **Anthony T. Cheung, PhD**[1,3], **Peter C. Chen, PhD**[3,4], **Xin Lin, PhD**[1], **Theodore Zwerdling, MD**[5], **Ted Wun, MD, FACP**[1,6,7], **Ralph Green, MD, PhD, FRC Path**[1,6]

[1]Department of Medical Pathology and Laboratory Medicine, University of California, Davis

[2]Department of Nutritional Sciences, Rutgers University, New Brunswick, NJ

[3]Institute for Biomedical Sciences, San Diego, CA

[4]Shiley Center for Orthopaedic Research and Education, Scripps Health, La Jolla, CA

[5]Department of Pediatrics (Hematology-Oncology), University of California, Davis (retired)

[6]Department of Internal Medicine (Hematology-Oncology), University of California, Davis

[7]UC Davis Clinical and Translational Science Center, Sacramento, CA

Summary

The pathophysiology of sickle cell disease (SCD) includes vasculopathy as well as anemia. Elevated plasma homocysteine is a risk factor for vascular disease and may be associated with increased risk of vascular complications in SCD patients. In the present study, microvascular characteristics were assessed in the bulbar conjunctiva of 18 pediatric and 18 adult SCD patients using the non-invasive technique of computer-assisted intravital microscopy. A vasculopathy severity index (SI) was computed to quantify the degree of microvasculopathy in each patient. Plasma homocysteine and several of its determinants [serum folate and vitamin B12, plasma pyridoxal-5'-phosphate (vitamin B6 status) and creatinine (kidney function)] were measured. Age was strongly correlated with microvasculopathy in the SCD patients, with the SI increasing about 0.1 unit per 1-year increase in age ($p<0.001$). After adjusting for age, sex, B vitamin status and creatinine, homocysteine concentration was directly correlated with severity index ($p<0.05$). Age and homocysteine concentration were independent predictors of microvasculopathy in SCD patients. It remains to be determined whether lowering homocysteine concentrations using

Corresponding Author: Ralph Green, MD, PhD; Department of Pathology and Laboratory Medicine, University of California Davis, 4400 V Street, PATH Building, Sacramento, California 95817, USA. rgreen@ucdavis.edu; Phone: 916-734-8078; Fax: 916-734-2652.

Authorship Contribution

Contributions: S.L.S. recruited patients, performed analyte assays, maintained the study database, interpreted data, and generated the first draft of the manuscript; J.W.M. helped design the study, supervised performance of the analyte assays, performed statistical analyses, interpreted data, and revised and generated the final draft of the manuscript; A.T.C. helped design the study, conducted the CAIM procedures, analyzed the CAIM data, and revised the manuscript; P.C.C. analyzed the CAIM data; X.L. performed analyte assays and revised the manuscript; T.Z. helped design the study and recruited patients; T.W. helped design the study, recruited patients, interpreted data, and revised the manuscript; R.G. served as Principal Investigator, designed the study, interpreted data, and revised the manuscript. All authors reviewed and provided final approval of the manuscript.

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appropriate B vitamin supplements (folate and vitamins B12 and B6), particularly if started early in life, could ameliorate microvasculopathy and its associated complications in SCD patients.

Keywords

Sickle Cell Disease; Homocysteine; Microvasculature; Intravital Microscopy; Folate Vitamin B12; Vitamin B6

Introduction

The pathophysiology of sickle cell disease (SCD) is a complex process that involves disturbances in blood flow, blood coagulation, endothelial function and vascular inflammation (Kato *et al,* 2018). Vaso-occlusion and ischemia-reperfusion injury, affecting both the micro- and macrovasculature, underlie many of the clinical manifestations of SCD. The clinical manifestations include painful vaso-occlusive crisis, acute chest syndrome, pulmonary hypertension, priapism, retinopathy and stroke, as well as chronic, irreversible damage to major organs, including the brain, heart, lungs, kidneys, eyes, and long bones.

Consistent, strong predictors of ischemic or hemorrhagic stroke in children with SCD include low hemoglobin and elevated systolic blood pressure, as well as increased cerebral blood flow velocity detected by transcranial Doppler ultrasound (TCD) (Strousse *et al*, 2011). However, these biomarkers have not been validated to the same extent in adult SCD patients (Strousse *et al*, 2011). The 1998 Cooperative Study of Sickle Cell Disease (CSSCD) reported that patients who were homozygous for the S genotype (HbSS) had chances of having a first cerebrovascular accident by 20, 30, and 45 years of age of 11%, 15%, and 24%, respectively (Ohene-Frempong *et al*, 1998). However, in the CSSCD post-trial follow-up study among their low-risk group who had experienced a cerebrovascular event, no predictive cerebrovascular biomarkers were identified (Lee *et al*, 2006). Additionally, a significant proportion of children with HbSS who have not had a clinically apparent neurologic event have nonetheless had silent cerebral infarctions as revealed by magnetic resonance imaging (Wang *et al*, 2000).

Elevated plasma homocysteine concentration (hyperhomocysteinemia) is a risk factor for vascular disease in the general population (Refsum *et al*, 1998), and often occurs in both pediatric and adult SCD patients (Houston *et al*, 1997; van der Dijs *et al*, 1998; Rodriguez-Cortes *et al*, 1999; Schnog *et al*, 2000; Rana *et al*, 2000; Wang, 2000; Lowenthal *et al*, 2000; Balasa *et al*, 2002; van der Dijs *et al*, 2002; Dhar *et al*, 2004; Pandey *et al*, 2012). The clinical significance of elevated homocysteine in SCD is unclear. Other than a correlation with stroke (Houston *et al*, 1997), no studies have assessed the association of hyperhomocysteinemia with microvascular or macrovascular events or damage in SCD. B vitamin deficiencies (folate and vitamins B12 and B6) and renal disease are primary causes of hyperhomocysteinemia (Refsum *et al*, 1998; Selhub & Miller, 1992). In SCD, folate is of particular interest because erythrocytes contain a high concentration of folate, and intravascular hemolysis leads to loss of folate in the urine and stool (Kato *et al*, 2017; Antony, 2017). Coupled with increased rates of DNA synthesis due to ineffective erythropoiesis (Jonsson *et al*, 1959; Chanarin *et al*, 1959), the requirement for folate in SCD,

as in other chronic hemolytic anemias, is increased and folic acid supplements are typically prescribed (Green, 2016).

Computer-assisted intravital microscopy (CAIM) has been developed in our laboratory as a tool to non-invasively study and quantify real-time microvascular characteristics in diseases that affect the vascular system such as SCD, diabetes, hypertension and Alzheimer's disease (Cheung *et al*, 2001; Cheung *et al*, 2001b; Cheung *et al*, 2002a; Cheung *et al*, 2002b; Smith *et al*, 2009; Cheung *et al*, 2010; Cheung *et al,* 2012). Previously, using CAIM, we demonstrated that microvascular abnormalities in SCD patients develop in childhood, and that the severity of microvasculopathy likely progresses with age (Cheung *et al*, 2010). In the present study we examined the associations of microvascular abnormalities, as measured by CAIM, with homocysteine and B vitamin status in pediatric and adult SCD patients.

Materials and Methods

Patients

Patient recruitment and procedures for the study were approved by the Institutional Review Board (IRB) Administration at the University of California, Davis. Signed informed consent was obtained from adult patients, and signed parental consent was obtained for all pediatric patients age 17 years and younger (along with assent from patients age 12–17 years). Patients diagnosed with SCD (HbSS and HbSC genotype), 4 years of age and older, were recruited from the UC Davis Medical Center (UCDMC) sickle cell clinics in Sacramento, CA during outpatient visits, or during participation in the Sacramento regional Sickle Cell Family Support Group and the annual California Sickle Cell Disease Symposia. Patients were recruited during routine clinical visits and assessed for eligibility. Patients who experienced a vaso-occlusive (painful) crisis or other acute complication of SCD within one month of the study were excluded. Patients receiving hydroxyurea were eligible. Those on chronic transfusion and/or exchanges were allowed to participate in the study, with their blood collections for analysis and other measurements performed at least 1 month after the most recent transfusion or exchange. Patients or guardians and their medical records were queried for the use of concurrent corticosteroids, nonsteroidal anti-inflammatory agents, hydroxyurea, folic acid, multivitamins, prophylactic antibiotics, past year immunizations and use of pain medications. Their medical records were also assessed for history of transfusions, asplenia, painful crisis and pulmonary complications, and any on-going, non-SCD-related health issues.

Over the course of this study (recruitment period 2007 – 2011) there was a cohort of 280 (120 adult, 160 pediatric) registered patients in the UCDMC Sickle Cell Clinics (Figure 1). Of the 280 patients, 34 pediatric (21% of patient pool) and 35 adult (29% of patient pool) patients were identified as having been seen by a hematologist within the previous 12 months. Among these, 19 pediatric (56%) and 19 adult (54%) patients consented to participate in the study, 13 pediatric (38%) and 11 adult (31%) declined to participate, and 2 pediatric (6%) and 5 adult (14%) were not eligible based on exclusion criteria. Reasons prospective participants refused to participate or were deemed ineligible for the study are provided in the legend to Figure 1.

Sample Collection and CAIM Analysis

Fasting venous blood was collected from each patient and wrapped in aluminum foil to minimize degradation of light-sensitive B vitamins during processing. Plasma (EDTA) tubes were immediately placed on ice; serum separator tubes were set at room temperature for 30 minutes to clot, and then placed on ice. Aliquots of EDTA whole blood were assayed for hemoglobin and hematocrit by Automated Coulter Counter (Beckman Coulter). After centrifugation at 4 ºC, plasma and serum were collected and stored at −80 ºC until analysis. Plasma creatinine was measured by the Jaffe rate reaction method using a SYNCHRON LX20 instrument (Beckman Coulter), and serum folate and vitamin B12 were measured using a competitive chemiluminescent immunoassay (Advia Centaur automated instrument, Siemens) by standardized clinical protocols at the UCDMC Department of Pathology and Laboratory Medicine Clinical Laboratory. Total plasma homocysteine (Gilfix *et al*, 1997) and plasma pyridoxal-5'-phosphate (Talwar *et al*, 2003) were measured by high pressure liquid chromatography in the investigators' research laboratory. Inter-assay coefficients of variation for all assays were <5%.

Directly following blood collections, patients had their bulbar conjunctiva visualized and recorded via CAIM. The CAIM system uses macro-optics in which image acquisition is based on video documentation of selected regions in the *in vivo* conjunctival microcirculation. Specific microvascular abnormalities were identified during viewing of selected video sequences and a severity index (SI) was computed to quantify the degree of microvasculopathy observed in each patient. SI values were calculated as the sum of the presence of any of 15 possible microvascular abnormalities (Supplemental Table) on a binary (yes = 1; no = 0) basis. SI values for each patient were determined by two independent evaluators who were blinded to the identity of the patients. Discrepancies between the two evaluators were adjudicated by a third party who also was blinded to the identity of the patients. The procedures of this technique, as well as descriptions and images of the 15 microvascular abnormalities, have been described in detail for SCD patients in previous publications (Cheung *et al*, 2001a; Cheung *et al*, 2002a; Cheung *et al*, 2010; Cheung *et al,* 2012).

Statistical Analysis

Data for blood analytes and SI are summarized as medians and ranges. Differences in median values between the pediatric and adult patient groups were assessed using the Mann-Whitney test. Associations between independent variables and SI were assessed by multivariable linear regression. First, a base regression model including only age and sex as independent variables was established. Subsequent models assessed the association of each additional independent variable with SI after adjusting for age and sex, as well as the change in the variance explained by the independent variable over and above the model that included age and sex alone, i.e. the change in R^2 of the model. Because homocysteine was not normally distributed it was natural log transformed before inclusion in the regression analysis. Statistical significance was defined as $p<0.05$. Statistics were carried out using Statview for Macintosh and Windows, version 5.0.1 (Abacus Concept, 1998).

Results

Summary patient characteristics are presented in Table 1. Thirty-eight SCD patients were recruited into the study. The patients were equally split between pediatric (age ≤18 years, n = 19) and adult patients (age >18 years, n = 19). The adult sex distribution was heavily weighted toward female patients - 79% females, 21% males; pediatric patients were 58% females and 42% males. The adult patients ranged from 22 to 61 years of age, with 7 patients (37%) age 22–29 years, 8 (42%) age 30–39 years, and 4 (21%) age 48–61 years. Of the pediatric patients, 6 (32%) were 4–8 years of age, 4 (21%) were 9–13 years of age, and 9 (47%) were 14–18 years of age.

Nine (47%) of the adult patients and 2 (10%) of the pediatric patients were on a stable dose of hydroxyurea treatment (Table 1). Transfusion frequency differed between the pediatric and adult patients: most pediatric patients (N=17) were on chronic transfusion therapy (approximately monthly). Adult patients were transfused as clinically indicated, and intervals were as infrequent as one per year to one per 5 years. Of the 15 adult patients who reported being prescribed a folic acid supplement (79%), 12 (80%) were non-adherent. Patients who took their pills at least 5 out of 7 days per week were considered as taking folic acid supplementation. Additional multivitamin supplementation was 16% among the pediatric patients and 37% among the adult patients.

One pediatric and one adult patient dropped out of the study prior to sample collection and CAIM procedure. B vitamin, homocysteine, creatinine, and CAIM data were available for the remaining subjects (N=18 per age group), except for B12, PLP, creatinine and CAIM data for 1 adult patient (Table 1). Median values for hemoglobin and hematocrit and the three B vitamins (folate, B12, and PLP) were not significantly different between the pediatric and adult patients. Median (range) homocysteine [5.5 (2.6–9.6) vs 7.0 (3.6–18.9) μmol/L; P=0.01], creatinine [0.4 (0.2–0.9) vs 0.6 (0.3–1.4) mg/dL; P=0.001], and SI values [4.0 (0.0–7.0) vs 7.0 (1.0–9.0); P<0.001] were lower in the pediatric patients compared with the adults. Only one pediatric patient had no microvascular abnormalities, while all the rest had 2 or more. Zero adults had no abnormalities, one had 1 abnormality, and all the rest had 4 or more. Specific microvascular abnormalities that were highly prevalent in both pediatric and adult patients (≥50% in both patient groups) included abnormal arteriovenous ratio, abnormal diameter, abnormal distribution, boxcar phenomenon, and sludging (Supplemental Table). Specific microvascular abnormalities that were more prevalent in adult than pediatric patients included abnormal vessel morphology, hemosiderin deposits, and vessel tortuosity.

In the multivariable linear regression model that included just age and sex, age was a strong determinant of microvasculopathy severity, with the SI increasing about 0.1 unit per 1-year increase in age (β=0.104, P<0.001) (Table 2). Sex was not a significant determinant of SI. Together, age and sex explained 43.8% (R^2=0.438) of the observed variance in SI among all the patients. Among the blood analytes, only homocysteine was significantly correlated with SI: β=3.187 (P=0.002) by simple regression (Figure 2) and β=2.376 (P=0.047) after adjustment for age and sex (Table 2). Addition of homocysteine to the regression model explained an additional 6.8% of the variance in SI over the model containing only age and sex (increasing the total R^2 for model to 0.506). Addition of determinants of homocysteine

concentration (B vitamins and creatinine concentrations) to the multivariable model that included age, sex, and homocysteine did not significantly change the homocysteine coefficient, thus indicating that the homocysteine association was independent of its known determinants. Hemoglobin, hematocrit, and multivitamin or folic acid supplement use were not associated with homocysteine concentration or severity index.

Discussion

The primary finding in the present study is that homocysteine, independent of age, sex, B vitamin status, and creatinine, is directly correlated with severity of microvasculopathy in the bulbar conjunctiva of SCD patients as measured by CAIM. In addition, the severity of microvasculopathy was significantly lower in the pediatric patients than in the adult patients [SI median values (range): 4.0 (0.0–7.0) and 7.0 (1.0–9.0), respectively]. For comparison purposes, typical SI values for healthy children and adults range from 0–2 (Cheung *et al*, 2002a; Smith *et al*, 2009). This latter finding confirms our previous report on a cohort of 14 pediatric and 8 adult SCD patients in which the severity of vasculopathy differed significantly between children and adults (Cheung *et al*, 2010). We also observed in the present study that the prevalence of abnormal vessel morphometry and vessel tortuosity was higher in the adult compared with the pediatric patients, again consistent with our previous report (Cheung *et al*, 2010). These results suggest microvascular abnormalities in the bulbar conjunctiva in SCD patients develop in childhood, their severity progresses with age, and that homocysteine may be a predictive biomarker. The results also indicate that CAIM may be a useful, non-invasive technology for assessing microvasculopathy in SCD patients as young as 4 years of age.

It is well documented and recognized that hyperhomocysteinemia is an independent risk factor for vascular disease in the general population (Refsum *et al*, 1998). The mechanistic role of homocysteine in the pathogenesis of vascular disease is poorly understood and is still to be determined in the general population, as well as the SCD patient population. A variety of mechanisms have been proposed, including homocysteine-induced oxidative stress, notably affecting the endoplasmic reticulum; epigenetic modifications causing altered transcription; and homocysteinylation of proteins (Zinellu *et al*, 2009; Fu *et al*, 2018). An additional hypothesis is that homocysteine has a thrombogenic effect on clotting factors and endothelial cell function, or their interactions (Houston *et al*, 1997).

There are few studies on homocysteine in SCD patients. One study (Dhar *et al*, 2004) demonstrated that mild hyperhomocysteinemia (>13.8 μmol/L in men and >12.5 μmol/L in women) was a common finding in adult SCD patients compared with healthy volunteer comparators (20% vs. 3%, respectively, $P<0.001$). Another study (Lowenthal *et al*, 2000) found similar results, reporting ~1.5-fold higher homocysteine levels in adult SCD patients compared with controls ($P<0.001$). In addition, SCD patients had 1.5-fold higher folate levels ($P<0.05$), but no difference in B12, compared with controls (Lowenthal *et al*, 2000). The high levels of folate may have resulted, in part, from hemolysis, and thus may not be reflective of adequacy of folate status in these patients as indicated by the prevalence of elevated homocysteine. In the present study, median serum folate for the adult patients (12.8 ng/ml) was about the same as previously reported (12.3 ng/ml) (Lowenthal *et al*, 2000).

Median serum folate of the pediatric patients in the present study was higher (19.2 ng/ml) than in the adult patients, but the difference was not statistically significant. After controlling for age and sex, plasma folate was inversely correlated with homocysteine, but plasma B12 was not (data not shown).

In a study of SCD children (Balasa *et al*, 2002), 38% of patients were classified with hyperhomocysteinemia (homocysteine levels greater than the 95th percentile of the corresponding level among similarly aged controls) versus 7% in controls. Moreover, elevated homocysteine is a biomarker for low folate, B12 and B6 status, and it has been demonstrated that SCD individuals have a high prevalence of B6 deficiency (Balasa *et al*, 2002; Claster *et al*, 2009; Flores *et al*, 1988) and inadequate dietary intake of folate (Kennedy *et al*, 2001). Balasa *et al* (2002) additionally found that hyperhomocysteinemia was more common in those with B6 deficiency (62%) than those with normal B6 levels (30%). In contrast, plasma PLP was not correlated with homocysteine in the present study (data not shown).

Our cross-sectional cohort study in SCD patients is the first to demonstrate that homocysteine is directly associated with the severity of microvasculopathy, as indicated by the CAIM severity index. To our knowledge, only one other study has found an association between homocysteine and vascular outcomes in SCD patients: elevated homocysteine (>10.1 μmol/L) was a risk factor for stroke in a cohort of 100 SCD patients (ages 1–58 years) (Houston *et al*, 1997). It is not known if elevated homocysteine is more strongly associated with microvascular or macrovascular pathology. It is possible that hyperhomocysteinemia affects vessels of all sizes. A limitation of the present study is that there is no clinical information available on microvasculopathy in other organs (e.g. kidneys, brain) and it is unknown if severity of microvasculopathy as determined in the bulbar conjunctiva correlates with severity in other organs within the study sample. It is also unclear what is the clinical significance of a unit difference or change in SI score. However, we have previously shown that microvascular blood flow velocity in the bulbar conjunctiva of pediatric SCD patients, as measured by CAIM, directly correlates with macrovascular blood flow velocity in the middle cerebral artery, as determined by TCD (Cheung *et al*, 2001a). In addition, homocysteine is directly correlated, and folate and PLP are inversely correlated, with carotid artery stenosis in non-SCD older adults (age 67–99 years) (Selhub *et al*, 1995; Selhub *et al*, 1996). It remains to be determined if homocysteine or B vitamins correlate with middle cerebral artery blood flow velocity or carotid artery stenosis in SCD patients.

In a study on B-vitamin supplementation and correlation with homocysteine levels in SCD pediatric patients (van der Dijs *et al*, 1998), it was determined that folate supplementation was associated with a 53% reduction in homocysteine levels. However, B6 and B12 supplementation did not change homocysteine levels. In a later, 82-week longitudinal supplementation study in SCD pediatric patients (van der Dijs *et al*, 2002), homocysteine was utilized as a biomarker to determine optimal folate, B12 and B6 doses. An optimal daily combination of 1 mg of folic acid, 6 μg of B12 and 6 mg of B6 led to maximal reduction in plasma homocysteine concentrations. In the present study, neither multivitamin nor folic acid supplement use was associated with plasma homocysteine or severity index. However,

the overall sample size of this study was small and only a minority of the cohort was regularly consuming supplements. Thus, there was likely insufficient power to see such associations if they existed. It is noted that plasma folate was inversely associated with plasma homocysteine suggesting that regular consumption of folic acid supplements might be effective in reducing homocysteine levels in these patients. No correlations with homocysteine were observed for B12 and PLP so that the potential benefit of supplements of these vitamins is less clear. However, it has been noted that folic acid supplementation may mask B12 deficiency, and that pernicious anemia may occur among the SCD patient population, even in younger individuals (Sinow *et al*, 1987). We therefore caution against indiscriminate supplementation with folic acid without assessment of B12 status.

An important determinant of plasma homocysteine concentration is renal function. In the general population, serum creatinine concentration serves as a sensitive biomarker of renal function and is inversely correlated with homocysteine concentration. However, correlation of serum creatinine with glomerular filtration rate (GFR) differs with age and our study included both pediatric and adult patients. Also, serum creatinine overestimates GFR in SCD patients and therefore neither creatinine nor estimated GFR (which is derived from serum creatinine) are ideal biomarkers of renal function in this patient population. A better measure of renal function would have been urine albumin/creatinine ratio, but this information was not available for the patients in our study. Another limitation of the study is that there was little data available on hematological parameters – only hemoglobin and hematocrit. Neither hemoglobin nor hematocrit was correlated with homocysteine or severity index. We did not have a measure of hemolysis, such as reticulocyte counts or serum lactate dehydrogenase. It will be of interest in future studies to determine if hemolysis is associated with microvasculopathy in SCD patients.

There are additional limitations of this study. Sizeable proportions of the active patients from which the participants were recruited refused to participate or were ineligible (Figure 1). Moreover, patients regularly seen in clinic were a small fraction of the total clinic population. Thus, the generalizability of the findings to the broader SCD patient population is unclear. Also, because of the relatively small sample size we were unable to adjust for potential confounders, such as SCD genotype (HbSS and HbSC), dose and duration of hydroxyurea treatment, transfusion history, and history of vascular symptoms and disease without significantly affecting the statistical power of the study for the primary *a priori* association of interest, i.e. homocysteine with severity index. Thus, it cannot be ruled out that elevated homocysteine is coincidental or a consequence of SCD-related factors that affect the pathogenesis of microvascular abnormalities, such as genetics, treatment protocols, and other pathophysiological factors.

It is known that elevated homocysteine levels are strongly correlated with vascular events in the general population, and studies of B vitamin supplementation, as a therapeutic intervention for lowering homocysteine levels to reduce vascular disease risk (including arterial vascular events and venous thromboembolism), have been conducted (Lonn *et al*, 2006; Pan *et al*, 2012; Ebbing *et al*, 2010; Clarke *et al*, 2011; Martí-Carvajal *et al*, 2017). There is a consensus among these studies that B vitamin supplementation lowers homocysteine concentrations, but does not reduce or improve vascular outcomes. However,

the mechanism of vascular disease in SCD is different than the general population and the vascular benefit of B vitamin supplementation and homocysteine lowering therapies is currently unknown. It remains to be determined if preemptive protection of the vasculature through low-risk homocysteine lowering B vitamin supplement therapy might be beneficial in SCD patients, particularly if begun early in life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Antony AC (2017) Evidence for potential underestimation of clinical folate deficiency in resource-limited countries using blood tests. Nutrition Reviews, 75, 600–615. [PubMed: 28969365]

Balasa BV, Kalinyak KA, Bean JA, Stroop D & Gruppo RA (2002) Hyperhomocysteinemia is associated with low plasma pyridoxine levels in children with sickle cell disease. Journal of Pediatric Hematology and Oncology, 24, 374–379.

Chanarin I, Dacie JV & Mollin DL (1959) Folic-acid deficiency in haemolytic anaemia. British Journal of Haematology, 5, 245–256. [PubMed: 13809098]

Cheung AT, Chen PC, Larkin EC, Duong PL, Ramanujam S, Tablin F & Wun T (2002a) Microvascular abnormalities in sickle cell disease: a computer-assisted intravital microscopy study. Blood, 99, 3999–4005. [PubMed: 12010800]

Cheung AT, Harmatz P, Wun T, Chen PC, Larkin EC, Adams RJ & Vichinsky EP (2001a) Correlation of abnormal intracranial vessel velocity, measured by transcranial Doppler ultrasonography, with abnormal conjunctival vessel velocity, measured by computer-assisted intravital microscopy, in sickle cell disease. Blood, 97, 3401–3404. [PubMed: 11369629]

Cheung AT, Miller JW, Craig SM, To PL, Lin X, Samarron SL, Chen PC, Zwerdling T, Wun T, Li CS & Green R (2010) Comparison of real-time microvascular abnormalities in pediatric and adult sickle cell anemia patients. American Journal of Hematology, 85, 899–901. [PubMed: 20872552]

Cheung AT, Miller JW, Miguelino MG, To WJ, Li J, Lin X, Chen PC, Samarron SL, Wun T, Zwerdling T, & Green R (2012) Exchange transfusion therapy and its effects on real-time microcirculation in pediatric sickle cell anemia patients: an intravital microscopy study. Journal of Pediatric Hematology and Oncology, 34, 169–174.

Cheung AT, Price AR, Duong PL, Ramanujam S, Gut J, Larkin EC, Chen PC & Wilson DM (2002b) Microvascular abnormalities in pediatric diabetic patients. Microvascular Research, 63, 252–258. [PubMed: 11969302]

Cheung AT, Ramanujam S, Greer DA, Kumagai LF, & Aoki TT (2001b) Microvascular abnormalities in the bulbar conjunctiva of patients with type 2 diabetes mellitus. Endocrine Practice, 7, 358–363. [PubMed: 11585371]

Clarke R, Halsey J, Bennett D & Lewington S (2011) Homocysteine and vascular disease: review of published results of the homocysteine-lowering trials. Journal of Inherited Metabolic Disorders, 34, 83–91.

Claster S, Wood JC, Noetzli L, Carson SM, Hofstra TC, Khanna R & Coates TD (2009) Nutritional deficiencies in iron overloaded patients with hemoglobinopathies. American Journal of Hematology, 84, 344–348. [PubMed: 19415722]

Dhar M, Bellevue R, Brar S & Carmel R (2004) Mild hyperhomocysteinemia in adult patients with sickle cell disease: a common finding unrelated to folate and cobalamin status. American Journal of Hematology, 76, 114–120. [PubMed: 15164375]

Ebbing M, Bønaa KH, Arnesen E, Ueland PM, Nordrehaug JE, Rasmussen K, Njølstad I, Nilsen DW, Refsum H, Tverdal A, Vollset SE, Schirmer H, Bleie Ø, Steigen T, Midttun Ø, Fredriksen A, Pedersen ER & Nygård O (2010) Combined analyses and extended follow-up of two randomized controlled homocysteine-lowering B-vitamin trials. Journal of Internal Medicine, 268, 367–382. [PubMed: 20698927]

Flores L, Pais R, Buchanan I, Arnelle D, Camp VM, Kutner M, Faraj BA, Eckman J & Ragab A (1988) Pyridoxal 5'-phosphate levels in children with sickle cell disease. American Journal of Pediatric Hematology and Oncology, 10, 236–240.

Fu Y, Wang X & Kong W (2018) Hyperhomocysteinaemia and vascular injury: advances in mechanisms and drug targets. British Journal of Pharmacology, 175, 1173–1189. [PubMed: 28836260]

Gilfix BM, Blank DW & Rosenblatt DS (1997) Novel reductant for determination of total plasma homocysteine. Clinical Chemistry, 43, 687–688. [PubMed: 9105275]

Green R Folate, cobalamin, and megaloblastic anemias (2016) In: Kaushansky K, Lichtman MA, Prchal JT, et al., eds. William's Hematology, 9th ed. New York, NY: McGraw-Hill; 583–616.

Houston PE, Rana S, Sekhsaria S, Perlin E, Kim KS & Castro OL (1997) Homocysteine in sickle cell disease: relationship to stroke. American Journal of Medicine, 103, 192–196. [PubMed: 9316551]

Jonsson U, Roath OS & Kirkpatrick CI (1959) Nutritional megaloblastic anemia associated with sickle cell disease. Blood, 14, 535–547. [PubMed: 13651327]

Kato GJ, Piel FB, Reid CD, Gaston MH, Ohene-Frempong K, Krishnamurti L, Smith WR, Panepinto JA, Weatherall DJ, Costa FF, & Vichinsky EP (2018) Sickle cell disease. Nature Reviews Disease Primers, 4, 18010.

Kato GJ, Steinberg MH & Gladwin MT (2017) Intravascular hemolysis and the pathophysiology of sickle cell disease. Journal of Clinical Investigation, 127, 750–760. [PubMed: 28248201]

Kennedy TS, Fung EB, Kawchak DA, Zemel BS, Ohene-Frempong K & Stallings VA (2001) Red blood cell folate and serum vitamin B12 status in children with sickle cell disease. Journal of Pediatric Hematology and Oncology, 23, 165–169.

Lee MT, Piomelli S, Granger S, Miller ST, Harkness S, Brambilla DJ, Adams RJ & STOP Study Investigators. (2006) Stroke Prevention Trial in Sickle Cell Anemia (STOP): extended follow-up and final results. Blood, 108, 847–852. [PubMed: 16861341]

Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, McQueen MJ, Probstfield J, Fodor G, Held C, Genest J Jr. & Heart Outcomes Prevention Evaluation (HOPE) 2 Investigators. (2006) Homocysteine lowering with folic acid and B vitamins in vascular disease. New England Journal of Medicine, 354, 1567–1577. [PubMed: 16531613]

Lowenthal EA, Mayo MS, Cornwell PE & Thornley-Brown D (2000) Homocysteine elevation in sickle cell disease. Journal of the American College of Nutrition, 19, 608–612. [PubMed: 11022874]

Martí-Carvajal AJ, Solà I, Lathyris D & Dayer M (2017) Homocysteine-lowering interventions for preventing cardiovascular events. Cochrane Database of Systematic Reviews, 8, CD006612. [PubMed: 28816346]

Ohene-Frempong K, Weiner SJ, Sleeper LA, Miller ST, Embury S, Moohr JW, Wethers DL, Pegelow CH & Gill FM (1998) Cerebrovascular accidents in sickle cell disease: rates and risk factors. Blood, 91, 288–294. [PubMed: 9414296]

Pan Y, Guo LL, Cai LL, Zhu XJ, Shu JL, Liu XL & Jin HM (2012) Homocysteine-lowering therapy does not lead to reduction in cardiovascular outcomes in chronic kidney disease patients: a meta-analysis of randomised, controlled trials. British Journal of Nutrition, 108, 400–407. [PubMed: 22244447]

Pandey S, Pandey HR, Mishra RM & Saxena R (2012) Increased homocysteine level in Indian sickle cell anemia patients. Indian Journal of Clinical Biochemistry, 27, 103–104. [PubMed: 23277722]

Rana S, Houston P & Castro OL (2000) Homocysteine levels and sickle cell anemia. Journal of Pediatric Hematology and Oncology, 22, 185.

Refsum H, Ueland PM, Nygard O & Vollset SE (1998) Homocysteine and cardiovascular disease. Annual Review of Medicine, 49, 31–62.

Rodriguez-Cortes HM, Griener JC, Hyland K, Bottiglieri T, Bennett MJ, Kamen BA & Buchanan GR (1999) Plasma homocysteine levels and folate status in children with sickle cell anemia. Journal of Pediatric Hematology and Oncology, 21, 219–223.

Schnog JB, van der Dijs FP, Brouwer DA, Duits AJ, Muskiet FD & Muskiet FA (2000) Plasma homocysteine levels in sickle cell disease and the need for folate supplementation. Journal of Pediatric Hematology and Oncology, 22, 184–185.

Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PW, Belanger AJ, O'Leary DH, Wolf PA, Schaefer EJ, & Rosenberg IH (1995) Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. New England Journal of Medicine, 232, 286–291.

Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PW, Belanger AJ, O'Leary DH, Wolf PA, Rush D, Schaefer EJ, & Rosenberg IH (1996) Relationship between plasma homocysteine, vitamin status and extracranial carotid-artery stenosis in the Framingham Study population. Journal of Nutrition, 126(4 Suppl), 1258S–1265S. [PubMed: 8642467]

Selhub J & Miller JW (1992) The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. American Journal of Clinical Nutrition, 55, 131–138. [PubMed: 1728812]

Sinow RM, Johnson CS, Karnaze DS, Siegel ME & Carmel R (1987) Unsuspected pernicious anemia in a patient with sickle cell disease receiving routine folate supplementation. Archives of Internal Medicine, 147, 1828–1829. [PubMed: 3662711]

Smith MM, Chen PC, Li CS, Ramanujam S & Cheung AT (2009) Whole blood viscosity and microvascular abnormalities in Alzheimer's Disease. Clinical Hemorheology and Microcirculation, 41, 229–239. [PubMed: 19318716]

Strousse JJ, Lanzkron S & Urrutia V (2011) The epidemiology, evaluation and treatment of stroke in adults with sickle cell disease. Expert Reviews of Hematology, 4, 597–606.

Talwar D, Quasim T, McMillan DC, Kinsella J, Williamson C & O'Reilly DS (2003) Optimisation and validation of a sensitive high-performance liquid chromatography assay for routine measurement of pyridoxal 5-phosphate in human plasma and red cells using pre-column semicarbazide derivatisation. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 792, 333–343. [PubMed: 12860041]

van der Dijs FP, Fokkema M, Dijck-Brouwer D, Niessink B, van der Wal TI, Schnog JJ, Duits AJ, Muskiet FD & Muskiet FA (2002) Optimization of folic acid, vitamin B(12), and vitamin B(6) supplements in pediatric patients with sickle cell disease. American Journal of Hematology, 69, 239–246. [PubMed: 11921017]

van der Dijs FP, Schnog JJ, Brouwer DA, Velvis HJ, van den Berg GA, Bakker AJ, Duits AJ, Muskiet FD & Muskiet FA (1998) Elevated homocysteine levels indicate suboptimal folate status in pediatric sickle cell patients. American Journal of Hematology, 59, 192–198. [PubMed: 9798656]

Wang WC (2000) Homocysteine levels and sickle cell anemia: response to Rana et al. Journal of Pediatric Hematology and Oncology, 22, 186–187.

Wang WC, Gallagher DM, Pegelow CH, Wright EC, Vichinsky EP, Abboud MR, Moser FG & Adams RJ (2000) Multicenter comparison of magnetic resonance imaging and transcranial Doppler ultrasonography in the evaluation of the central nervous system in children with sickle cell disease. Journal of Pediatric Hematology and Oncology, 22, 335–339.

Zinellu A, Sotgia S, Scanu B, Pintus G, Posadino AM, Cossu A, Deiana L, Sengupta S & Carru C (2009) S-homocysteinylated LDL apolipoprotein B adversely affects human endothelial cells in vitro. Atherosclerosis, 206, 40–46. [PubMed: 19249051]

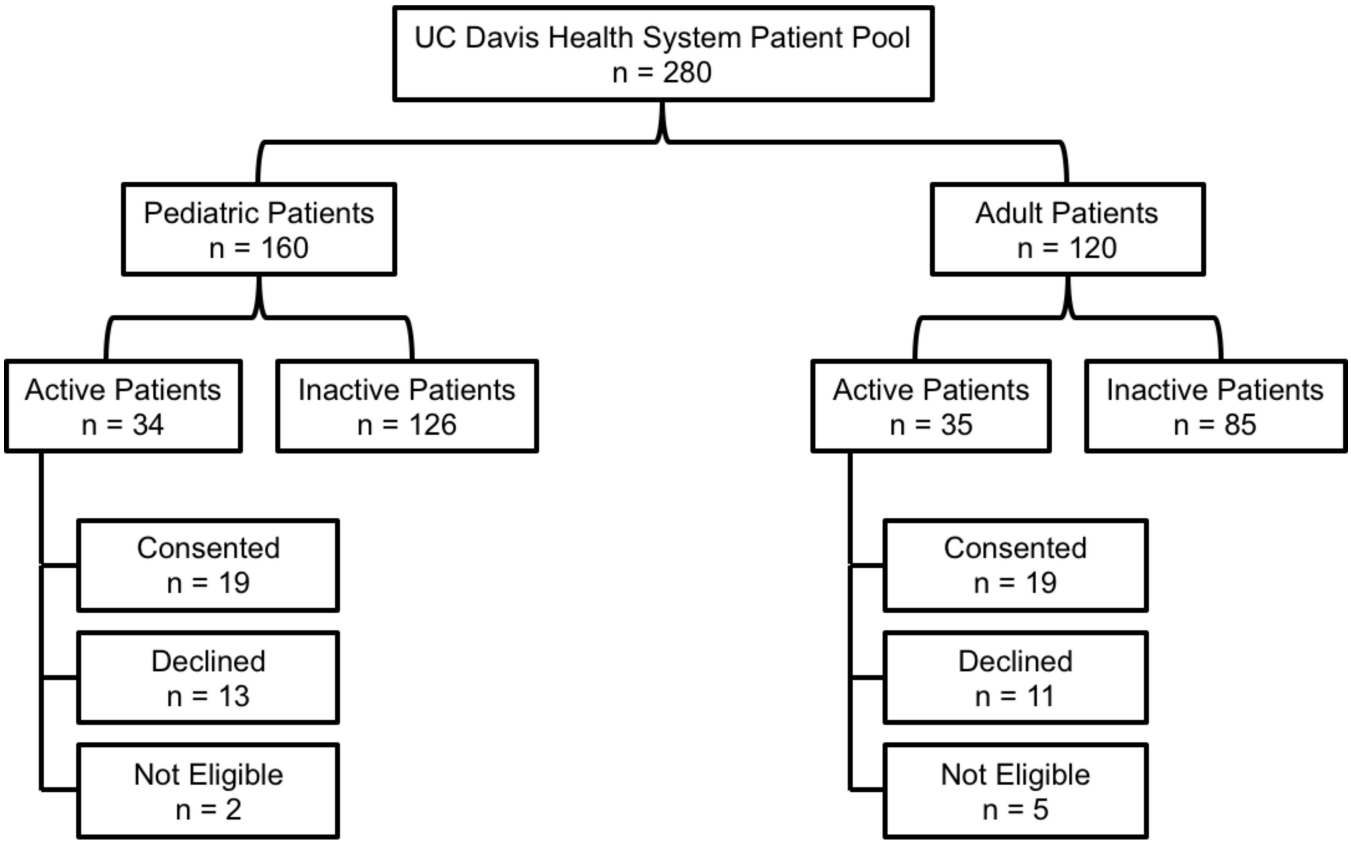

Figure 1: Flow Chart of Sickle Cell Disease Patient Recruitment.

Pediatric patients are defined as ≤18 years, and adult patients as >18 years. Active patients are defined as patients who have been seen by a hematologist within the previous 12 months, and inactive patients as those not seen by a hematologist in the last 12 months. For the pediatric active patients, reasons given for declining to participate included ‘not interested’ (N=4), ‘out of health system network’ (N=1), ‘moving out of state’ (N=2), and ‘no reason given’ (N=6). For the adult active patients, reasons given for declining to participate included ‘not interested’ (N=3), ‘out of health system network’ (N=4), ‘deceased’ (N=1), and ‘no reason given’ (N=3). Reasons that pediatric active patients were deemed not eligible for the study included ‘no definitive SCD diagnosis’ (N=1) and ‘out of area’ (N=1). Reasons that adult active patients were deemed not eligible for the study included ‘related to another participant enrolled in the study’ (N=5).

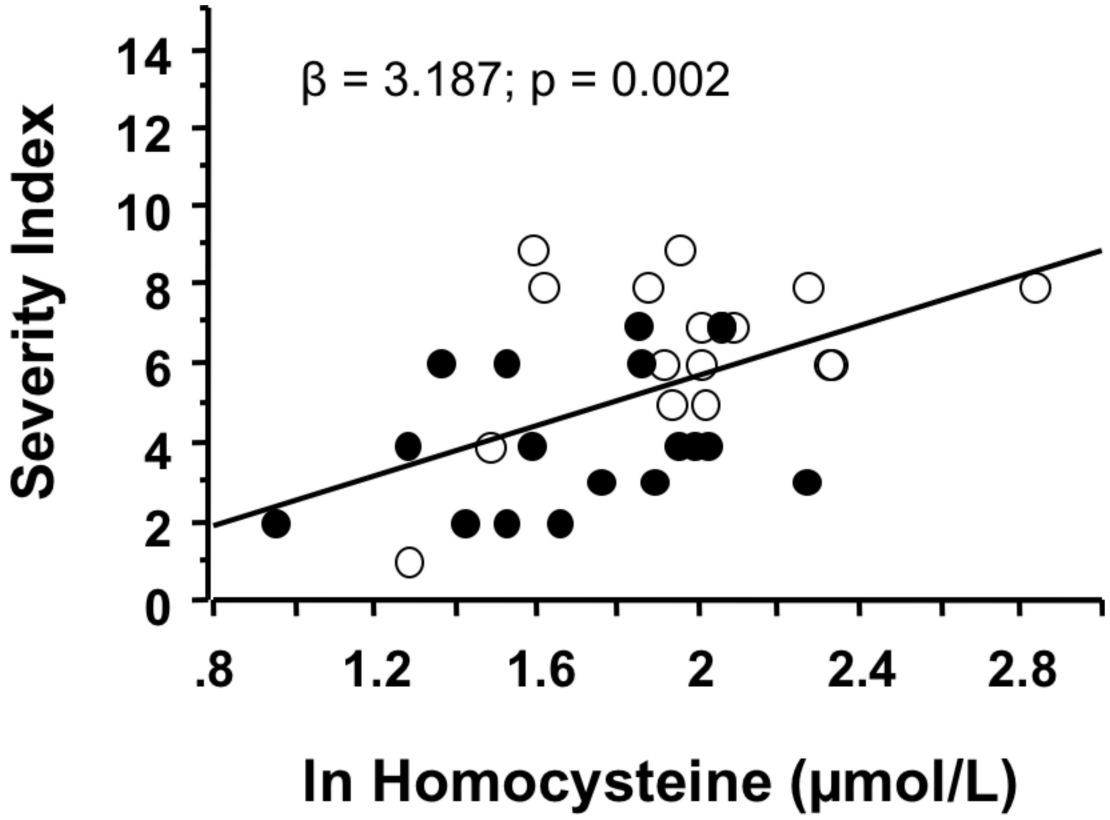

Figure 2: Correlation between Homocysteine and Severity Index.

Includes both pediatric (closed circles) and adult (open circles) sickle cell disease patients (n = 35). β-value and P-value are for simple correlation without controlling for potential confounding factors.

Table 1:

Sickle Cell Disease Patient Characteristics*

	Pediatric (n=19)¶	Adult (n=19)¶	P-value**
Male/Female, n/n	7/12	4/15	
Age, y	13 (4–18)	31 (22–61)	
Transfused, n (%)	17 (89)	8 (42)	
Hydroxyurea, n (%)	2 (11)	9 (47)	
Folic Acid Supplement, n (%)	3 (16)	3 (16)	
Multivitamin Supplement, n (%)	3 (16)	7 (37)	
Hemoglobin, g/dL[a]	9.4 (6.1–10.9)	9.5 (7.7–11.5)	0.45
Hematocrit, %[a]	27.3 (17.5–32.9)	27.6 (23.3–35.3)	0.48
Serum Folate, ng/mL[a]	19.2 (4.1– >20)	12.8 (4.9– >20)	0.32
Serum B12, pg/mL[a]	549 (313–793)	460 (321–1744)	0.57
Plasma PLP, nmol/L[b]	40.3 (22.6–162)	36.6 (11.6–103)	0.26
Total Plasma Hcy, μmol/L[a]	5.5 (2.6–9.6)	7.0 (3.6–18.9)	**0.01**
Serum Creatinine, mg/dL[a]	0.4 (0.2–0.9)	0.6 (0.3–1.4)	**0.001**
Severity Index	4 (0–7)	7 (1–9)	**<0.001**

*Values are count [n (%)] or median (range).

**P-values are pediatric vs. adult by the Mann-Whitney test.

¶One pediatric and 1 adult patient dropped out after initial recruitment and collection of demographic, clinical, and supplement use data. Sample sizes for the blood analytes and severity index determinations were n=18 for both pediatric and adult groups, except for B12, PLP, creatinine and CAIM for the adults (n=17) due to missing data for one subject.

[a]UCDMC Department of Pathology and Laboratory Medicine Clinical Laboratory reference ranges: serum folate – ≥7.0 ng/ml; serum B12 – 213–816 pg/ml; total plasma Hcy – 5.0–11.7 μmol/L (men) and 3.8–11.0 μmol/L (women); serum creatinine – 0.4–1.3 mg/dL

[b]Reference range for plasma PLP: >20 nmol/L (30)

Abbreviations: PLP, pyridoxal-5′-phosphate; Hcy, homocysteine

Table 2:

Correlations with Severity Index in Sickle Cell Disease Patients*

Variable	n	β	P-Value	ΔR^{2}**
Age	35	0.104	**<0.001**	---
Sex	35	−0.544	0.42	---
Folate	34	−0.047	0.44	0.009
B12	34	−0.001	0.55	0.007
PLP	34	−0.013	0.18	0.033
ln Hcy	35	2.376	**0.047**	0.068
Creatinine	34	0.848	0.70	0.003

*R^2 for base regression model including age and sex is 0.438. Regression models for all blood analytes are adjusted for age and sex.

**ΔR^2 is the increase in R^2 value of the model with inclusion of the independent variable compared with the model including age and sex alone.

Abbreviations: PLP, pyridoxal-5′-phosphate; ln Hcy, natural log of homocysteine.