Cross-Sectional Relationships Between Biomarkers of B Vitamin Status, Inflammation, Vascular Adhesion, and Microvascular Characteristics in Sickle Cell Anemia Patients Under Steady-State Conditions

By

SANDRA LEE SAMARRON

B.S. (University Of California, Davis) 2003
M.S. (University Of California, Davis) 2011

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

NUTRITIONAL BIOLOGY

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

_____
Joshua W. Miller, PhD., Chair

_____
Ralph Green, M.D., PhD

_____
Theodore Wun, M.D., FACP

Committee in Charge

2013

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

Acknowledgements

I would like to dedicate this dissertation to my daughter Alisha Marie Miller for her unconditional love and many sacrifices during my journey from my first class in junior college to the completion of my dissertation. She spent the better part of her childhood with a mom who had her head buried in the books day and night. I would like to thank my parents Lester Leroy and Cecilia Garner, Jr. for their persistent prodding, “Are you done yet!” But even more so for their unconditional love, support, encouragement and belief I would someday attain my doctorate. I would like to acknowledge my brother David Michael Garner, although he has since left this earthly world, for his inspiration to begin this journey.

Drs. Miller, Green and Wun were instrumental in my education and guidance on this dissertation. Therefore, I would like to thank my mentor Dr. Joshua W. Miller for his gentle nudges and encouragement over the years, and showing me the patience and persistence needed for the study of science. Additionally, for the many hours spent teaching me to critically think and write. I would also like to thank Dr. Ralph Green for sharing his unending depth of knowledge and guiding me through the exploration of science. I would like to thank Dr. Ted Wun for sharing his depth of knowledge in hematology and the complexity of sickle cell disease.

I would also like to express my gratitude to Soraya Foutouhi and Dr. Xin Lin for their many hours of encouragement, instruction and assistance in the laboratory.

Without each and every one of them I would not have achieved this accomplishment—thank you from the bottom of my heart.

Table of Contents

List of Tables

List of Figures

List of Figures
– continued –

Cross-Sectional Relationships Between Biomarkers of B Vitamin Status, Inflammation, Vascular Adhesion, and Microvascular Characteristics in Sickle Cell Anemia Patients Under Steady-State Conditions

Abstract

1. Background

Sickle cell anemia (SCA) was the first recognized inherited “molecular disease”. SCA is one of several polygenetic sickle cell diseases (SCD), which also includes sickle-hemoglobin β-thalassemia and C diseases. SCA is caused by a single DNA base mutation, leading to a single amino acid substitution in the β-globin chain, resulting in hemoglobin with altered oxygen-binding and solubility properties and myriad clinical consequences. The complications of SCA (as wells as all forms of SCD) are characterized by acute clinical manifestations of painful crisis, acute and chronic pulmonary hypertension, priapism, other end-organ dysfunction and ischemic stroke as a result of chronic vascular damage. The vascular damage resulting from vaso-occlusion by sickled red blood cells affects all organs, particularly the heart, lungs, kidneys, eyes, spleen and femoral heads.

The vascular injuries caused by repeated vaso-occlusions increase vascular inflammation, signified by increased circulating levels of vascular adhesion molecules and cytokines that further mediate erythrocyte and leukocyte adhesion to the vascular endothelium. In addition to the interplay of vascular adhesion molecules and cytokines on endothelial activation and inflammation, the levels of several of the B vitamins and related molecules may influence the progression of SCD pathophysiology. The

hemolysis of the sRBC and ineffective erythropoiesis leads to increased rates of DNA synthesis and demands for folate, vitamin B12 (B12) and vitamin B6 (B6), resulting in relative deficiencies, particularly of folate.

Deficiency in any of these vitamins can lead to elevated blood levels of homocysteine. An elevated plasma level of homocysteine (hyperhomocysteinemia) may be associated with increased risk of vascular complications in SCD patients. In addition, plasma homocysteine is a functional marker of folate, vitamin B6 and vitamin B12 status.

A nutritional biochemical characteristic of sickle cell subjects, both adult and pediatric, is a high prevalence of low vitamin B6 plasma levels (exceeding 50% in some cohorts), as indicated by low plasma pyridoxal-5'phosphate levels (PLP). In the general population, B6 deficiency is a risk factor for cardiovascular disease, peripheral vascular disease and stroke, but it is unknown if low B6 status affects vascular morbidity in SCD subjects. Preliminary data indicated that a low B6 level is associated with increased levels of adhesion molecules that mediate erythrocyte and leukocyte adhesion to the vascular endothelium, key events in the pathogenesis of vascular occlusion in SCD subjects.

2. Objective

The objective of this study was to identify predictive biomarkers of microvascular abnormalities in SCA patients with the long-term goal of designing novel interventions to reduce morbidity and mortality caused by vascular damage in this patient population. The specific aims were to assess and quantify microvasculature abnormalities in SCA

patients, and to determine the cross-sectional relationships between B vitamin status, inflammatory cytokines, vascular adhesion molecules, and microvascular characteristics in SCA patients under steady-state conditions.

3. Design

Thirty eight SCA patients (19 pediatric patients and 19 adult patients) were enrolled in a cross-sectional study. B vitamin status and circulating levels of adhesion molecules and inflammatory cytokines known to be involved in the adhesion of sickle erythrocytes and leukocytes to the vascular endothelium were measured. In addition, real-time microvascular characteristics (vessel morphometry and red-cell flow velocity) in the microcirculation of the bulbar conjunctiva were assessed in these patients using the non-invasive technique of computer-assisted intravital microscopy (CAIM). The CAIM system uses macro-optics for real-time, *in vivo* image acquisition of a selected region within the conjunctival microcirculation. The captured images are analyzed to identify morphometric microvascular abnormalities. From the analysis a severity index (SI) is computed from a list of 15 qualitative and quantitative microvascular abnormalities.

4. Results

The data showed age is a strong determinant of microvasculopathy severity, with severity index increasing about 0.1 units per 1 year increase in age ($P < 0.001$). When reviewing the B-vitamins and related analytes, the only variable that was significantly associated with severity index after adjusting for age and sex was homocysteine ($P = 0.047$). The only vascular and inflammatory biomarker variable that

was significantly associated with severity index after adjusting for age and sex was monocyte chemotactic protein 1 (MCP-1) ($P = 0.026$).

The severity of microvasculopathy, as indicated by severity index (SI), was significantly lower in the pediatric patients than in the adult patients (4.2 ± 1.8 vs. 6.6 ± 2.4, $P = 0.028$). For comparison, the mean SI values for both the pediatric and adult SCD patients were significantly higher than the mean SI value determined for a previous cohort of healthy non-SCD subjects ($n = 10$; $SI = 0.31 \pm 0.72$; $P < 0.05$). In pediatric patients undergoing transfusion therapy, a significant decrease in average vessel diameter occurred immediately following transfusion ($n = 6$; 36.76 ± 7.23 μm; $P = 0.015$).

5. Conclusions

It is concluded that age, homocysteine and MCP-1 are significantly correlated with microvasculopathy severity in SCD. These markers should be considered when assessing potential sickle cell disease progression and microvasculopathy severity. The microvascular abnormalities are fewer in children than in adults, suggesting SCD is a progressive microvascular disease. Therefore, intervention therapies designed to ameliorate or prevent microvascular disease should probably start very early in life.

As elevated homocysteine levels have been correlated with increased risk of vascular disease, it is therefore postulated that maintaining lower levels of homocysteine in SCD patients may be linked to lower risk of vascular damage. Studies have demonstrated that B-vitamin supplementation, specifically folate, B12 and B6, lowers homocysteine levels in the general population and in SCD patients. It remains to be determined, however, whether B-vitamin supplementation to lower homocysteine levels could effectively ameliorate or reduce microvascular damage, and subsequently extend

life expectancy in SCD patients. Hence, B vitamin placebo-controlled trials in pediatric SCD patients should be conducted. Furthermore, in these supplementation trials, microvascular abnormalities as assessed using CAIM and circulating MCP-1, may be useful biomarkers of the efficacy of the B vitamin supplementation.

Chapter 1: Background

A. Introduction

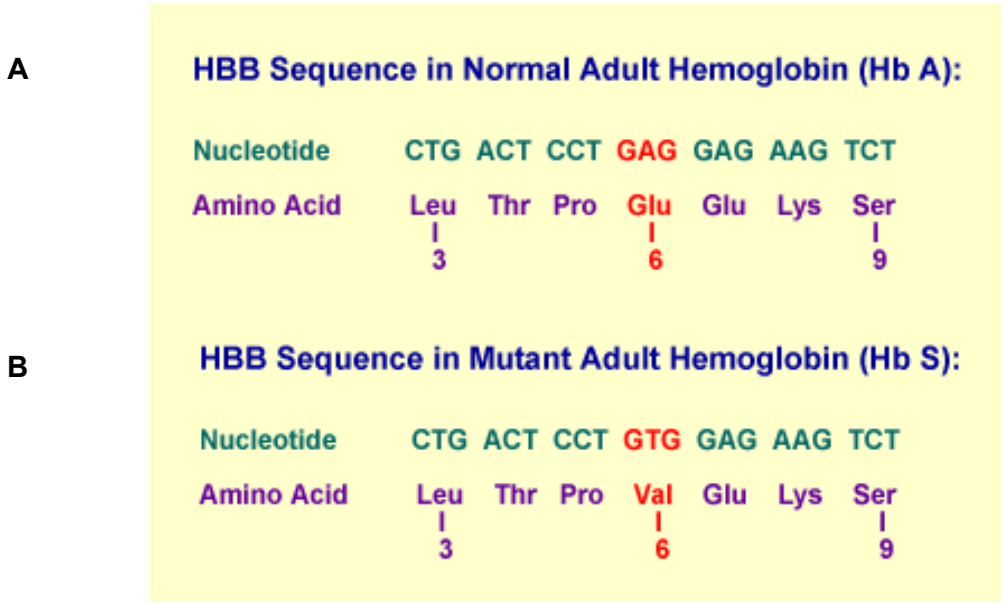
1. Sickle Cell Disease

Sickle Cell Anemia (SCA) is a monogenic disease in which a single DNA base substitution (adenine to thymine) in the hemoglobin (1) gene results in a single amino acid substitution (glutamate to valine) (**Figure 1-1**) causing deformation or "sickling" of the red blood cell under conditions of decreased oxygen tension. SCA is one of several polygenetic sickle cell diseases (SCD), which also includes sickle-hemoglobin β-thalassemia and sickle-hemoglobin C diseases. SCD affects millions of humans worldwide—between 72,000 and 98,000 in the US (2). It is estimated that approximately 2 million Americans have a sickle allele. SCD commonly occurs in individuals of African descent, as well as people of Mediterranean and Middle Eastern backgrounds and some Indian tribal (3, 4). The sickle cell hemoglobin mutation is thought to have persisted evolutionarily through selective pressure by affording protection against *falciparum* malaria, and thus the ethnic distribution of the sickle cell allele reflects endemic regions of malarial infection throughout the world (**Figure 1-2**) (5-7).

SCD was the first discovered molecularly-based disease and is the paradigm of hemoglobinopathies. In 1910 James Herrick observed "peculiar elongated sickle shaped RBCs" in the blood of an anemic black dental student (8). However, it was not until 1949 when Linus Pauling et al published "Sickle Cell Anemia, A Molecular Disease" that there was proof that a human disease could be caused by abnormal proteins (9). Pauling et al noted that certain individuals have erythrocytes that undergo morphological changes in response to changing partial pressure of oxygen. Under low oxygen

pressure (below 40 to 45 mm Hg) the RBCs change to a sickled shape (sRBC) (**Figure 1-3**). The sickle phenotype causes a multisystem disease with recurring episodes of acute illness and progressive organ damage which significantly affect morbidity and mortality (10-13).

Figure 1-1: Normal and Altered Sickle Hemoglobin Nucleotide and Amino Acid Sequences

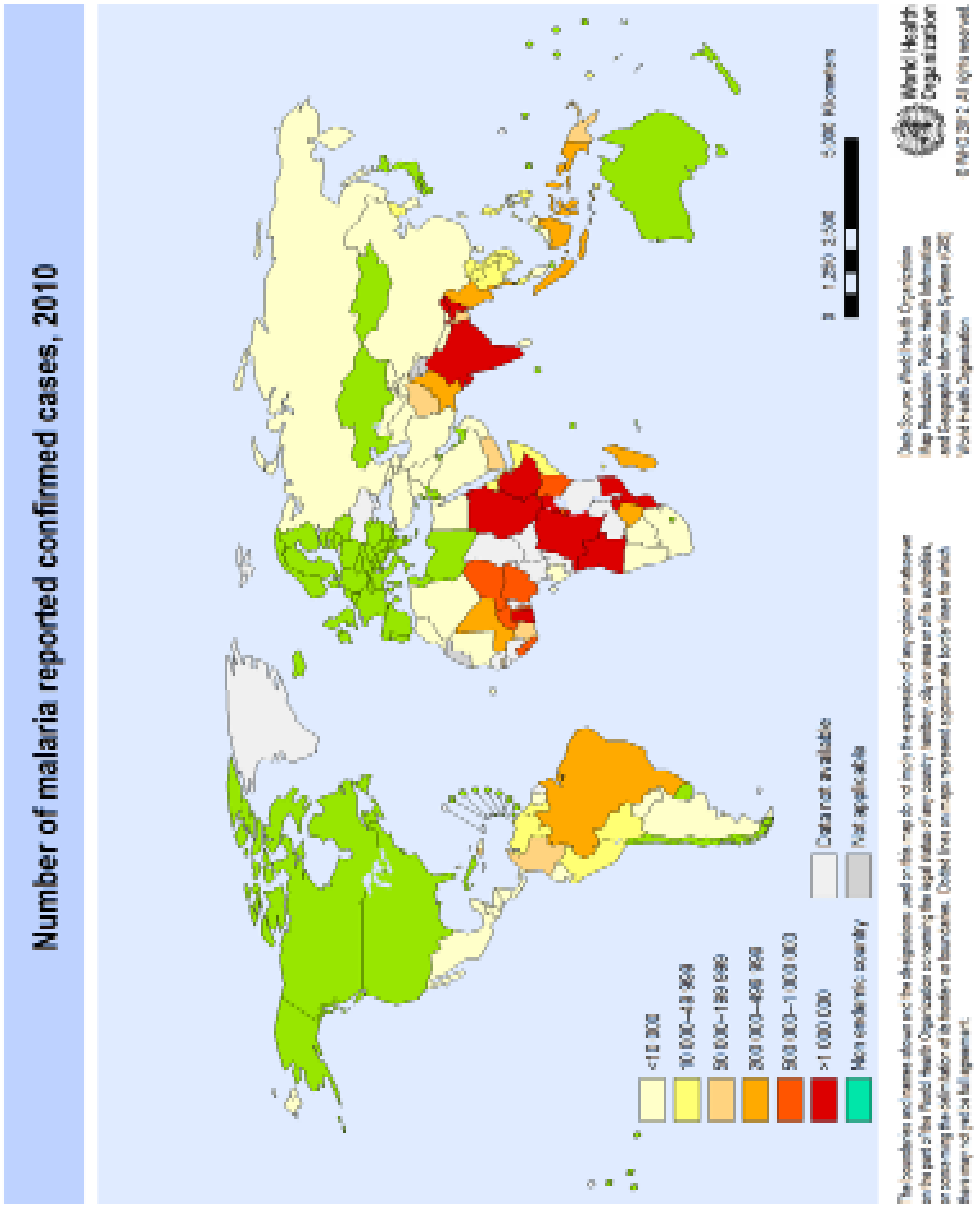


(A) The B hemoglobin chain nucleotide and translated amino acid sequences in a normal adult (HbB)

(B) The altered B hemoglobin chain nucleotide and translated amino acid sequences in a sickle cell adult (HbS)

Source: Genomics.Energy.Gov (*http://www.ornl.gov/sci/techresources/HumanGenome/posters/chromosome/hbb.shtml*); *accessed September 2012 (14) .*

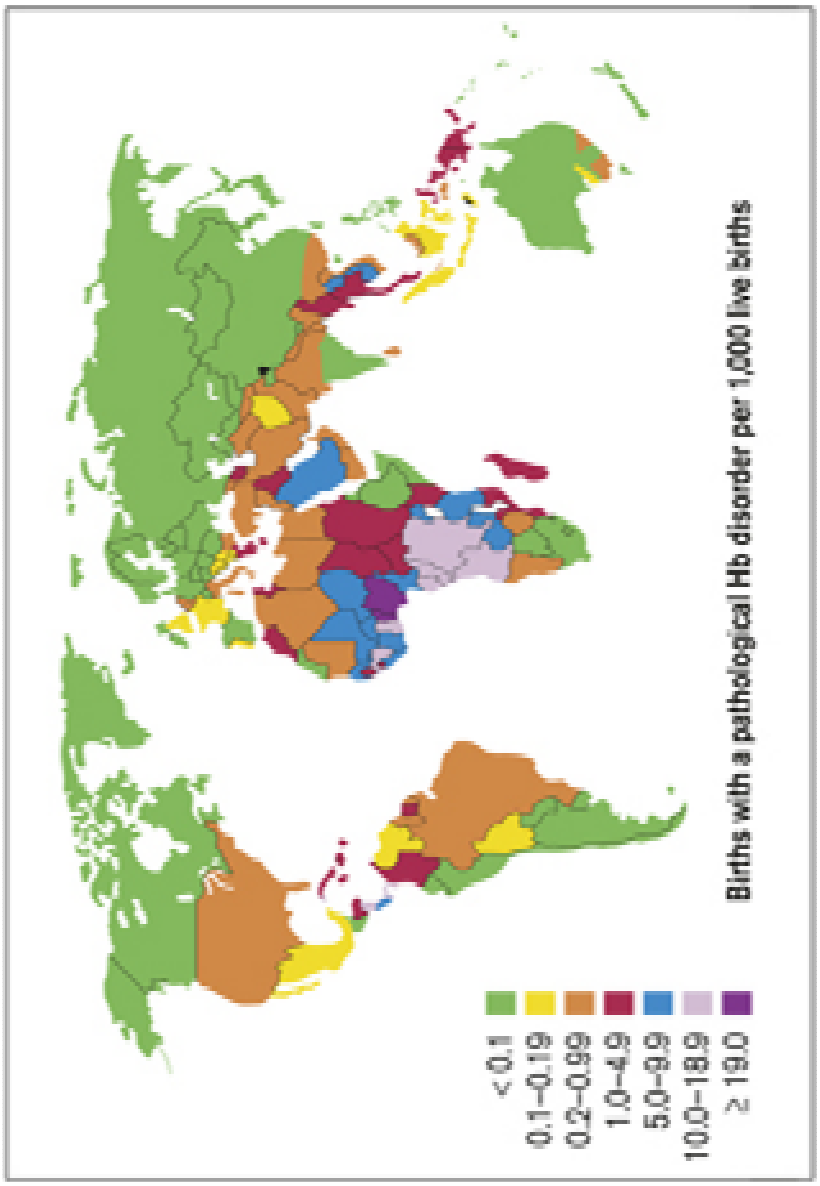
Figure 1-2: Modern Distribution of Malaria and Pathological Hb Disorders



Global distribution map of reported confirmed cases of malaria as of 2010.

Source: Based on data from World Health Organization (WHO), 2012, (http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_ReportedCases_2010.png); accessed October 2012 (15).

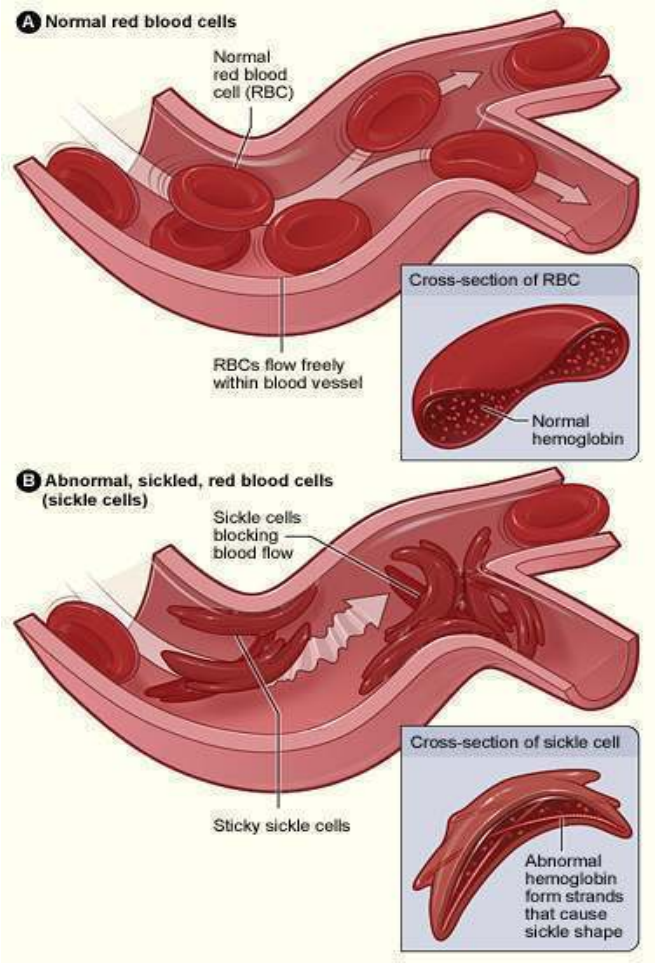
Figure 1-2: Modern Distribution of Malaria and Pathological Hb Disorders—continued



Global distribution of births with a pathological Hb disorder per 1,000 live births.

Source: Based on Data from WHO, 1996, (http://www.who.int/genomics/public/Maphaemoglobin.pdf), accessed October 12, 2012 (16).

Figure 1-3: Sickle Cell Disease: Diagram of Normal and Sickled Red Blood Cells



A: Normal RBC flowing freely in a blood vessel. The inset image to the right shows a cross-section of an RBC with normal hemoglobin.

B: Abnormal sRBC blocking blood flow in a blood vessel. The inset image to the right shows a cross-section of a sickle cell with abnormal (sickle) hemoglobin.

Source: Based on data from National Heart, Lung and Blood Institute (http://www.nhlbi.nih.gov/health/health-topics/topics/sca/); accessed: April 2012 (17).

2. Sickle Cell Disease is a Vascular Disease

Importantly, much of the morbidity and mortality in SCD is vascular in nature. The complications of SCD are characterized by clinical manifestations of painful crisis, acute and chronic pulmonary hypertension, end-organ failure and ischemic stroke as a result of chronic irreversible vascular damage. The vascular damage results from vaso-occlusion by sickled red blood cells primarily within the microvascular system, and affects all organs, particularly the heart, lungs, kidneys, eyes, spleen and femoral heads.

Frenette et al (18) proposed a novel multistep model where sickle cells or secondary inflammatory stimuli induce endothelial activation and recruitment of leukocytes. The interactions of leukocytes with sickled erythrocytes impede microvascular flow. The sickled erythrocytes become trapped and result in vaso-occlusion. The vascular injuries caused by repeated vaso-occlusions increase vascular inflammation, thus elevating circulating levels of vascular adhesion molecules. In turn, the endothelial adhesion molecules are upregulated and mediate erythrocyte and leukocyte adhesion to the vascular endothelium—key events in the development of vascular disease. It is therefore important to understand the overall mechanisms of vascular disease and how it relates to SCD.

3. B-vitamins and 1-Carbon Metabolism in SCD

SCD is characterized by hemolysis and ineffective erythropoiesis, which leads to increased rates of DNA synthesis. Effective DNA synthesis is dependent on 1-carbon metabolic pathways, which are involved in the shunting of single carbon units between biological substrates. Several key B vitamins, including folate, cobalamin (B12) and pyridoxine (B6), participate in 1-carbon metabolic pathways, either as cofactors or substrates. The increased rates of DNA synthesis in SCD may increase demand for

these B vitamins which results in relative deficiencies, particularly of folate. Relative folate deficiency (as well as B12 deficiency) may result in disruption of DNA synthesis leading to the development of megaloblastic anemia. Such megaloblastic anemia, though rare in SCD, may nonetheless further aggravate ineffective erythropoiesis and hemolysis in SCD. Perturbations of 1-carbon metabolic pathways caused by B vitamin deficiencies may also lead to deficiencies of key amino acids and nucleotides as well as accumulation of homocysteine (an intermediated in 1-carbon metabolism) and other potentially harmful metabolites implicated in atherothrombosis. Elevated homocysteine, termed hyperhomocysteinemia, is an independent risk factor for vascular diseases, including atherosclerosis, venous thrombosis, pulmonary embolism and stroke (19-29). Hyperhomocysteinemia may aggravate atherothrombosis and vascular complications seen in SCD (30).

While B vitamin deficiencies may contribute to ineffective erythropoiesis in SCD, it is also the case that folate and B6 and B12 (31) status can be affected by this disorder, as reflected by low circulating levels of the B vitamins. Current data shows a high prevalence of folate deficiency in SCD children. Additionally, it has been demonstrated that more than 50% had inadequate dietary intake of folate (32). Moreover, SCD patients have a high prevalence of B6 deficiency (26-28), which may be associated with inflammation in this population. Thus, there may be a vicious circle in SCD in which the disorder leads to relative or absolute deficiencies in B vitamins, particularly folate and B6, which in turn may exacerbate the ineffective erythropoiesis and development of vascular disease that are the hallmarks of the disorder.

Because of the vascular manifestations of SCD, there is a markedly increased risk of morbidity and mortality (3). Furthermore, recurring sickle cell painful crises have

major social and economic implications related to interference with education, work and psychosocial development. Therefore, in this introductory chapter, what is known about CVD in the general population, the mechanisms and components responsible for its development, and how they relate to SCD patients and disease progression is reviewed. The roles and relationships of B vitamins and homocysteine in SCD patients are reviewed, as well.

B. Basics of Cardiovascular Disease in the General Population

1. Overview

CVD is the leading cause of death worldwide, according to the World Health Organization on International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10), even in the midst of two decades of declining CVD mortality rates (33-36). A primary pathogenetic mechanism of CVD is the development of atherosclerosis, which is marked by fatty streaks within the tunica intima of the vascular wall, causing narrowing and hardening of the arteries and consequent endothelial dysfunction. Atherosclerosis is a chronic inflammatory condition of the large and medium arteries, caused by genetic predispositions and what has been termed the "atherosclerotic lifestyle", which can begin to develop as early as the first year of life, remaining asymptomatic for many decades with the accumulated impact experienced by older adults (37, 38).

Although the exact mechanism of atherosclerosis is unknown, there is much literature that supports a cholesterol-based hypothesis where lifestyle and clinical factors are known to exacerbate the condition. An atherosclerotic lifestyle consists of a

sedentary physical activity level, a diet high in saturated fats and low in fiber, and smoking (34, 39-42). Clinical factors that also contribute to the development of atherosclerosis are hypertension, high blood levels of low-density lipoproteins (LDL), and diabetes. Elevated blood levels of the sulfur amino acid, homocysteine, may also contribute to the pathogenesis of atherosclerosis (39, 43).

2. Mechanisms of Atherosclerosis Development

Under normal conditions of lipid metabolism and homeostasis, low density lipoprotein (LDL) particles are responsible for delivering cholesterol to the cells of the body. LDLs are taken up by arterial endothelial cells through receptor (proteoglycans) mediated endocytosis, facilitated by apolipoprotein B (apoB) proteins on the LDL. As a normal adaptive process, when blood lipid concentrations increase, the endothelial cells become laden with LDLs (44-46).

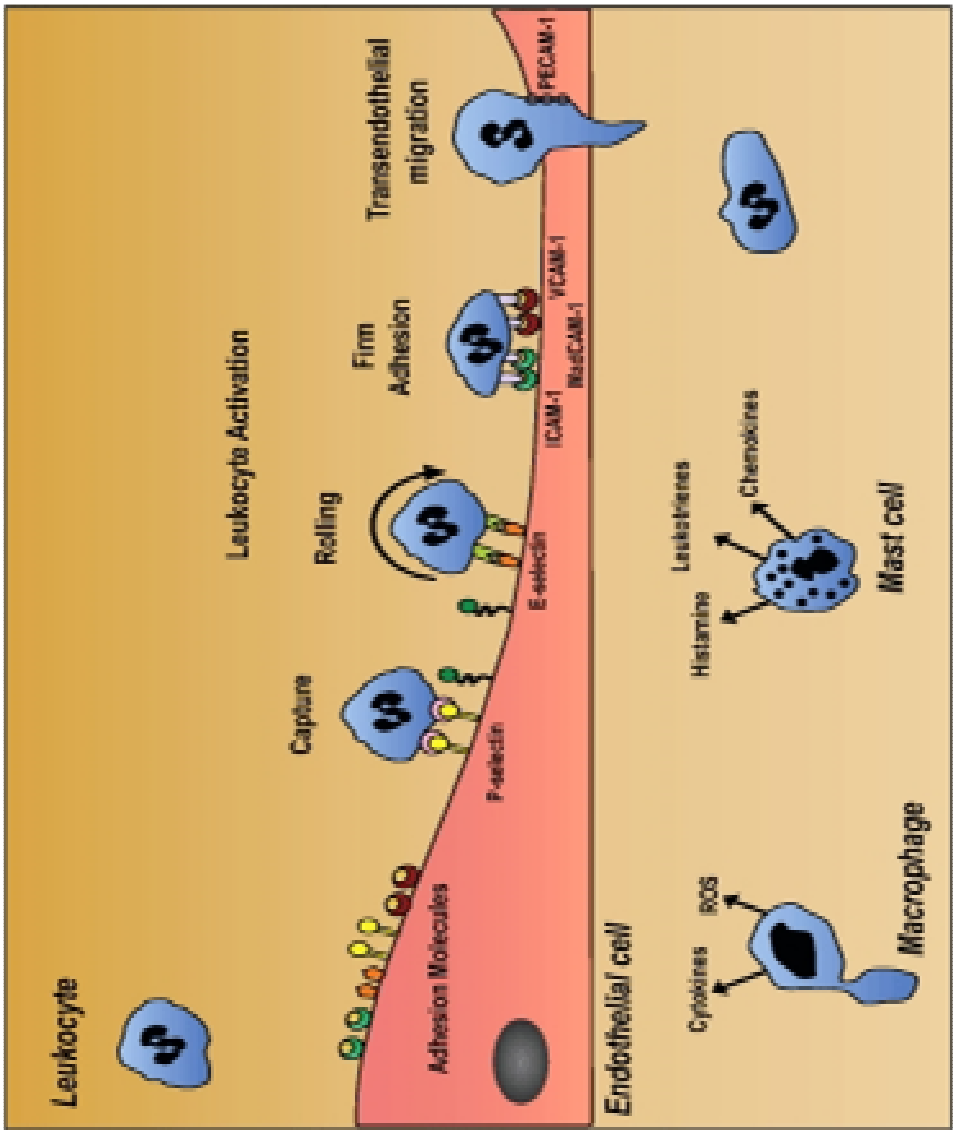
Following retention by the endothelial cells, LDL has been shown *in vitro* to undergo several modifications, including oxidation (47, 48). The oxidized LDLs are chemoattractive to leukocytes, which in turn stimulate local blood vessel inflammation. Various cytokines and other inflammatory mediators recruit and direct circulating monocytes to transmigrate into the sub-endothelial space through recognition and tethering to proteoglycans (adhesion molecules) on the endothelial cells. Transmigration of monocytes drive their differentiation to macrophages which function to engulf oxidized LDLs through scavenger receptor mediated endocytosis (**Figure 1-4**) (49-51).

The binding of leukocytes (predominately monocytes) to adhesion molecules on the endothelial cells activates the tissue and initiates the vascular inflammation process (52). The activated endothelium signals functional changes, and upregulates expression

of adhesion molecules. The increased expression of vascular adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin, recruits and directs circulating monocytes to invaginate the sub-endothelial space (52-54).

As the aggregate of oxidized LDL-laden macrophages grow, they also take up calcium and cellular debris, which develop into foam cells. The foam cells are pathologically recognized as fatty streaks, which encroach into the arterial luminal space and are associated with thickening of the artery wall, thus initiating the multistep progression of vascular damage. The damaged endothelial cells form a fibrous capsule, and develop into an atherosclerotic plaque which, together with calcium deposition, leads to hardening of the arteries and further endothelial dysfunction—the hallmarks of atherosclerosis (2, 35, 55). Vessels affected by this process mainly include large and medium-size arteries, such as the aorta, and cerebral, coronary, mesenteric and peripheral arteries.

Figure 1-4: The Leukocyte-Extravasation Cascade



Leukocyte extravasation from the blood into inflamed tissues is a multistep process of tethering (capturing), rolling, firm adhesion and activation, and then transmigration of monocytes that differentiate into macrophages.

Source: Ernest (http://www.daftblogger.com/leukocyte-response-in-acute-inflammation-extravasation/); accessed October 2012.

3. Cardiovascular Disease in Sickle Cell Disease is Different

As described above, the paradigm of vascular disease development in the general population is a cholesterol-based hypothesis. However, the development of vascular disease in SCD is in many respects different from atherosclerosis. Vascular damage in SCD is initiated by vaso-occlusion caused by the sickling, clumping and sticking of the red blood cells to the endothelial cells within the microvasculature.

C. Cardiovascular Disease in SCD

1. Overview

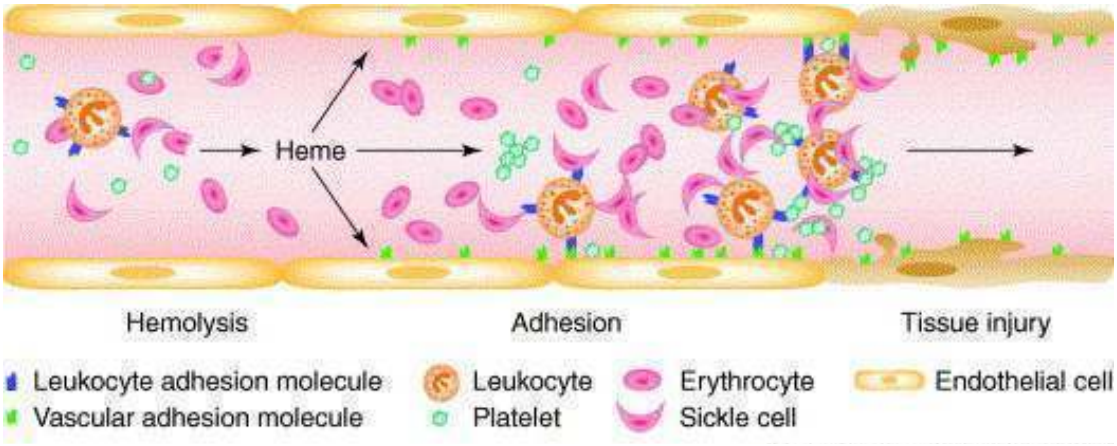
Current experimental evidence suggests that sickled erythrocytes (sRBC) adhere to the microvascular endothelium, mediated by vascular adhesion molecules and other cytokines, and may be the initiating event in sickle cell vaso-occlusion (56-58). However, animal models have also suggested leukocyte adhesion may be the initial event with secondary red cell capture (18, 59). The resulting decreased blood flow leads to local hypoxemia, hemoglobin polymerization, and subsequent further red cell sickling (60). Persistent adherence of sRBC to activated endothelium, along with recurrent episodes of ischemia/reperfusion injury, eventually results in permanent vaso-stenosis and vascular damage (61).

2. Current Mechanistic Paradigm in the Microvascular Disease SCD

Microvasculature vaso-occlusion by sRBC results from complex interactions with the vascular endothelium that lead to obstruction of blood flow, and result in a sickle cell crisis (intermittent exacerbations of acute pain or pulmonary complications called acute chest syndrome). As sRBC enter the microvasculature they adhere to the endothelial

and activate the endothelial cells. In turn, the activated cells upregulate vascular adhesion molecules, recruiting leukocytes that transmigrate the tissue. As vascular inflammation increases, blood flow is diminished, causing oxygen tension to drop. The reduced oxygen tension causes further red cells to become sickled in shape, and rigid and sticky. This results in polymerization of the hemoglobin and causes the red cells to become distorted and sickled. This distortion induces adhesion of the sRBC to vascular endothelial cells mediated by vascular adhesion molecules (**Figure 1-5**) (58, 62-64). Some sRBCs undergo hemolysis. Subsequently, the adhesion of sRBC to the endothelium significantly decreases blood flow, causing pain, infarction and organ damage. The resulting damage activates the endothelium, leading to increased expression of endothelial adhesion molecules and increased recruitment of leukocytes (18, 63).

Figure 1-5: Role of Circulating Free Heme in SCD



SCD erythrocytes are susceptible to hemolysis, which results in the release of free heme into circulation. Free heme induces expression of adhesion molecules on endothelial cells, increasing binding of erythrocytes and decreasing blood flow (65).

Source: Wagener, et.al. (http://www.sciencedirect.com/science/article/pii/S0165614700016096); accessed October 20, 2012 (65)

3. Visualization of Microvascular Pathology in Sickle Cell Disease

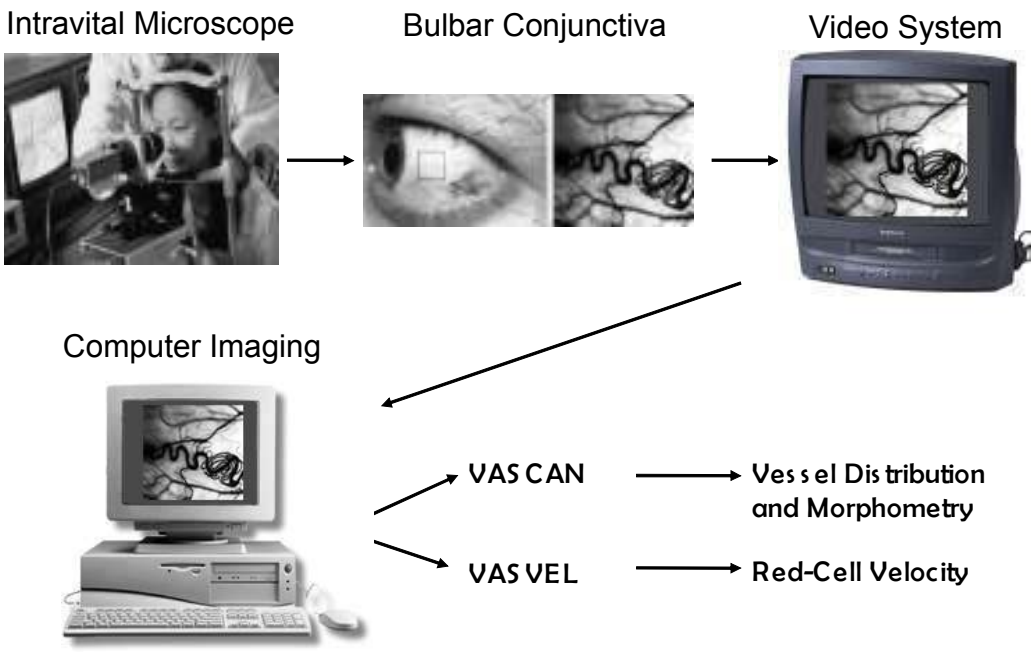
While anemia accounts for some of the morbidity in SCD, vascular abnormalities underlie most of the serious acute and chronic complications (66). Therefore, it is vital to characterize the changes to the microvasculature. In recent years a novel noninvasive technique has been developed to detect microvascular abnormalities. The technique, called computer-assisted intravital microscopy (CAIM), was developed by Anthony Cheung, PhD, and is a powerful, non-invasive tool for assessment of microvascular

morphology and pathology. CAIM has been used in various disease populations, including patients with diabetes, hypertension, Alzheimer's disease, and SCD (67-73).

The CAIM system uses macro-optics for real-time, *in vivo* image acquisition of a selected region within the conjunctival microcirculation. The video sequences are analyzed to identify morphometric microvascular abnormalities in the conjunctival microcirculation (**Figure 1-6**). From the analysis a severity index (SI) is computed from a list of 15 qualitative and quantitative microvascular abnormalities (**Table 1-1**). The full procedural details of this technique have been described in detail in previous publications (68, 69, 71).

In a previous study on the microvascular abnormalities in SCA by Cheung et al, 18 homozygous HbSS patients were investigated under steady-state, painful crisis and post-crisis conditions using CAIM. The SCA patients were compared to non-SCD controls of similar age and gender distributions; all SCA patients exhibited some morphometric abnormalities and the severity of these abnormalities was significantly greater than in the non-SCD healthy controls (**Figure 1-7**). It was determined that CAIM is a noninvasive tool that provides qualitative and quantitative real-time characterization of microvascular abnormalities in vascular diseases, including sickle cell disease (72).

Figure 1-6: Schematic Diagram of Computer–Assisted Intravital Microscopy (CAIM) Process



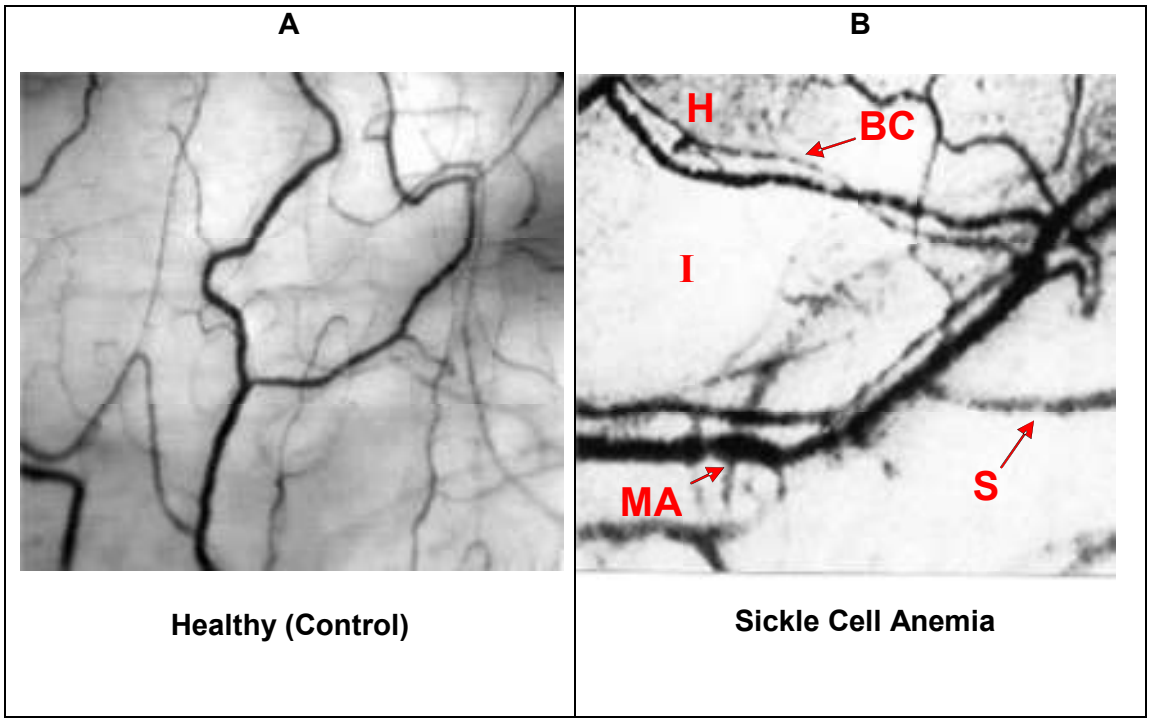
In the CAIM process, the subject's head is placed on a chin-and-head rest. The microscope is then focused on the microvessels of the white of the eye (bulbar conjunctiva). The images are recorded in real-time, and then analyzed by computer using specialized in-house developed software (VASCAN and VASVEL) to assess vessel distribution, morphometry, and red-cell flow velocity. Courtesy of Dr. Anthony Cheung (72, 74).

Table 1-1: CAIM Abnormality Index

1. Abnormal diameter
2. Abnormal distribution
3. Uneven thickness
4. Abnormal morphometry
5. Distended Vessels
6. Damaged vessels
7. Sludging (flow)
8. Tortuosity
9. Ischemia
10. Abnormal AV ratio
11. Microaneurysms
12. Boxcar flow pattern (same as trickled flow)
13. Hemosiderin deposits
14. “Comma” sign
15. Blood flow velocity

Presence of any vascular abnormality in a CAIM image is assigned a point value of 1. A severity index score on a scale of 0-15 points is then calculated, with increasing points indicative of increasing severity of microvasculopathy.

Figure 1-7: Comparison of Bulbar Conjunctival Microvasculature between a Healthy Control Subject and a Patient with Sickle Cell Anemia



Images were captured using CAIM at equal magnification. **A)** Conjunctival microvasculature of a control subject without any history of vascular disease. Note the normal (even) vessel distribution pattern. **B)** Conjunctival microvasculature of a sickle cell anemia patient. Note the presence of hemosiderin deposits [H], "boxcar" blood flow phenomenon [BC], microaneurysms [MA], vessel sludging [S], and ischemic areas [I] lacking capillaries and small arterioles ("blanching" phenomenon). Courtesy of Dr. Anthony Cheung (72, 74).

4. Common Pathophysiological Characteristics of Atherosclerosis and Microvascular Occlusion

The pathophysiology of SCD is a complex process that involves coagulation, endothelial function and inflammation. The initiating mechanism—microvascular occlusion—is very different from the transmigration of monocytes into the sub-endothelial space that is the initiating event in atherosclerosis. However, both pathophysiological mechanisms lead to common pathways that contribute to vascular damage and end-organ pathology. Specifically, these include upregulation of vascular adhesion molecules and inflammatory cytokines, which are putative contributors to vascular morbidity in both atherosclerosis and SCD.

D. Vascular Adhesion Molecules and Inflammation in Sickle Cell Disease

1. Overview

As previously stated, endothelial activation and inflammation lead to vascular occlusion in SCD, and contributes to vascular disease progression. Various adhesion molecules and cytokines participate in this process. Their roles and mechanisms in vascular adhesion in the atherosclerotic population and SCD are reviewed here.

2. Vascular Adhesion Molecules and Other Vascular Factors

A. Vascular Cell Adhesion Molecule 1

Vascular cell adhesion molecule 1 (VCAM-1), or cluster of differentiation 106 (CD106), is a protein that is expressed on vascular endothelial cells in response to stimulation by the inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β). VCAM-1 functions to mediate the adhesion of leukocytes

(monocytes, eosinophils, and basophils) and lymphocytes to vascular endothelium. An elevated level of VCAM-1 in SCD is a reflection of endothelial activation and inflammation. More importantly, VCAM-1 is significantly elevated in SCD patients experiencing end-organ disease and pulmonary hypertension, a major contributor to SCD morbidity and mortality (75, 76).

In a study by Kato et al (76), endothelium-derived adhesion molecules and markers for pulmonary hypertension and organ dysfunction were measured in 160 adult SCD patients during steady state, and in 52 healthy controls. Plasma soluble VCAM-1 (sVCAM-1) levels were found to be higher in those with SCD (median 811 vs. 334 ng/mL, $P < 0.001$), and were associated with markers indicating renal dysfunction and hepatic impairment. Additionally, sVCAM-1 levels were directly associated with markers of hemolysis, and pulmonary arterial pressure.

A later study by Kling et al in 2008 (75) focused on pulmonary hypertension (PH), left-sided heart disease (LHD), an etiology of PH, and their link to VCAM-1 levels in SCD patients. Of the 97 adult SCD patients, 43% had PAH and 10% had LHD. The SCD patients were age- and racially-matched to 23 healthy volunteer controls without cardiopulmonary disease. VCAM-1 levels in SCD subjects were two-fold higher compared with controls.

B. Intercellular Adhesion Molecule 1

Intercellular adhesion molecule 1 (ICAM-1), also known as cluster of differentiation 54 (CD54), is a cell surface immunoglobulin ligand (a glycoprotein) that is constitutively expressed on endothelial cells. As with VCAM-1, ICAM-1 expression is upregulated by TNF-α and IL-1β. ICAM-1 is expressed on endothelium and binds to an

integrin expressed on the leukocyte. The interaction of ICAM-1 and integrin mediates firm adhesion and immobilization of the leukocyte to the endothelium (49, 77, 78). ICAM-1, like VCAM-1, has been shown to correlate with the severity of cardiovascular disease in the general population (79) and pulmonary arterial hypertension in SCD patients (75, 76).

ICAM-1 is less well studied in SCD than VCAM-1. Kato et al (76) found that the median soluble ICAM-1 (sICAM-1) concentration was not significantly different than in healthy controls. However, sICAM-1 did show an association with multiple markers of liver dysfunction. Additionally, it was suggested that elevated sICAM-1 levels observed in some of the SCD patients were because of pathology in the bone potentially due to vaso-occlusive damage. Similar to Kato et al (66), Kling et al (75) found sICAM-1 values were not different between SCD patients and controls.

C. E-selectin

E-selectin, also known as leukocyte-endothelial cell adhesion molecule 2, is constitutively expressed on endothelial cells and upregulate by TNF-α and IL-1β. E-selectin mediates the initial tethering and rolling of leukocytes on activated endothelial cells. Soluble E-selectin (sE-selectin) has been shown to be elevated in SCD patients with pulmonary hypertension, and the levels are correlated with the severity of the condition (75, 80, 81). In an additional study, Mohan et al proposed that elevated sE-selectin and may contribute to the prothrombotic/hypercoagulable state and vascular occlusion in SCD (81).

In the Kato et al (76) study, sE-selectin concentrations were associated with inflammatory stress. sE-selectin was found to be independently linked to C-reactive

protein (CRP) ($P < 0.031$). In contrast, in the study by Mohan et al (81), despite elevation of sE-selectin in SCD patients compared with healthy controls, there was a lack of correlation of sE-selectin with inflammatory markers (IL-6 and CRP).

D. P-selectin

P-selectin is an adhesion molecule expressed on activated endothelial cells and is part of the tethering process for leukocyte transmigration when there is integrin activation and capture on VCAM-1 and ICAM-1. P-selectin differs as it is stored in granules called Weibel-Palade bodies of healthy, quiescent (unactivated) endothelial cells, and upon endothelial activation moves to the cell surface within minutes. P-selectin is also important in platelet monocyte aggregation, and leads to promotion of a prothrombotic phenotype often seen in SCD (82, 83).

Blann et al (82) measured soluble P-selectin (sP-selectin) and vascular endothelial growth factor (VEGF, a marker of platelet activation) in 27 HbSS and 37 HbSC patients, and compared them to 42 age and race matched normal subjects. sP-selectin was higher in HbSS than in HbSC ($P = 0.025$), but there was no difference in VEGF. The correlation between P-selectin and VEGF in SCD was consistent with the observation that VEGF is released from platelet granules during *in vivo* activation.

Mohan et al (83) measured sP-selectin, platelet indices mass, volume and component in 16 HbSS SCD patients, and compared values to 29 healthy subjects matched for age and ethnicity. The SCD patients had lower sP-selectin and mean platelet volume (MPV), but elevated component. Mohan et al concluded that the differences may promote a prothrombotic state in SCD.

E. Vascular Endothelial Growth Factor

VEGF is a signaling protein involved in both vasculogenesis (the *de novo* formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature) (84, 85). VEGF is also an important mediator in normal kidney function. Elevated levels of VEGF are proposed to be associated with renal dysfunction and pulmonary hypertension (85, 86). VEGF stimulates surface expression of ICAM-1, VCAM-1, E-selectin and P-selectin molecules on endothelial cells during inflammation (87).

Recently, it was shown that activation of endothelial cells in SCD contributes to vaso-occlusion events. VEGF has been shown to contribute to increased adhesion to endothelial cells in SCD patients by increasing expression of ICAM-1 and VCAM-1 (88). During an SCD pain crisis, VEGF has also been shown to have a sustained elevation (88, 89).

In a 2005 study by Gurkan et al (88), VEGF levels were investigated in 37 patients with SCD during vaso-occlusion events (n=22) and steady state (n=15), compared with 22 healthy controls. Nine subjects with Hb SS provided samples for measurement of serum VEGF both in their steady state and during acute painful episodes. VEGF levels were found to be significantly elevated during vaso-occlusion events (703.1 +/- 119.0 pg/ml) when compared with those at steady state (258.0 +/- 57.8 pg/ml) and healthy controls (196.6 +/- 21.9 pg/ml) ($P < 0.001$). However, no difference was observed between VEGF concentrations in sickle patients at steady state and the healthy subjects ($P > 0.05$). From this study, it was suggested VEGF levels might be helpful in monitoring disease severity.

In a subsequent study, Qari et al (89) measured several chemokines and cytokines including VEGF in SCA patients. They were divided into 3 groups; 36 SCA patients during painful crisis, 30 SCA patients under steady state, and compared them with 35 healthy controls. Their findings from both SCA patient groups showed a distinct and statistically significant elevated level of VEGF (2- to 3-fold increase) either during painful crisis or at steady state, compared with healthy controls.

F. Brain Natriuretic Peptide

Brain natriuretic peptide (BNP), a 32 amino acid polypeptide, is important in normal cardiovascular homeostasis. BNP works through several hormonal and autonomic mechanisms producing hemodynamic effects, such as diuresis and natriuresis (90). It is released in response to cardiomyocyte stretch, and pressure overload is reflected by high blood concentrations (91-93). BNP is quickly removed from the circulation ($t_{1/2}$ = 22 min) by binding to NPR-C (a clearance receptor) and neutral endopeptidases (present throughout the body) (94). Paradoxically, the natriuretic effect of BNP is blunted in heart disease. It has been proposed that in heart failure, BNP undergoes an alternate post-translational modification that alters its clearance and thus may be less biologically effective (95-99).

In recent years, BNP has been associated with other vascular conditions, including pulmonary arterial hypertension in SCD patients. During a sickle cell vaso-occlusive crisis, BNP level and pulmonary arterial pressure are increased, in particular during life-threatening complications, such as acute chest syndrome. There are many studies demonstrating that an elevated level of BNP is associated with an increase in the risk of death in SCD patients. In some studies, NT-pro-BNP blood concentration (the

BNP precursor) was as much as 5-fold higher in SCD compared with healthy controls (93, 100).

Three prospective studies by Machado et al (93, 101), Agheli et al (100) and Haaf et al (102), have all shown BNP as a strong and independent predictor of mortality in SCD patients. Recently, Machado et al (101) obtained frozen plasma samples from the Cooperative Study of Sickle Cell Disease (CSSCD), a multi-center registry study. NT-pro-BNP was measured in 892 samples (467 pediatric and 395 adult SCD patients). They revealed that elevated BNP (>160 pg/mL) was correlated with many classically defined mortality risk factors in SCD, including pain crisis, acute chest syndrome, sepsis, and leg ulcers.

3. Cytokines and Inflammatory Markers

Cytokines also play a role in vascular activation and thus participate in vaso-occlusive crises. Activated endothelial cells produce inflammatory cytokines (Interleukin-1 (IL-1), IL-2, IL-6, IL-8, monocyte chemotactic protein 1, and TNF- α) which induce cell adhesion to and activation of the vascular endothelium (103, 104).

A. Monocyte Chemotactic Protein 1

Monocyte chemotactic protein 1 (MCP-1) is a chemokine involved in the recruitment of monocytes to sites of endothelial activation or injury, and thus contributes to the pathophysiology of vascular disease (105). In SCD patients, circulating MCP-1 concentrations are elevated during both steady state and vaso-occlusive crisis (89). As previously described by Qari et al (89), biomarkers of inflammation were determined in 36 SCD patients during painful crisis, 30 SCD patients under steady state, and 35 healthy volunteers. MCP-1 concentrations were significantly higher (20- to 30-fold

elevation) in SCD patients, regardless of whether they were in painful crisis or in steady state, compared with the healthy controls.

B. Interleukin-1 beta

Interleukin-1 beta (IL-1β), also known as catabolin, is a proinflammatory cytokine produced by activated macrophages, and is involved in a variety of cellular activities such as proliferation, differentiation, and apoptosis (106). IL-1β may also play a role in leukocyte transmigration. It has been demonstrated that IL-1β increases endothelial surface receptors that mediate the adherence of sRBC to the endothelium (67, 89, 107).

Belcher et al (107) hypothesized that SCD monocytes are activated and can enhance vaso-occlusion by activating endothelium. They collected peripheral blood monocytes from SCD patients and incubated them with human microvascular endothelial cells. The results demonstrated that SCD monocytes had 34% more IL-1β ($P = 0.002$) per cell than normal monocytes.

Wun et al (67) tested whether circulating monocytes were activated in SCD as defined by intracellular expression of the cytokines TNF-α and intracellular IL-1β. Whole blood was collected from 13 SCD patients (7 of which were obtained during a pain crisis), 12 African-American and 11 Caucasian controls. The data showed a non-significant difference in the percentage of monocytes expressing IL-1β between the SCD patients during pain crisis [15 (1.3–44.8) ($n = 7$)] and those in steady state [8.7 (3.3–16.6) ($n = 8$)]. It was postulated that a significant difference may be observed with larger samples sizes.

In a 2012 study by Qari et al (89) they found plasma levels of IL-1β to be significantly elevated during pain crisis by 100- to 200-fold above the levels in healthy, age-matched controls ($P < 0.01$). However, they found much higher levels at steady state ($P < 0.05$) as compared to levels during painful crisis.

C. Interleukin-2

Interleukin-2 (IL-2) is a signaling molecule expressed by leukocytes and is necessary for the growth, proliferation and differentiation of T cells, and the development of T-memory cells (108). IL-2 has been shown to be elevated in SCD, as compared with healthy, non-sickle cell controls. However, IL-2 has not been shown to differ among patients with SCD in steady-state and those experiencing a vaso-occlusive crisis (109, 110).

Pathare et al (109) and Musa et al (110) studied the cytokine profiles, including IL-2, in SCD patients at steady state and during vaso-occlusive crisis, and in healthy controls. IL-2 levels in the Pathare et al study were 64 pg/mL (range 51–97 pg/mL) for control subjects (n = 20), 67 pg/mL (range 53–97 pg/mL) for steady state SCD patients (n = 26) and 65 pg/mL (range 53–104 pg/mL) for those in vaso-occlusive crisis (n = 34). In the Musa et al study, the mean level of IL-2 in normal healthy control subjects (n = 20) was 31 +/- 11 pg/ml (range, 6 to 60 pg/ml); in steady state SCD patients (n = 20) it was 83 +/- 21 pg/ml (range, 48 to 120 pg/ml); and in SCD patients experiencing vaso-occlusive crisis it was 87 +/- 27 pg/ml (range, 53 to 145 pg/ml). Thus, Musa et al demonstrated a significant difference between SCD patients and normal healthy controls ($P < 0.05$), but no difference between steady state and vaso-occlusive crisis, while Pathare et al saw no differences between SCD patients and healthy controls.

D. Interleukin-6

Interleukin-6 (IL-6) acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response during infection or trauma, and is a mediator of the acute phase response to injury and infection (111, 112). SCD patients in crisis exhibit elevated IL-6 compared with healthy non-SCD subjects (109).

The primary study on cytokines from Pathare et al (109) showed that the mean values for IL-6 were 20- to 70-fold lower in healthy controls compared with SCD patients ($P < 0.0001$), with values higher in patients in vaso-occlusive crisis than in steady-state ($P < 0.024$).

E. Interleukin-8

Interleukin-8 (IL-8) is secreted in response to oxidative stress, and is a marker of inflammation. It regulates the production of TNF-α and IL-1 produced by macrophages, which in turn stimulate the production of chemokines that enhance the adhesion of leukocytes (103). IL-8 functions as a chemoattractant to target neutrophil granulocytes, mast cells and macrophages, and induces phagocytosis (113). IL-8 promotes monocyte activation, which in turn enhances the endothelial inflammatory response and leukocyte adhesion. It has been demonstrated that IL-8 is elevated in SCD patients as compared to healthy controls, and may enhance vaso-occlusion (89, 107, 109).

In a cell culture study, Belcher et al (107) incubated microvascular endothelial cells with sickle and normal leukocytes, and found that SCD monocytes had 34% more IL-8 ($P = 0.002$) per cell than normal monocytes.

In two separate cross-sectional studies, both Qari et al (89) and Pathare et al (109), showed higher mean serum IL-8 levels compared with healthy controls. Qari et al demonstrated that IL-8 was 30- to 50-fold above that for healthy, age-matched control subjects ($P < 0.01$), but there was no difference between steady state and vaso-occlusive crisis SCD patients. Pathare et al echoed those results, with the lowest IL-8 levels in the control group, higher levels in the SCD steady state group ($P < 0.09$), and highest levels in the vaso-occlusive crisis group ($P < 0.05$). No significant difference between the SCD steady state and vaso-occlusive crisis groups was observed ($P = 0.21$).

F. Tumor Necrosis Factor-alpha

Tumor necrosis factor-alpha (TNF-α) is a cytokine secreted by leukocytes in response to local vascular endothelium damage, and induces an acute phase reaction in a systemic inflammatory response. TNF-α has also been shown to be associated with increase endothelial adhesion molecules that mediate the adherence of sRBC to the endothelium, and increased vaso-occlusion in SCD patients (67, 107).

In a 2002 study by Wun et al (67), the median percentage of monocyte expression of TNF-α in SCD patients was 14.1 (1.3–44.8) compared with 0.3 (0.1–0.5) and 0.2 (0.1–0.5) in African-American and Caucasians controls, respectively ($P < 0.0001$). Belcher et al (107) incubated sickle and normal leukocytes with human microvascular endothelial cells to test whether monocytes are activated in sickle cell disease and can enhance vaso-occlusion by activating endothelium. Their results showed peripheral blood monocytes from SCD patients had 139% more TNF-α ($P = 0.002$) per cell than normal monocytes.

G. C-Reactive Protein

C-reactive protein (CRP) is a fast responding acute-phase protein synthesized by the liver in response to an IL-6 signal secreted by macrophages occurring in response to infection, inflammation and tissue damage. Its physiological role is to bind to dead and dying cells, and in turn activate the inflammatory complement system (114). CRP levels may to be a biomarker for tissue ischemia in the early (prodromal) stage of sickle cell crisis and used to confirm crisis resolution (115, 116).

To identify suitable acute phase proteins as a biomarkers of tissue ischemia during painful vaso-occlusive crises, Stuart et al (115) longitudinally measured CRP over the course of vaso-occlusive crises in SCD patients (n = 10). Comparable to the overall heterogeneity of symptoms in SCD, their data showed a wide range in clinical severity and inflammatory response to tissue ischemia, as demonstrated by CRP levels.

In a more extensive study, Mohammed et al (116) tested if vaso-occlusive crisis was associated with increased levels of CRP as an inflammatory mediator. In their case-control longitudinal study, CRP levels in 104 vaso-occlusive SCA patients were compared with a second group of 40 SCA patients in steady-state. The median and range for the vaso-occlusive and steady-state groups were 31.3 (1.14–363.0) and 5.0 (0.16–185.0), ($P < 0.001$), respectively.

4. Other Plasma/Serum Biomarkers

A. Renal Function

As one of the end-organs affected in SCD, chronic and acute renal failure is frequently seen as a result of recurrent episodes of vaso-occlusion (117, 118). Renal

dysfunction leads to elevated plasma levels of homocysteine, which in turn may promote vascular disease. Creatinine (Cr) is a measure of renal function and can be used to calculate the glomerular filtration rate (estimated-GFR, or eGFR) to monitor renal function in SCD patients (119).

eGFR is a measure of renal plasma flow and glomerular function. African-Americans have higher eGFR than Caucasians at the same level of serum creatinine due to higher average muscle mass and creatinine generation rate (120). It has been noted that SCD patients may have hyperfiltration (increased GFR) in response to renal vasodilating substances increased in response to medullary ischemia and injury (119, 121).

Day et al (119) reviewed whether renal dysfunction in SCD is linked to hemolysis-associated vasculopathy. The eGFR was positively correlated with reticulocyte count, suggesting increased eGFR was associated with increased hemolysis. Marouf et al (121) were unable to show significant correlations between hematological variables and GFR. Creatinine and eGFR values within the reference range for healthy individuals in a patient with SCD may not reflect normal renal function.

B. Alkaline Phosphatase

Alkaline phosphatase (ALP) is a dephosphorylation enzyme primarily found in the liver, bone, kidney and RBC. Of importance in SCD, ALP functions to dephosphorylate pyridoxal-5'-phosphate (PLP), pyridoxamine-5'-phosphate (PMP) and pyridoxine-5'-phosphate (PNP) in the RBC (122). From a clinical standpoint, ALP is measured to check for bone and liver disease. Notwithstanding the standard clinical reasoning for measuring, Mohammed et al (123) demonstrated that reduced PLP may be related to

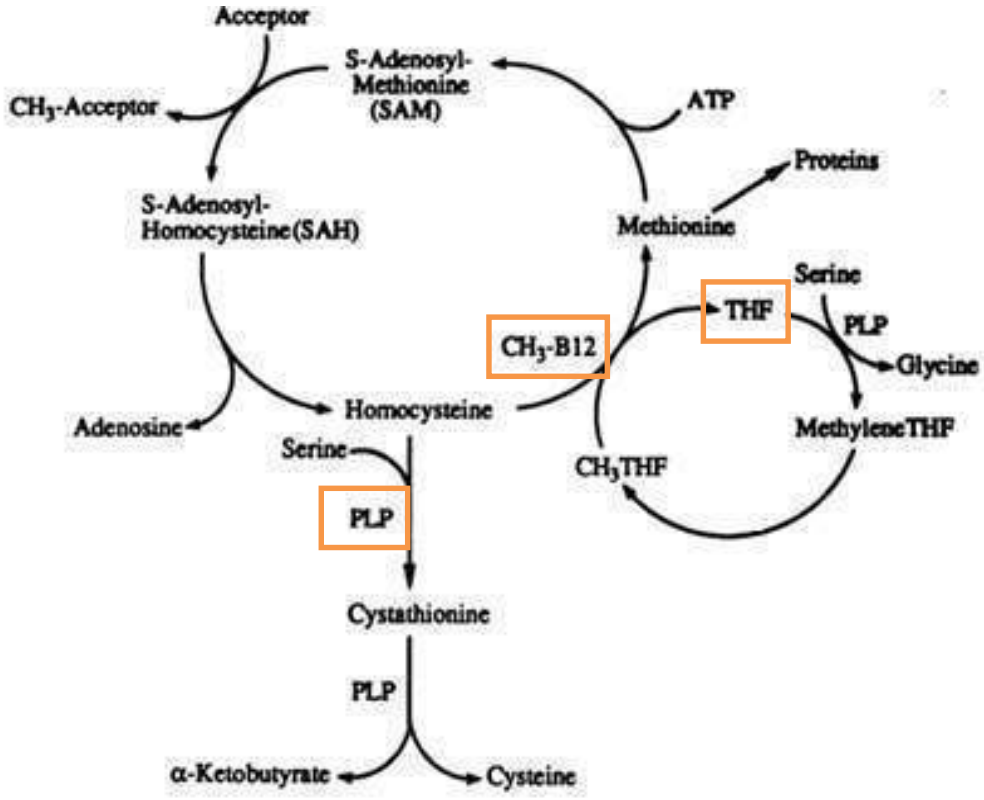
increased activity of alkaline phosphatase, and elevated alkaline phosphatase is common in SCD.

E. B Vitamins and Related Analytes

In addition to the interplay of vascular growth factors and cytokines on endothelial activation and inflammation, the status of several of the B vitamins and related molecules may influence the progression of SCD pathogenesis. SCD is characterized by hemolysis and ineffective erythropoiesis. The hemolysis and ineffective erythropoiesis leads to increased rates of DNA synthesis and therefore increased demand for B vitamins, which putatively results in relative deficiencies, particularly of folate.

The B vitamins, folate, riboflavin (B2), vitamin B12 (B12) and vitamin B6 (B6), serve as substrates or cofactors in 1-carbon and homocysteine metabolism (**Figure 1-8**) (26). Deficiency in any of these vitamins can lead to elevated blood levels of homocysteine. An elevated plasma level of homocysteine (hyperhomocysteinemia) is associated with increased risk of vascular disease in the general population (1, 39, 124, 125). In addition, plasma homocysteine is a functional marker of folate, vitamin B6 and vitamin B12 status (1, 19, 23, 39, 43, 126, 127). Below is a brief review of B vitamin functions and their relevance to SCD.

Figure 1-8: Biochemical Pathways of 1-Carbon Metabolism



The cofactors, B12 and PLP (B6), and coenzyme THF (folate) are highlighted. Courtesy of Dr. Joshua W. Miller [15].

1. Folate

Folate is an essential substrate utilized in the cyclic 1-carbon metabolic pathways involved in amino acid (methionine, homocysteine, serine) and nucleotide (thymidine, purines) metabolism. Folate also serves as a methyl donor for a variety of methyl acceptors, including DNA, RNA, histones, membrane phospholipids, proteins,

neurotransmitters, and other metabolites. With respect to thymidine and purine synthesis, folate is especially important in cells that are rapidly dividing and growing. Therefore, a relative folate deficiency results in disruption of DNA synthesis and may cause megaloblastosis, further aggravating the ineffective erythropoiesis and hemolysis in SCD. This is characterized by large immature and dysfunctional RBCs in bone marrow progenitor cells (**Figure 1-9**) (128).

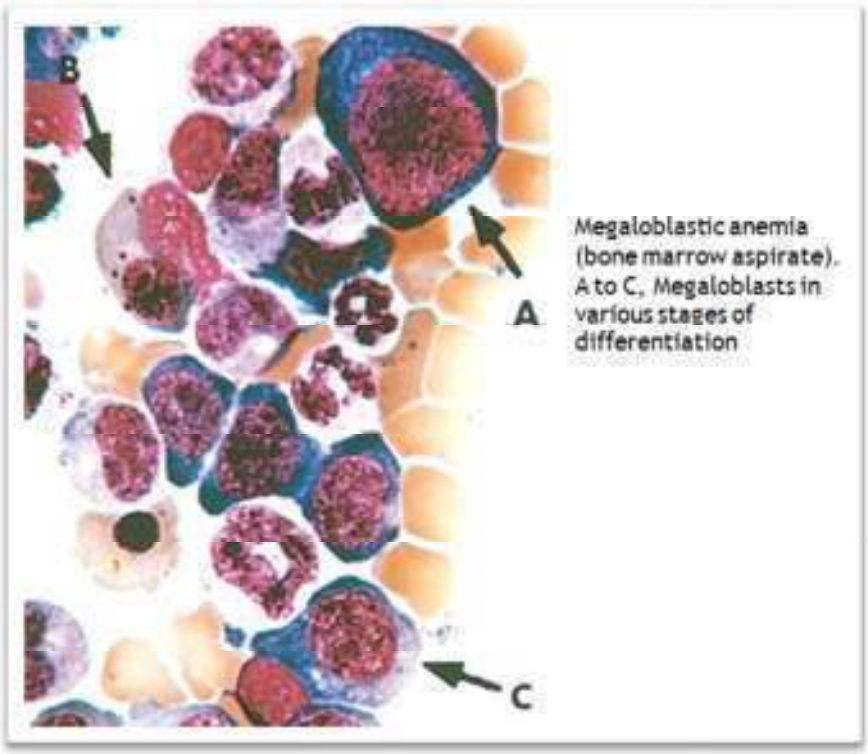
For its role in methionine metabolism, folate (in the form of methyltetrahydro-folate) serves as a 1-carbon donor to homocysteine to resynthesize methionine. Under the condition of low folate, and/or B6 and B12 deficiency, homocysteine blood levels will rise, resulting in hyperhomocysteinemia. Although the direct mechanism of homocysteine on the development of CVD is still to be determined, if any, it has been well document that hyperhomocysteinemia is an independent risk factor for CVD (23, 31, 39, 43, 124, 125, 129-136).

As SCD is a chronic hemolytic disease with increased RBC and folate turnover, the folate requirement in SCD patients is higher than that of the general population. This was demonstrated by Kennedy et al (32) where folate and B12 intake and status in 70 SCD pediatric patients were studied. More than half of the patients had inadequate intake of folate from food, and 15% had low RBC folate levels (32). Folic acid supplementation has also been shown to reduce homocysteine levels in SCD patients (137). Accordingly, a folic acid supplement is routinely prescribed (127, 137, 138).

Despite this evidence, however, there remains much controversy on folate supplementation recommendations. In a 1-year double blind controlled trial of folic acid supplementation of 117 homozygous SCA children, Rabb et al found no effect on RBC

size, suggesting supplementation is not required (138). However, Hoffer (139) argues that it has not been proven that the lack of supplementation is innocuous.

Figure 1-9: Megaloblastic Pronormoblast



Source: PathPedia.com (http://medicinembbs.blogspot.com/2011/01/ megaloblastic-anemia.html), permissions granted; accessed October 2012 (140).

The perturbations in DNA synthesis that result from lack of 1-carbon donors leading to megaloblastic anemia is due to a lack of vitamin B12 or folate, both of which are needed for DNA synthesis. In deficiency of either or both, cells cannot complete DNA synthesis, arresting cell development in mitoses (three arrowheads in Figure 1-9).

These cells ultimately undergo apoptosis within the marrow (ineffective erythropoiesis), and subsequently cause deficiency of normal red cell production and anemia.

2. Vitamin B12

Vitamin B12 (B12, also called cobalamin), like folate, is essential for 1-carbon metabolism, and is important for normal hematological and nervous system function (141). Clinical manifestations of B12 deficiency include megaloblastic anemia and neurological degeneration caused by neuronal demyelination (142). As B12 is utilized in the 1-carbon metabolic pathways, it is also a determinant of homocysteine levels and increased risk of vascular disease (43, 143), but has been less studied in SCD.

Three studies have measured B12 status in SCD patients. In a 10-year study by Kamineni et al of 217 subjects (112 controls), 105 SCD patients had significantly lower serum B12 levels as compared to patients without SCD with 7% having a subclinical deficiency (as defined by a serum cobalamin level of < 200 pg/ml). The mean ages of the low-B12 SCD and non-SCD patients were 28.1 and 62.9, respectively (144). In a study by al-Momen (145), 37 of 85 SCD patients (43.5%) had serum vitamin B12 levels below the reference range (178-897 pmol/L). Notably, none had macrocytosis, a sign of B12 deficiency.

In a 2002 longitudinal study of 21 pediatric SCD patients by van der Dijs et al, optimal supplementation levels of folic acid, B6 and B12 in SCD pediatric patients were determined using homocysteine as a functional marker. Blood was collected 9 times for measurements of RBC and serum folate, plasma B12, whole-blood B6, and homocysteine. Over an 82-week dose-escalation, it was determined that the optimal

dosage levels, as demonstrated by the lowest plasma homocysteine level achieved, was a combination of 1 mg folic acid, 6 µg B12 and 6 mg B6 (137).

3. Homocysteine

Homocysteine is a non-protein amino acid intermediate produced in 1-carbon metabolism as a byproduct of the transmethylation reaction of methionine. Homocysteine metabolism involves multiple enzymes, cofactors and coenzymes where it is either remethylated to form methionine, or metabolized via the transsulfuration pathway to form cysteine. These steps are interdependent on folate, B12 and B6, which serve as substrates or cofactors in the reactions (146, 147). A suboptimal level of these B vitamins can lead to perturbation of 1-carbon metabolic pathways, and accumulation of homocysteine and other potentially harmful metabolites implicated in atherothrombosis (28, 29, 148).

Elevated homocysteine has been demonstrated as a biomarker for low folate, B12 and B6 status. Although the mechanistic role of homocysteine in the pathogenesis of CVD is still poorly understood, hyperhomocysteinemia is now recognized as an independent risk factor for essentially all forms of vascular disease and their consequences, including heart attack and stroke (3, 23, 39, 129, 131). Consensus within the literature is that plasma homocysteine >15.0 µmol/L is associated with increased risk for cardiovascular events (19, 131, 143, 149, 150). Hoogeveen et al determined that a 5 µmol/L increase in plasma homocysteine is associated with a 60% increase in cardiovascular mortality (151).

Zinellu et al (124) proposed that homocysteine binds to LDL, inducing a high level of reactive oxygen species, resulting in reduced endothelial cell proliferation and viability.

An earlier study by Rodriguez-Cortes et al (152) assessed plasma homocysteine levels as a marker of folate status. They found homocysteine levels in SCD patients and controls were similar and that there was no correlation with RBC folate. However, more recent studies have contradicted these findings.

Dhar et al (153) found that mild hyperhomocysteinemia was a common finding in adult SCD patients compared to control subjects ($P = 0.03$), independent of folate and cobalamin status. The study also found that creatinine levels directly correlated significantly with homocysteine ($P < 0.0001$). Lowenthal et al (154) found ~1.5-fold higher plasma levels of homocysteine in SCD patients compared with controls ($P < 0.001$), despite 1.5-fold higher folate levels in the SCD patients compared with controls ($P < 0.05$).

4. Vitamin B6

Vitamin B6 (B6), a water-soluble vitamin, was first isolated in the 1930s, and in 1942 Snell and co-workers observed multiple forms of B6, later determined to be pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM). The phosphate ester derivative pyridoxal-5'-phosphate (PLP) is the primary coenzyme form that participates in approximately 100 catabolic and anabolic enzymatic reactions. General reaction categories include macronutrient metabolism, synthesis of neurotransmitters, heme synthesis and erythrocyte metabolism and function (155).

A majority of ingested B6 is irreversibly converted to 4-pyridoxic acid—the end product of B6 metabolism. The liver is the primary organ which metabolizes B6, performing the interconversion of B6 to the coenzyme PLP (156). PLP synthesized in the liver is released into circulation bound to albumin, which serves to protect PLP from degradation (157).

It has also been demonstrated that B6 in erythrocytes affects oxygen binding (158, 159). In the RBC, PL binds to the α-chain of hemoglobin and increases O_2 binding. In an antagonistic reaction, PLP binds to the β-chain of hemoglobin, decreasing O_2 binding (160), and in SCD higher levels of B6 have been shown to have anti-sickling properties (161, 162).

It has been demonstrated that patients with chronic inflammatory diseases (rheumatoid arthritis, inflammatory bowel disease, diabetes, obesity, cardiovascular disease and SCD) have significantly lower plasma PLP levels (27, 163, 164). However, in these populations, the red cell PLP concentration is higher (164-167). It is hypothesized that the apparent B6 deficiency in SCD may be due to the increase demand for erythropoiesis and lymphocyte production due to systemic vascular disease and asplenia.

B6 is also required for the two-step catabolism of homocysteine through cystathionine synthesis in a condensation reaction involving serine (**Figure 1-8**). This latter reaction is potentially relevant because elevated plasma homocysteine (hyperhomocysteinemia), among other causes, can result from vitamin B6 deficiency. Of note, however, low B6 status in and of itself has been shown to be an independent risk factor for vascular disease in multivariate regression analysis even after adjusting for

hyperhomocysteinemia. Specifically, B6 deficiency has been associated with coronary artery disease (1, 125), increased risk of stroke (1, 168), and peripheral vascular disease in the general population (1). However, the pathogenic mechanism through which vitamin B6 deficiency contributes to the risk of vascular disease remains to be determined in the general population and SCD.

SCD patients have been shown to have elevated levels of homocysteine and low levels of B6. In the longitudinal study by Segal et al (169), SCD pediatric patients were selected to measure B6 status, as measured by serum PLP concentration and urinary 4-pyridoxic acid (4-PA) concentration, and compared with healthy controls of similar age, gender, ethnicity, and geographic residential area. Eighty-four of the 109 subjects (77%) had PLP concentrations less than 20 nmol/L, the criterion for deficiency. Though low B6 status may be an indicator of poor nutrition, Nelson et al (161) proposed that the low status may in fact be related to increased hemolysis in SCD pediatric patients. Additionally, low B6 may be due to increased protein metabolism, which increases B vitamin requirements (170).

Notably, we and others have shown that low vitamin B6 levels observed in SCD likely arise out of increased requirements for the vitamin as detailed below. In a preliminary study, vitamin B6 status, homocysteine, C-reactive protein (CRP), and adhesion molecules were measured in a case-control study of 18 adult SCA patients (homozygous HbSS) and 20 age-, sex-, and ethnic-matched controls (171). Summary data from this study are presented in **Table 1-2**.

The SCA patients had a lower median PLP concentration than the control subjects ($P < 0.001$), and a greater prevalence of low (<20 nmol/L) PLP levels (53% vs.

10%, respectively; P = 0.009). These findings are consistent with previous studies in which low PLP was observed in pediatric and adult SCD patients (161, 162, 172). The SCD patients also had a higher median homocysteine concentration than the controls (P = 0.02).

The prevalence of hyperhomocysteinemia (>12 μmol/L) was higher in the SCA patients, but this was not statistically significant (33% vs. 10%; P = 0.08). No correlation was found between PLP and homocysteine in the SCA patients or controls. No difference was observed in creatinine, though two patients had creatinine levels >1.3 mg/dL, indicative of significantly impaired renal function. The SCA patients had higher median values for CRP (P = 0.01) and sVCAM-1 (P < 0.001) than controls. Median sICAM-1 was higher in the SCA patients, but this was not statistically significant (P = 0.08). These findings are consistent with endothelial activation and support previous findings of chronic inflammation, and monocyte, platelet, and endothelium activation in SCA patients (67).

Multiple regression analyses, adjusting for age and sex, were used to assess correlations between PLP, homocysteine, and creatinine (independent variables) and CRP, sICAM-1, and sVCAM-1 (dependent variables) in the SCA patients. No associations were found between homocysteine and CRP or either of the adhesion molecules. Creatinine was directly correlated with sVCAM-1 (P = 0.017), thus connecting impaired renal function with endothelial activation. This association may be reflective of renal clearance of these metabolites. PLP also was inversely correlated with sVCAM-1 in the SCA patients (P = 0.009) (**Figure 1-10**).

No significant correlations were observed between PLP and CRP or sICAM-1. The significant inverse association between PLP and sVCAM-1 suggests that low vitamin B6 status may contribute to vascular complications in SCA patients, directly or indirectly, through a mechanism mediated by sVCAM-1, and independent of homocysteine. Alternatively, low B6 may result from inflammation associated with vascular complications.

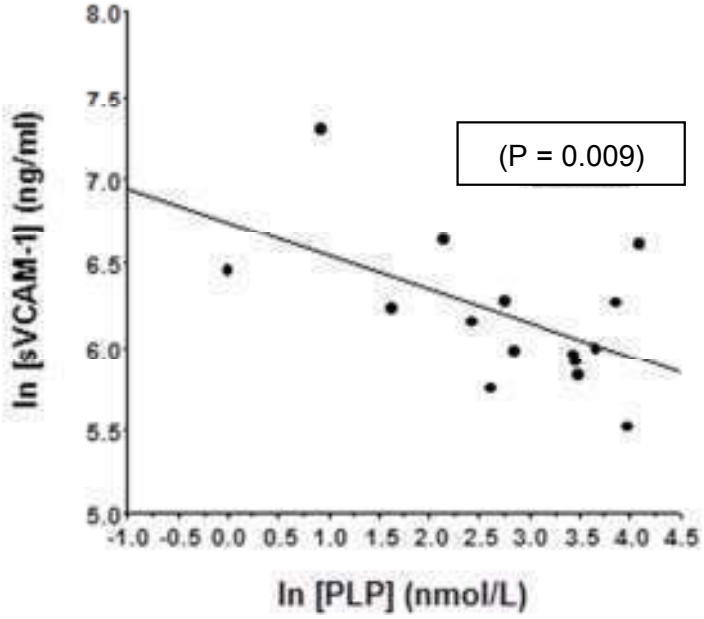
Table 1-2: Characteristics of SCA Patients and Healthy, Ethnic-Matched Controls

	Healthy Controls	SCA Patients
N	20	18
Age (y)	31 (20-65)	31 (18-61)
Gender (% Female)	70	61
PLP (nmol/L)	72.4 (11.4-240)	17.2 (1.0-60.4)**
Homocysteine (μmol/L)	9.0 (3.7-12.9)	11.2 (7.2-18.4)**
Creatinine (mg/dL)	0.7 (0.5-1.1)	0.7 (0.3-8.3)
CRP (mg/dL)	0.20 (0-0.95)	0.38 (0.08-1.75)**
sICAM-1 (ng/ml)	207 (79-317)	258 (0-555)
sVCAM-1 (ng/ml)	258 (215-387)	516 (251-1671)**

* Values are medians (ranges) except for sample size (N) and gender.

** Significantly different from controls, $P \leq 0.02$ (Mann-Whitney U test).

Figure 1-10: PLP vs. VCAM-1



Association between circulating levels of pyridoxal-5'-phosphate (PLP) and soluble vascular cell adhesion molecule-1 (sVCAM-1) in SCA patients. Lower sVCAM-1 levels are associated with higher PLP levels. The p-value is from a multiple regression analysis controlling for potential confounding by age, gender, homocysteine, and creatinine (171).

F. References

1. Robinson K, Arheart K, Refsum H, Brattstrom L, Boers G, Ueland P, Rubba P, Palma-Reis R, Meleady R, Daly L, Witteman J, Graham I. *Low circulating folate and vitamin B6 concentrations: risk factors for stroke, peripheral vascular disease, and coronary artery disease. European COMAC Group*. Circulation 1998;97(5):437-43.
2. World Health Organization. *WHO Disease and Injury Country Estimates*. Internet: http://www.who.int/healthinfo/global_burden_disease/estimates_country/en/index.html. (accessed April 2012).
3. World Health Organization. *Genomic resource centre: Genes and human disease*. Internet: http://www.who.int/genomics/public/geneticdiseases/en/index2.html; http://www.who.int/entity/genomics/public/Maphaemoglobin.pdf. (accessed April 2012).
4. Hassell KL. *Population estimates of sickle cell disease in the U.S*. Am J Prev Med 2010;38(4 Suppl):S512-21.
5. Carlson J NG, Gabutti V, al-Yaman F, Wahlgren M. *Natural protection against severe Plasmodium falciparum malaria due to impaired rosette formation*. Blood 1994;84:3909-814.
6. Friedman MJ, Trager W. *The biochemistry of resistance to malaria*. Sci Am 1981;244(3):154-5, 8-64.
7. Friedman MJ. *Erythrocytic mechanism of sickle cell resistance to malaria*. Proc Natl Acad Sci U S A 1978;75(4):1994-7.
8. Herrick J. *Peculiar elongated and sickle shaped red blood corpuscles in a case of severe anemia.* . Arch Intern Med 1910;6:517.
9. Pauling L, Itano, H.A., Singer, S. J., Wells, I.C. *Sickle Cell Anemia, a Molecular Disease*. Science 1949;110(2865):543-8.
10. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, Klug PP. *Mortality In Sickle Cell Disease -- Life Expectancy and Risk Factors for Early Death*. New England Journal of Medicine 1994;330(23):1639-44.
11. Silliman CC, Fung YL, Bradley Ball J, Khan SY. *Transfusion-related acute lung injury (TRALI): Current concepts and misconceptions*. Blood reviews 2009;23(6):245-55.
12. Mekontso Dessap A, Leon R, Habibi A, Nzouakou R, Roudot-Thoraval F, Adnot S, Godeau B, Galacteros F, Brun-Buisson C, Brochard L, Maitre B. *Pulmonary Hypertension and Cor Pulmonale during Severe Acute Chest Syndrome in Sickle Cell Disease*. Am J Respir Crit Care Med 2008;177(6): 646-53.
13. Steinberg MH, McCarthy WF, Castro O, Ballas SK, Armstrong FD, Smith W, Ataga K, Swerdlow P, Kutlar A, DeCastro L, Waclawiw MA. *The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: A 17.5 year follow-up*. American Journal of Hematology;85(6):403-8.

14. *HBB: The Gene Associated with Sickle Cell Anemia*. Retreived 2011. Internet: http://www.ornl.gov/sci/techresources/Human_Genome/posters/chromosome/hbb.shtml. U.S. Department of Energy (DOE) Human Genome Project Information Web site.).

15. *Number of malaria reported confirmed cases, 2010*. Internet: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_Reported Cases_2010.png. World Health Organization. (accessed October 2012).

16. *WHO Technical Report Series*. WHO, Geneva, Switzerland: World Health Organization, 1996.

17. National Heart Lung and Blood Institute. *What Is Sickle Cell Anemia?* September 28, 2012. Internet: http://www.nhlbi.nih.gov/health/health-topics/topics/sca/. (accessed October 12,2012).

18. Frenette PS. *Sickle cell vaso-occlusion: multistep and multicellular paradigm*. Current Opinion in Hematology 2002;9(2):101-6.

19. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. *A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes*. JAMA 1995;274(13):1049-57.

20. Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, McQueen MJ, Probstfield J, Fodor G, Held C, Genest J, Jr. *Homocysteine lowering with folic acid and B vitamins in vascular disease*. N Engl J Med 2006;354(15):1567-77.

21. Marti-Carvajal AJ, Sola I, Lathyris D, Salanti G. *Homocysteine lowering interventions for preventing cardiovascular events*. Cochrane Database Syst Rev 2009(4):CD006612.

22. Smach MA, Naffeti S, Charfeddine B, Ben Abdallah J, Othmen LB, Letaef A, Limem K. *Homocysteine, vitamin B-12, folic acid and the cognitive decline in the elderly.* Pathol Biol (Paris) 2012.

23. Collaboration HS. *Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis*. JAMA 2002;288(16):2015-22.

24. Baik HW, Russell RM. *Vitamin B12 deficiency in the elderly*. Annu Rev Nutr 1999;19:357-77.

25. Bates C, Pentieva K, Prentice A, Mansoor A, Finch S. *Plasma pyridoxal phosphate and pyridoxic acid and their relationship to plasma homocysteine in a representative sample of British men and women aged 65 years and over*. British Journal of Nutrition 1999;81:191-201.

26. Fox JT, Stover PJ. *Folate-mediated one-carbon metabolism*. Vitam Horm 2008;79:1-44.

27. Friso S, Girelli D, Martinelli N, Olivieri O, Lotto V, Bozzini C, Pizzolo F, Faccini G, Beltrame F, Corrocher R. *Low plasma vitamin B-6 concentrations and modulation of coronary artery disease risk*. The American journal of clinical nutrition 2004;79(6):992-8.

28. Hou CT, Wu YH, Cheng CH, Huang PN, Huang YC. *Higher plasma homocysteine is associated with lower vitamin b6 status in critically ill surgical patients*. Nutr Clin Pract 2012;27(5):695-700.

29. Zsori KS, Csiki Z, Katona E, Bereczky Z, Shemirani AH. *Vitamin B(12) level in peripheral arterial disease*. J Thromb Thrombolysis 2012.

30. Goldstein LB, Bushnell CD, Adams RJ, Appel LJ, Braun LT, Chaturvedi S, Creager MA, Culebras A, Eckel RH, Hart RG, Hinchey JA, Howard VJ, Jauch EC, Levine SR, Meschia JF, Moore WS, Nixon JV, Pearson TA. *Guidelines for the primary prevention of stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association*. Stroke 2011;42(2):517-84.

31. Carmel R, Bellevue R, Kelman Z. *Low cobalamin levels associated with sickle cell disease: Contrasting origins and clinical meanings in two instructive patients*. Am J Hematol 2010;85(6):436-9.

32. Kennedy TS, Fung EB, Kawchak DA, Zemel BS, Ohene-Frempong K, Stallings VA. *Red blood cell folate and serum vitamin B12 status in children with sickle cell disease*. J Pediatr Hematol Oncol 2001;23(3):165-9.

33. Mendis S, Puska P, Norrving B. *Global Atlas on cardiovascular disease prevention and control*. World Health Organization, World Heart Federation and World Stroke Organization, 2011:16-70.

34. Lusis AJ. *Atherosclerosis*. Nature 2000;407(6801):233-41.

35. World Health Organization. *World Health Statistics*. 2011. Internet: http://www.who.int/gho/publications/world_health_statistics/EN_WHS2011_Full.pdf.

36. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J. *Heart disease and stroke statistics--2010 update: a report from the American Heart Association*. Circulation 2010;121(7):e46-e215.

37. Vanhecke TE, Miller WM, Franklin BA, Weber JE, McCullough PA. *Awareness, knowledge, and perception of heart disease among adolescents*. European Journal of Cardiovascular Prevention & Rehabilitation 2006;13(5):718-23.

38. Ross R. *Atherosclerosis--an inflammatory disease*. N Engl J Med 1999;340(2):115-26.

39. Choy PC, Mymin D, Zhu Q, Dakshinamurti K, O K. *Atherosclerosis risk factors: the possible role of homocysteine*. Mol Cell Biochem 2000;207(1-2):143-8.

40. Daniels TF, Killinger KM, Michal JJ, Wright RW, Jr., Jiang Z. *Lipoproteins, cholesterol homeostasis and cardiac health*. Int J Biol Sci 2009;5(5):474-88.

41. Ross R. *The pathogenesis of atherosclerosis: a perspective for the 1990s*. Nature 1993;362(6423):801-9.

42. Clinton SK, Libby P. *Cytokines and growth factors in atherogenesis*. Arch Pathol Lab Med 1992;116(12):1292-300.

43. Refsum H, Ueland PM, Nygard O, Vollset SE. *Homocysteine and cardiovascular disease*. Annu Rev Med 1998;49:31-62.

44. Brown MS GJ. *A receptor-mediated pathway for cholesterol homeostasis*. Science 1986;232:34-47.

45. Goldstein JL, Brown MS. *The LDL receptor*. Arterioscler Thromb Vasc Biol 2009;29(4):431-8.

46. Goldstein JL, Brown MS. *The LDL receptor and the regulation of cellular cholesterol metabolism*. J Cell Sci Suppl 1985;3:131-7.

47. Aviram M. *Macrophage foam cell formation during early atherogenesis is determined by the balance between pro-oxidants and anti-oxidants in arterial cells and blood lipoproteins*. Antioxid Redox Signal 1999;1(4):585-94.

48. Galle J, Hansen-Hagge T, Wanner C, Seibold S. *Impact of oxidized low density lipoprotein on vascular cells*. Atherosclerosis 2006;185(2):219-26.

49. Weber C, Fraemohs L, Dejana E. *The role of junctional adhesion molecules in vascular inflammation*. Nature Reviews Immunology 2007(7):467-77.

50. Adams MR, Kinlay S, Blake GJ, Orford JL, Ganz P, Selwyn AP. *Atherogenic lipids and endothelial dysfunction: mechanisms in the genesis of ischemic syndromes*. Annu Rev Med 2000;51:149-67.

51. Favaloro EJ. *Differential expression of surface antigens on activated endothelium*. Immunol Cell Biol 1993;71 (Pt 6):571-81.

52. Deem TL C-MJ. *Vascular cell adhesion molecule 1 (VCAM-1) activation of endothelial cell matrix metalloproteinases: role of reactive oxygen species*. Blood 2004;104:2385-93.

53. Blann AD SM, Steiner M, Miller JP, McCollum CN. *Circulating ICAM-1 and VCAM-1 in Peripheral Artery Disease and Hypercholesterolaemia: Relationship to the Location of Atherosclerotic Disease, Smoking, and in the Prediction of Adverse Events*. Thromb Haemost 1998;76(6):1080-5.

54. Videm V AM. *Soluble ICAM-1 and VCAM-1 as markers of endothelial activation.* Scand J Immunol 2008;67(5):523-31.

55. *International Classification of Diseases (ICD)*. World Health Organization Retreived 2011.

56. Swerlick RA EJ, Kumar A, Jeitler M, Wick TM. *Alpha 4 beta 1-integrin expression on sickle reticulocytes: vascular cell adhesion molecule-1-dependent binding to endothelium*. Blood 1993;82(6):1891-9.

57. Kumar A, Eckmam JR, Swerlick RA, Wick TM. *Phorbol ester stimulation increases sickle erythrocyte adherence to endothelium: a novel pathway involving alpha 4 beta 1 integrin receptors on sickle reticulocytes and fibronectin*. Blood 1996;88(11):4348-58.

58. Hebbel RP, Osarogiagbon R, Kaul D. *The Endothelial Biology of Sickle Cell Disease: Inflammation and a Chronic Vasculopathy*. Microcirculation 2004;11(2):129-51.

59. Kaul DK, Hebbel RP. *Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice*. The Journal of clinical investigation 2000;106(3):411-20.

60. Bunn HF. *Pathogenesis and Treatment of Sickle Cell Disease*. New England Journal of Medicine 1997;337(11):762-9.

61. Natarajan M, Udden MM, McIntire LV. *Adhesion of sickle red blood cells and damage to interleukin-1 beta stimulated endothelial cells under flow in vitro*. Blood 1996;87(11):4845-52.

62. Hebbel RP, Yamada O, Moldow CF, Jacob HS, White JG, Eaton JW. *Abnormal adherence of sickle erythrocytes to cultured vascular endothelium: possible mechanism for microvascular occlusion in sickle cell disease*. J Clin Invest 1980;65(1):154-60.

63. Chiang EY, Frenette PS. *Sickle cell vaso-occlusion*. Hematol Oncol Clin North Am 2005;19(5):771-84.

64. Schlager A, Zamir G, Barshtein G, Yedgar S, Arbell D. *Plasma factor in red blood cells adhesion to endothelial cells: humans and rats*. Cell Biochem Biophys 2010;58(3):157-61.

65. Wagener FA, Abraham NG, van Kooyk Y, de Witte T, Figdor CG. *Heme-induced cell adhesion in the pathogenesis of sickle-cell disease and inflammation*. Trends Pharmacol Sci 2001;22(2):52-4.

66. Francis RB JC. *Vascular Occlusion in Sickle Cell Disease: Current Concepts and Unanswered Questions*. Blood 1991;77(7):1405-14.

67. Wun T, Cordoba M, Rangaswami A, Cheung AW, Paglieroni T. *Activated monocytes and platelet-monocyte aggregates in patients with sickle cell disease*. Clin Lab Haem 2002;24:81-8.

68. Smith MM, Chen PC, Li CS, Ramanujam S, Cheung AT. *Whole blood viscosity and microvascular abnormalities in Alzheimer's Disease*. Clin Hemorheol Microcirc 2009;41(4):229-39.

69. Devaraj S, Cheung AT, Jialal I, Griffen SC, Nguyen D, Glaser N, Aoki T. *Evidence of increased inflammation and microcirculatory abnormalities in patients with type 1 diabetes and their role in microvascular complications*. Diabetes 2007;56(11):2790-6.

70. Chew SH, Meighan Smith Tomic M, Cheung AT. *Alzheimer's disease: more than amyloid*. Clin Hemorheol Microcirc 2010;46(1):69-73.

71. Cheung A, Tomic M, Chen P, Miguelino E, Li C, Devaraj S. *Correlation of microvascular abnormalities and endothelial dysfunction in Type-1 Diabetes Mellitus (T1DM): a real-time intravital microscopy study*. Clin Hemorheol Microcirc 2009;42(4):285-95.

72. Cheung AT, Chen PC, Larkin EC, Duong PL, Ramanujam S, Tablin F, Wun T. *Microvascular abnormalities in sickle cell disease: a computer-assisted intravital microscopy study*. Blood 2002;99(11):3999-4005.

73. Cheung AT, Chan MS, Ramanujam S, Rangaswami A, Curl K, Franklin P, Wun T. *Effects of poloxamer 188 treatment on sickle cell vaso-occlusive crisis: computer-assisted intravital microscopy study*. J Investig Med 2004;52(6): 402-6.

74. Cheung AT, Harmatz P, Wun T, Chen PC, Larkin EC, Adams RJ, Vichinsky EP. *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 2001;97(11):3401-4.

75. Klings ES, Anton Bland D, Rosenman D, Princeton S, Odhiambo A, Li G, Bernard SA, Steinberg MH, Farber HW. *Pulmonary arterial hypertension and left-sided heart disease in sickle cell disease: clinical characteristics and association with soluble adhesion molecule expression*. Am J Hematol 2008;83(7):547-53.

76. Kato GJ, Martyr S, Blackwelder WC, Nichols JS, Coles WA, Hunter LA, Brennan ML, Hazen SL, Gladwin MT. *Levels of soluble endothelium-derived adhesion molecules in patients with sickle cell disease are associated with pulmonary hypertension, organ dysfunction, and mortality*. Br J Haematol 2005;130(6):943-53.

77. Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. *ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow*. Blood 2005;106(2):584-92.

78. Rothlein R, Dustin ML, Marlin SD, Springer TA. *A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1*. J Immunol 1986;137(4):1270-4.

79. Hwang S, Ballantyne C, Sharrett A, Smith L, Davis C, Jr AG, Boerwinkle E. *Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study*. Circulation 1997;96:4219-25.

80. Zorca S, Freeman L, Hildesheim M, Allen D, Remaley AT, Taylor JGt, Kato GJ. *Lipid levels in sickle-cell disease associated with haemolytic severity, vascular dysfunction and pulmonary hypertension*. Br J Haematol 2010;149(3):436-45.

81. Mohan JS, Lip GY, Wright J, Bareford D, Blann AD. *Plasma levels of tissue factor and soluble E-selectin in sickle cell disease: relationship to genotype and to inflammation*. Blood Coagul Fibrinolysis 2005;16(3):209-14.

82. Blann AD, Mohan JS, Bareford D, Lip GY. *Soluble P-selectin and vascular endothelial growth factor in steady state sickle cell disease: relationship to genotype*. J Thromb Thrombolysis 2008;25(2):185-9.

83. Mohan JS, Lip GY, Bareford D, Blann AD. *Platelet P-selectin and platelet mass, volume and component in sickle cell disease: relationship to genotype*. Thromb Res 2006;117(6):623-9.

84. Claesson-Welsh L. *Arterioscler Thromb Vasc Biology*. Edtion ed. VEGF-B Taken to Our Hearts: Specific Effect of VEGF-B in Myocardial Ischemia, 2008:1575-6.

85. Liu E, Morimoto M, Kitajima S, Koike T, Yu Y, Shiiki H, Nagata M, Watanabe T, Fan J. *Increased expression of vascular endothelial growth factor in kidney leads to progressive impairment of glomerular functions*. J Am Soc Nephrol 2007;18(7):2094-104.

86. Ataga KI, Brittain JE, Jones SK, May R, Delaney J, Strayhorn D, Desai P, Redding-Lallinger R, Key NS, Orringer EP. *Association of soluble fms-like tyrosine kinase-1 with pulmonary hypertension and haemolysis in sickle cell disease*. Br J Haematol 2011;152(4):485-91.

87. Kim I MS, Kim SH, Kim HJ, Koh YS, Koh GY. *Vascular Endothelial Growth Factor Expression of Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), and E-selectin through Nuclear Factor-κB Activation in Endothelial Cells*. J Biol Chem 2001;276(10):7614-20.

88. Gurkan E, Tanriverdi K, Baslamisli F. *Clinical relevance of vascular endothelial growth factor levels in sickle cell disease*. Ann Hematol 2005;84(2):71-5.

89. Qari MH, Dier U, Mousa SA. *Biomarkers of inflammation, growth factor, and coagulation activation in patients with sickle cell disease*. Clin Appl Thromb Hemost 2012;18(2):195-200.

90. Rocca HB-L, Kaye D, Woods R, Hastings J, Esler M. *Effects of intravenous brain natriuretic peptide on regional sympathetic activity in patients with chronic heart failure as compared with healthy control subjects*. J Am Coll Cardiol 2001;37(7):1221-8.

91. Hall C. *Essential biochemistry and physiology of (NT-pro)BNP*. Eur J Heart Fail 2004;6(3):257-60.

92. Levin E, Gardner D, Samson W. *Natriuretic Peptides*. Mechanisms of Disease 2004;339(5):321-8.

93. Machado RF AA, Steinberg MH, Bonds D, Sachdev V, Kato GJ, et al. . *N-terminal pro-brain natriuretic peptide levels and risk of death in sickle cell disease.* JAMA 2006;296:310-19.

94. Charles C, Espiner E, Nicholls M, al. e. *Clearance receptors and endopeptidase 24.11: equal role in natriuretic peptide metabolism in conscious sheep*. Am J Physiol 1996;271:R373-9.

95. Baerts L, Gomez N, Vanderheyden M, De Meester I, Mc Entee K. *Possible mechanisms for brain natriuretic peptide resistance in heart failure with a focus on interspecies differences and canine BNP biology*. Vet J 2012.

96. Thireau J, Karam S, Fauconnier J, Roberge S, Cassan C, Cazorla O, Aimond F, Lacampagne A, Babuty D, Richard S. *Functional evidence for an active role of B-type natriuretic peptide in cardiac remodelling and pro-arrhythmogenicity*. Cardiovasc Res 2012;95(1):59-68.

97. Eindhoven JA, van den Bosch AE, Jansen PR, Boersma E, Roos-Hesselink JW. *The Usefulness of Brain Natriuretic Peptide in Complex Congenital Heart Disease: A Systematic Review*. J Am Coll Cardiol 2012.

98. Maisel AS, Krishnaswamy P, Nowak RM, McCord J, Hollander JE, Duc P, Omland Tr, Storrow AB, Abraham WT, Wu AHB, Clopton P, Steg PG, Westheim A, Knudsen CW, Perez A, Kazanegra R, Herrmann HC, McCullough PA. *Rapid Measurement of B-Type Natriuretic Peptide in the Emergency Diagnosis of Heart Failure*. New England Journal of Medicine 2002;347(3): 161-7.

99. Tsutamoto T, Wada A, Maeda K, Hisanaga T, Maeda Y, Fukai D, Ohnishi M, Sugimoto Y, Kinoshita M. *Attenuation of Compensation of Endogenous Cardiac Natriuretic Peptide System in Chronic Heart Failure : Prognostic Role of Plasma Brain Natriuretic Peptide Concentration in Patients With Chronic Symptomatic Left Ventricular Dysfunction*. Circulation 1997;96(2):509-16.

100. Agheli A, Rathnasabapathy C, Sangal A, He Z, Steier W, Rafii S, Kalavar M. *Changes of BNP Level and Pulmonary Arterial Pressure during An Acute Sickle Cell Crisis, Comparison with Steady State*. 50th ASH Annual Meeting and Exposition, 2008.

101. Machado R, Hildesheim M, Mendelsohn L, Remaley A, Kato G, Gladwin M. *NT-pro brain natriuretic peptide levels and the risk of death in the cooperative study of sickle cell disease*. Br J Haematol 2011;154(4):512-20.

102. Haaf P RT, Corson N, Twerenbold R, Reiter M, Steuer S, Bassetti S, Winkler K, Stelzig C, Heinisch C, Drexler B, Freidank H, Mueller C. *B-type Natriuretic Peptide in the Early Diagnosis and Risk Stratification of Acute Chest Pain*. Am J Med 2011;124(5):444-52.(5):444-52.

103. Makis AC, Hatzimichael EC, Bourantas KL. *The role of cytokines in sickle cell disease*. Ann Hematol 2000;79(8):407-13.

104. Francis RB, Jr., Haywood LJ. *Elevated immunoreactive tumor necrosis factor and interleukin-1 in sickle cell disease*. J Natl Med Assoc 1992;84(7):611-5.

105. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. *Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant*. Proc Natl Acad Sci U S A 1994;91(9):3652-6.

106. Bensi G, Raugei G, Palla E, Carinci V, Tornese Buonamassa D, Melli M. *Human interleukin-1 beta gene*. Gene 1987;52(1):95-101.

107. Belcher JD, Marker PH, Weber JP, Hebbel RP, Vercellotti GM. *Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion*. Blood 2000;96(7):2451-9.

108. Cantrell DA, Smith KA. *The interleukin-2 T-cell system: a new cell growth model*. Science 1984;224(4655):1312-6.

109. Pathare A, Kindi SA, Daar S, Dennison D. *Cytokines in sickle cell disease*. Hematology 2003;8(5):329-37.

110. Musa BO, Onyemelukwe GC, Hambolu JO, Mamman AI, Isa AH. *Pattern of serum cytokine expression and T-cell subsets in sickle cell disease patients in vaso-occlusive crisis*. Clin Vaccine Immunol 2010;17(4):602-8.

111. Bastard JP, Jardel C, Delattre J, Hainque B, Bruckert E, Oberlin F. *Evidence for a link between adipose tissue interleukin-6 content and serum C-reactive protein concentrations in obese subjects*. Circulation 1999;99(16):2221-2.

112. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. *Principles of interleukin (IL)-6-type cytokine signalling and its regulation*. Biochem J 2003;374(Pt 1):1-20.

113. Utgaard JO, Jahnsen FL, Bakka A, Brandtzaeg P, Haraldsen G. *Rapid secretion of prestored interleukin 8 from Weibel-Palade bodies of microvascular endothelial cells*. J Exp Med 1998;188(9):1751-6.

114. Pepys MB, Hirschfield GM. *C-reactive protein: a critical update*. J Clin Invest 2003;111(12):1805-12.

115. Stuart J, Stone PC, Akinola NO, Gallimore JR, Pepys MB. *Monitoring the acute phase response to vaso-occlusive crisis in sickle cell disease*. J Clin Pathol 1994;47(2):166-9.

116. Mohammed FA, Mahdi N, Sater MA, Al-Ola K, Almawi WY. *The relation of C-reactive protein to vasoocclusive crisis in children with sickle cell disease*. Blood Cells Mol Dis 2010;45(4):293-6.

117. Ballas SK. *Defining the phenotypes of sickle cell disease*. Hemoglobin 2011;35(5-6):511-9.

118. Silva Junior GB, Liborio AB, Vieira AP, Bem AX, Lopes Filho AS, Figueiredo Filho AC, Guedes AL, Souza JH, Costa CM, Costa R, Daher EF. *Evaluation of renal function in sickle cell disease patients in Brazil*. Braz J Med Biol Res 2012;45(7):652-5.

119. Day TG, Drasar ER, Fulford T, Sharpe CC, Thein SL. *Association between hemolysis and albuminuria in adults with sickle cell anemia*. Haematologica 2012;97(2):201-5.

120. Textor SC, Gloviczki ML, Flessner MF, Calhoun DA, Glockner J, Grande JP, McKusick MA, Cha SS, Lerman LO. *Association of filtered sodium load with medullary volumes and medullary hypoxia in hypertensive African Americans as compared with whites*. Am J Kidney Dis 2012;59(2):229-37.

121. Marouf R, Mojiminiyi O, Abdella N, Kortom M, Al Wazzan H. *Comparison of renal function markers in Kuwaiti patients with sickle cell disease*. J Clin Pathol 2006;59(4):345-51.

122. Leklem JE. *Handbook of Vitamins*: Marcel Dekker, 2001; 339-396.

123. Mohammed SM, Suleiman SA, Addae SK, Annobil SH, Adzaku FK, Kadoummi OF, Richards JT. *Urinary hydroxyproline and serum alkaline phosphatase in sickle cell disease*. Clin Chim Acta 1991;203(2-3):285-94.

124. Zinellu A, Sotgia S, Scanu B, Pintus G, Posadino AM, Cossu A, Deiana L, Sengupta S, Carru C. *S-homocysteinylated LDL apolipoprotein B adversely affects human endothelial cells in vitro*. Atherosclerosis 2009;206(1):40-6.

125. Robinson K, Mayer EL, Miller DP, Green R, van Lente F, Gupta A, Kottke-Marchant K, Savon SR, Selhub J, Nissen SE, et al. *Hyperhomocysteinemia and low pyridoxal phosphate. Common and independent reversible risk factors for coronary artery disease*. Circulation 1995;92(10):2825-30.

126. Miller J, Green R, Mungas D, Reed B, Jagust W. *Homocysteine, vitamin B6, and vascular disease in AD patients*. Neurology 2002;58:1471-5.

127. van der Dijs FP, Schnog JJ, Brouwer DA, Velvis HJ, van den Berg GA, Bakker AJ, Duits AJ, Muskiet FD, Muskiet FA. *Elevated homocysteine levels indicate suboptimal folate status in pediatric sickle cell patients*. Am J Hematol 1998;59(3):192-8.

128. Brody T, Shane B. *Folic Acid*. In Handbook of Vitamins. Third ed, 2001; 427-462.

129. Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PWF, Belanger AJ, O'Leary DH, Wolf PA, Schaefer EJ, Rosenberg IH. *Association between Plasma Homocysteine Concentrations and Extracranial Carotid-Artery Stenosis*. New England Journal of Medicine 1995;332(5):286-91.

130. Jardine MJ, Kang A, Zoungas S, Navaneethan SD, Ninomiya T, Nigwekar SU, Gallagher MP, Cass A, Strippoli G, Perkovic V. *The effect of folic acid based homocysteine lowering on cardiovascular events in people with kidney disease: systematic review and meta-analysis*. BMJ 2012;344:e3533.

131. Stubbs PJ, Al-Obaidi MK, Conroy RM, Collinson PO, Graham IM, Noble IM. *Effect of plasma homocysteine concentration on early and late events in patients with acute coronary syndromes*. Circulation 2000;102(6):605-10.

132. Houston PE, Rana S, Sekhsaria S, Perlin E, Kim KS, Castro OL. *Homocysteine in sickle cell disease: relationship to stroke*. Am J Med 1997;103(3):192-6.

133. Lonn E. *Homocysteine in the prevention of ischemic heart disease, stroke and venous thromboembolism: therapeutic target or just another distraction?* Curr Opin Hematol 2007;14(5):481-7.

134. Wolfgang H, Markus H, Jacob K, Suresh T. *Homocysteine, brain natriuretic peptide and chronic heart failure : a critical review*. Clin Chem Lab Med 2007;45(12):1633-45.

135. Haynes R, Clarke R. *Homocysteine, the kidney, and vascular disease*. BMJ 2012;344:e3925.

136. Cheng X. *Updating the Relationship Between Hyperhomocysteinemia Lowering Therapy and Cardiovascular Events*. Cardiovasc Ther 2012.

137. van der Dijs FP, Fokkema M, Dijck-Brouwer D, Niessink B, Wal Tvd, Schnog J, Duits A, Muskiet F, Muskiet F. *Optimization of folic acid, vitamin B(12), and vitamin B(6) supplements in pediatric patients with sickle cell disease*. Am J Hematol 2002;69(4):239-46.

138. Rabb LM, Grandison Y, Mason K, Hayes RJ, Serjeant B, Serjeant GR. *A trial of folate supplementation in children with homozygous sickle cell disease*. Br J Haematol 1983;54(4):589-94.

139. Hoffer L. *Folate Supplementation in Sickle Cell Anemia*. New England Journal of Medicine 2003;349(8):813.

140. PathPedia: Global Online Pathology Resource. *PathPedia; Global Online Pathology Resource. Megaloblastic anemia*. Internet: http://www.pathpedia.com/education/eatlas/histopathology/bone_marrow/megaloblastic_anemia.aspx. (accessed October 2012).

141. Green R, Miller JW. *Handbook of Vitamins*. 4th ed: New York: CRC Press (Taylor & Fracis Group), 2007; 413-457.

142. Beck WS. *Handbook of Vitamins*: Marcel Dekker, 2001; 463-512.

143. Toole JF, Malinow MR, Chambless LE, Spence JD, Pettigrew LC, Howard VJ, Sides EG, Wang CH, Stampfer M. *Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death: the Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial*. JAMA 2004;291(5):565-75.

144. Kamineni P, Chirla S, Dinh K, Hasan S, Nidhiry E, Kwagyan J, Naab T, Lombardo F, Castro O, Dawkins F. *Low cobalamin levels in African Americans with and without sickle cell disease*. J Natl Med Assoc 2006;98(3):352-6.

145. al-Momen A. *Diminished vitamin B12 levels in patients with severe sickle cell disease*. J Intern Med 1995;237(6):551-5.

146. Martinov MV, Vitvitsky VM, Banerjee R, Ataullakhanov FI. *The logic of the hepatic methionine metabolic cycle*. Biochim Biophys Acta 2010;1804(1):89-96.

147. Chen NC, Yang F, Capecci LM, Gu Z, Schafer AI, Durante W, Yang XF, Wang H. *Regulation of homocysteine metabolism and methylation in human and mouse tissues*. FASEB J 2010;24(8):2804-17.

148. McNeil CJ, Beattie JH, Gordon MJ, Pirie LP, Duthie SJ. *Differential effects of nutritional folic acid deficiency and moderate hyperhomocysteinemia on aortic plaque formation and genome-wide DNA methylation in vascular tissue from ApoE-/- mice*. Clin Epigenetics 2011;2(2):361-8.

149. Stauffenberg MT, Lange RA, Hillis LD, Cigarroa J, Hsu RM, Devaraj S, Jialal I. *Hyperhomocysteinemia measured by immunoassay: a valid measure of coronary artery atherosclerosis*. Arch Pathol Lab Med 2004;128(11):1263-6.

150. Rauh M, Verwied S, Knerr I, Dorr HG, Sonnichsen A, Koletzko B. *Homocysteine concentrations in a German cohort of 500 individuals: reference ranges and determinants of plasma levels in healthy children and their parents*. Amino Acids 2001;20(4):409-18.

151. Hoogeveen EK, Kostense PJ, Jakobs C, Dekker JM, Nijpels G, Heine RJ, Bouter LM, Stehouwer CD. *Hyperhomocysteinemia increases risk of death, especially in type 2 diabetes : 5-year follow-up of the Hoorn Study*. Circulation 2000;101(13):1506-11.

152. Rodriguez-Cortes HM, Griener JC, Hyland K, Bottiglieri T, Bennett MJ, Kamen BA, Buchanan GR. *Plasma homocysteine levels and folate status in children with sickle cell anemia*. J Pediatr Hematol Oncol 1999;21(3):219-23.

153. Dhar M, Bellevue R, Brar S, Carmel R. *Mild hyperhomocysteinemia in adult patients with sickle cell disease: a common finding unrelated to folate and cobalamin status*. Am J Hematol 2004;76(2):114-20.

154. Lowenthal EA, Mayo MS, Cornwell PE, Thornley-Brown D. *Homocysteine elevation in sickle cell disease*. J Am Coll Nutr 2000;19(5):608-12.

155. Dakshinamurti S DKVBIZJ, Rucker RB, McCormick DB, Suttie JW, eds. *Handbook of Vitamins*: New York: CRC Press (Taylor & Fracis Group), 2007; 315-359.

156. Merrill AH, Jr., Henderson JM. *Vitamin B6 metabolism by human liver*. Ann N Y Acad Sci 1990;585:110-7.

157. Anderson BB, Newmark PA, Rawlins M, Green R. *Plasma binding of vitamin B6 compounds*. Nature 1974;250(5466):502-4.

158. Fonda ML, Harker CW. *Metabolism of pyridoxine and protein binding of the metabolites in human erythrocytes*. The American journal of clinical nutrition 1982;35(6):1391-9.

159. Ink SL, Mehansho H, Henderson LM. *The binding of pyridoxal to hemoglobin*. J Biol Chem 1982;257(9):4753-7.

160. Leklem JE. Vitamin B6. In: Shils M OJ, Shike M, Ross AC, eds. *Modern Nutrition in Health and Disease*: Baltimore: Williams & Wilkins, 1999; 413-422.

161. Nelson MC ZB, Kawchak DA, Barden EM, Frongillo EA Jr, Coburn SP, Ohene-Frempong K, Stallings VA. *Vitamin B6 status of children with sickle cell disease.* J Pediatr Hematol Oncol 2002;26:24(6):463-9.

162. Natta C, Reynolds R. *Apparent vitamin B6 deficiency in sickle cell anemia.* Am J Clin Nutr 1984;40:235-9.

163. Friso S, Jacques PF, Wilson PW, Rosenberg IH, Selhub J. *Low circulating vitamin B(6) is associated with elevation of the inflammation marker C-reactive protein independently of plasma homocysteine levels*. Circulation 2001;103(23):2788-91.

164. Leung EY, Roxburgh CS, Talwar D, O'Reilly DS, McKee RF, Horgan PG, McMillan DC. *The relationships between plasma and red cell vitamin B2 and B6 concentrations and the systemic and local inflammatory responses in patients with colorectal cancer*. Nutr Cancer 2012;64(4):515-20.

165. Vasilaki AT, McMillan DC, Kinsella J, Duncan A, O'Reilly DS, Talwar D. *Relation between pyridoxal and pyridoxal phosphate concentrations in plasma, red cells, and white cells in patients with critical illness*. The American Journal of Clinical Nutrition 2008;88(1):140-6.

166. Hou CT, Wu YH, Huang PN, Cheng CH, Huang YC. *Higher plasma pyridoxal 5'-phosphate is associated with better blood glucose responses in critically ill surgical patients with inadequate vitamin B-6 status*. Clin Nutr 2011;30(4): 478-83.

167. Quasim T, McMillan DC, Talwar D, Vasilaki A, St JORD, Kinsella J. *The relationship between plasma and red cell B-vitamin concentrations in critically-ill patients*. Clin Nutr 2005;24(6):956-60.

168. Kelly PJ, Shih VE, Kistler JP, Barron M, Lee H, Mandell R, Furie KL. *Low vitamin B6 but not homocyst(e)ine is associated with increased risk of stroke and transient ischemic attack in the era of folic acid grain fortification*. Stroke 2003;34(6):e51-4.

169. Segal JB, Miller ER, 3rd, Brereton NH, Resar LM. *Concentrations of B vitamins and homocysteine in children with sickle cell anemia*. South Med J 2004;97(2):149-55.

170. Borel MJ, Buchowski MS, Turner EA, Goldstein RE, Flakoll PJ. *Protein turnover and energy expenditure increase during exogenous nutrient availability in sickle cell disease*. The American Journal of Clinical Nutrition 1998;68(3):607-14.

171. Miller JW, Medina MV, Wun T, Green R. *Plasma pyridoxal-5'-phosphate is inversely correlated with VCAM levels in adult sickle cell disease patients*. FASEB J, 2004.

172. Balasa BV, Kalinyak KA, Bean JA SD, Gruppo RA. *Hyperhomocysteinemia is associated with low plasma pyridoxine levels in children with sickle cell disease*. J Pediatr Hematol Oncol 2002;24:374-9.

Chapter 2 : Statement of Purpose

Sickle cell anemia (SCA) is characterized by acute and chronic hemolysis and anemia with clinical manifestations of intermittent painful crisis, acute chest syndrome, priapism, and vascular damage as demonstrated by ischemic stroke and irreversible damage to all organs, particularly heart, lung, kidneys, spleen, eyes and femoral heads.

Elevated homocysteine has been demonstrated as a risk factor for cardiovascular disease, peripheral vascular disease, and stroke in the general population. B vitamin deficiencies are important causes of elevated homocysteine and are associated with vascular disease.

There is increasing evidence that microvascular abnormalities underlie the morbidity in SCA patients. Elevated homocysteine and low B vitamin status, particularly folate and vitamin B6, are highly prevalent in SCA patients. Preliminary data suggest low vitamin B6 status is associated with increased levels of soluble adhesion molecules, the membrane bound forms of which mediate adhesion of erythrocytes and leukocyte to the vascular endothelium—key events in the pathophysiology of vascular occlusion in SCA patients. However, it is not known if elevated homocysteine and low B vitamin status affect vascular morbidity in SCA patients.

The objective of this dissertation is to identify predictive biomarkers of microvascular abnormalities in SCA patients with the long-term goal of designing novel interventions to reduce morbidity and mortality caused by vascular damage in this patient population.

The specific aims are as follows:

Specific Aim #1: To assess and quantify microvasculature abnormalities in SCA patients.

Specific Aim #2: To determine the cross-sectional relationships between biomarkers of B vitamin status (including homocysteine, folate, vitamin B12, and vitamin B6), inflammation, vascular adhesion, and microvascular characteristics in SCA patients under steady-state conditions.

Chapter 3: Biomarkers of Vitamin B6, Folate, Vitamin B12, Inflammation, Vascular Adhesion, and Microvasculopathy in Sickle Cell Anemia Patients under Steady-State Conditions

A. Background

The pathophysiology of sickle cell anemia (SCA) is a complex process that alters coagulation, endothelial function and vascular inflammation. Vaso-occlusion and vascular injury, affecting both the micro- and macrovasculature, underlie most of the clinical manifestations of SCA. The clinical manifestations include painful crisis, acute chest syndrome, priapism, retinopathy and stroke as well as chronic, irreversible damage to all organs.

Adherence of blood cells, including sickle erythrocytes (sRBC), leukocytes and platelets, to the microvascular endothelium with secondary capture is mediated by vascular adhesion molecules and cytokines. The capture of blood cells impedes microvascular blood flow and is the initiating event in sickle cell vaso-occlusion (1-3).

Vaso-occlusion results in decreased blood flow, local hypoxemia, hemoglobin polymerization, and subsequent further red cell sickling (4). This leads to further endothelial activation and upregulation of inflammatory and vascular adhesion molecules, and contributes to vascular disease progression. Adherence of sRBC to activated endothelium, with recurrent episodes of ischemia/reperfusion injury, lead to hyperplasia, and eventual stenosis and permanent vascular damage (5).

In addition to the interplay of vascular factors and cytokines on endothelial activation and inflammation, the status of several other micronutrients including

B vitamins and their related metabolites that may influence the progression of SCD pathogenesis (6-9). The various hemoglobinopathies comprising the spectrum of SCD are characterized by hemolysis and ineffective erythropoiesis (10-12). The hemolysis and ineffective erythropoiesis leads to increased rates of DNA synthesis and demands for B vitamins resulting in relative deficiencies, particularly of folate (13).

The B-vitamins, folate, vitamin B12 (B12), riboflavin (B2) and vitamin B6 (B6) serve as substrates or cofactors in one-carbon and homocysteine metabolism (14). Deficiency in any of these vitamins can lead to elevated blood levels of homocysteine. An elevated plasma level of homocysteine (hyperhomocysteinemia) is associated with increased risk of vascular disease in SCD patients (15-17). In addition, plasma homocysteine is a functional marker of folate, vitamin B6 and vitamin B12 status (18-24).

A nutritional biochemical characteristic of sickle cell patients, both adult and pediatric, is a high prevalence of low vitamin B6 status (exceeding 50% in some cohorts), as indicated by low plasma pyridoxal-5'phosphate levels (PLP) (<20–30 nmol/L) (25-28). In the general population, B6 deficiency is a risk factor for cardiovascular disease, peripheral vascular disease and stroke (23, 29, 30), but it is unknown if low B6 status affects vascular morbidity in SCD patients. Preliminary data from our laboratory indicated that low B6 status was associated with increased circulating levels of adhesion molecules that, in the form present on endothelial cells, mediate erythrocyte and leukocyte adhesion to the vascular endothelium, key events in the pathogenesis of vascular occlusion in SCD patients (28).

The focus of this chapter is to present details of study patient recruitment, review sample collection procedures and outline the analyte assays conducted for the study

that underlies the further investigation of the association of vascular endothelial dysfunction with abnormalities in B vitamin status and circulating levels of adhesion molecules. The chapter concludes with presentation of the baseline, cross-sectional data collected, including summaries of demographic, blood analyte concentrations, and microvascular characteristics. In chapter 4, correlation analysis of the cross-sectional relationships between vitamin B6 levels, soluble circulating vascular adhesion molecules, and microvascular characteristics in SCD patients under steady-state conditions is presented.

B. Patients and Methods

1. Patient Recruitment and Criteria

Patient recruitment and procedures for the study were approved by the Office of the Vice Chancellor for Research Institutional Review Board (IRB) Administration at the University of California, Davis. Written informed consent (along with ascent for patients age 12-17) was obtained from all study patients.

Patients diagnosed with SCD (HbSS and HbSC genotype), greater than 4 years of age were selected for potential participation into the study. Those receiving hydroxyurea and/or blood transfusions or exchanges were not excluded. Those on chronic transfusion were allowed to participate in this study, with their blood collections for analysis and other measurements performed at least 1 month after the most recent transfusion.

The patients were recruited from the Pediatric and Adult Sickle Cell Clinics at the University of California, Davis Medical Center (UCDMC) during outpatient clinic visits, or

participation in the Sacramento regional Sickle Cell Family Support Group and the Annual California Sickle Cell Disease Symposiums. Theodore Wun, MD, Division Chief of Hematology and Oncology at UCDMC, and Theodore Zwerdling, MD, of the Adult and Pediatric Sickle Cell Clinics, respectively, at the University of California, Davis Medical Center (UCDMC) reviewed each potential patient's medical history, performed a physical examination, and reviewed medical records to assess each patient's eligibility for the study. The patients were also assessed to ensure none had clinical manifestations of vitamin B6 deficiency (evidence of dermatitis or history of epileptiform convulsions), none were having any acute complications (i.e., in steady-state condition) and none had suffered a vaso-occlusive (painful) crisis for at least a month before the study.

Patients or guardians were questioned for use of concurrent steroids, non-steroidal anti-inflammatory agents, hydroxyurea, folic acid, multivitamins, prophylactic antibiotics, past year immunizations and use of pain medications. Their medical records were also surveyed for history of transfusions, asplenia, pain crisis and pulmonary pain episodes, and any on-going, non-SCD issues.

2. Sample Collection and Analysis

Fasting blood of ~15mL was collected while the patient was free of acute complications into plasma (EDTA) and serum (SST) tubes from each subject at the Pediatric Comprehensive Hemoglobinopathy and the Adult Sickle Cell Disease clinics at UCDMC. Immediately upon collection, blood samples were wrapped in aluminum foil to protect the B-vitamins from photo-degradation during handling and transporting. Plasma (EDTA) tubes were placed directly on ice; serum separator tubes (SST) tubes were held at room temperature for 30 minutes, then placed on ice.

Directly following their blood collections, patients had their bulbar conjunctiva visualized and recorded via Computer-Assisted Intravital Microscopy (CAIM) as previously described (31-34). A Severity Index (SI) was computed to quantify the degree of vasculopathy in each patient based on the arithmetic summation of the presence of any of 15 possible microvascular abnormalities on a binary (yes = 1; no = 0) basis. The SI ranges from a score of 0 (no abnormalities present) to 15 (all 15 abnormalities present), as described (35).

The UCDMC Department of Pathology and Laboratory Medicine Clinical and Chemistry Laboratories processed and measured the following serum and plasma analytes by standardized clinical procedures: beta-human chorionic gonadotropin (serum β-hCG) by chemiluminescent immunoassay; complete blood count (CBC) and automated differential by automated cell count with flow cell differential; creatinine and albumin by spectrophotometry; glomerular filtration rate calculated from creatinine-based approximations; alkaline phosphatase by kinetic rate reaction (p-nitrophenyl phosphate); total iron, total iron binding capacity (TIBC), and transferrin by turbidimetric testing; iron saturation by spectrophotometry; ferritin by chemiluminometric immunoassay; C-reactive protein by immunoassay (near infrared particle immunoassay rate); serum folate and vitamin B12 by Siemens Advia Centaur Competitive Chemiluminescent Immunoassay; and plasma brain natriuretic peptide (BNP) levels by immunoenzymatic assay.

The remainder of the blood was transported to the UCDMC Pathology Research laboratory for further processing. A portion of the whole blood was aliquoted from plasma EDTA tubes and stored frozen at –80 °C for later analysis. The remainder of the EDTA whole blood and additional tubes of whole blood collected without anticoagulant

were centrifuged at 2,200 rpm for 10 minutes. The plasma and serum supernatants were then aliquoted and stored at –80 °C for later analysis.

Plasma homocysteine was measured by the high-performance liquid chromatography (HPLC) method of Gilfix et al (35a); plasma and RBC pyridoxal-5'-phosphate (PLP), and plasma and RBC pyridoxal (PL) concentrations were determined by the HPLC method described by Talwar et al (36, 37), plasma 4-pyridoxic acid (4-PA) by HPLC described by Gregory et al (38) and plasma kynurenine and tryptophan by HPLC described by Zheng et al (39).

Erythrocyte PLP and PL values were calculated from whole blood PLP and PL values by taking the whole blood PLP value, adjusting for dilution, subtracting the plasma PLP value, multiplying the difference of 100 percent less the hematocrit value, and dividing this value by the hematocrit.

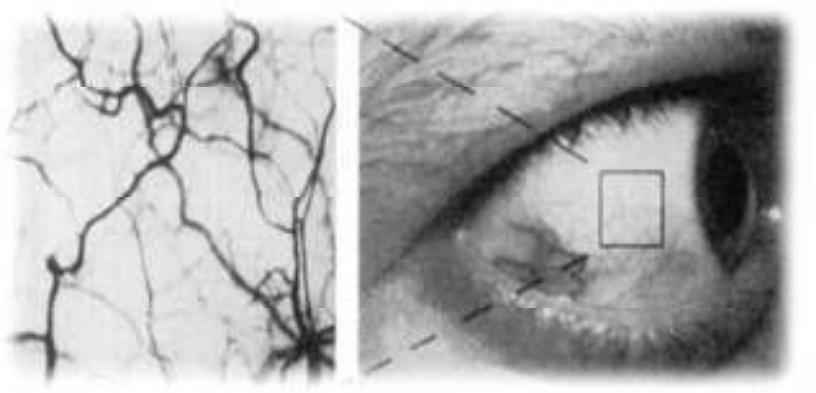
Cytokines (IL-1β, IL-2, IL-6, IL-8, MCP-1, TNF-α and VEGF) and adhesion molecules (sICAM, sP-selectin and sE-selectin) concentrations were measured by Luminex multiple analyte profiling (Life Technologies, Grand Island, NY) (40, 41). The adhesion molecule sVCAM-1 was measured by commercial ELISA assay (R&D Systems, Minneapolis, MN).

3. Computer-Assisted Intravital Microscopy (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 (**Figure 3-1**). A severity index on a scale of 0 – 15 was calculated to quantify the degree

of microvasculopathy observed among the patients. The procedural details of this technique have been described in detail in previous publications by Cheung et al (35, 42-44).

Figure 3-1: A View of the Bulbar Conjunctiva



The view of the bulbar conjunctiva shows the conjunctival microcirculation (optical magnification 4.5x and on-screen magnification 125x.) Kind permission of Dr. Anthony T. Cheung.

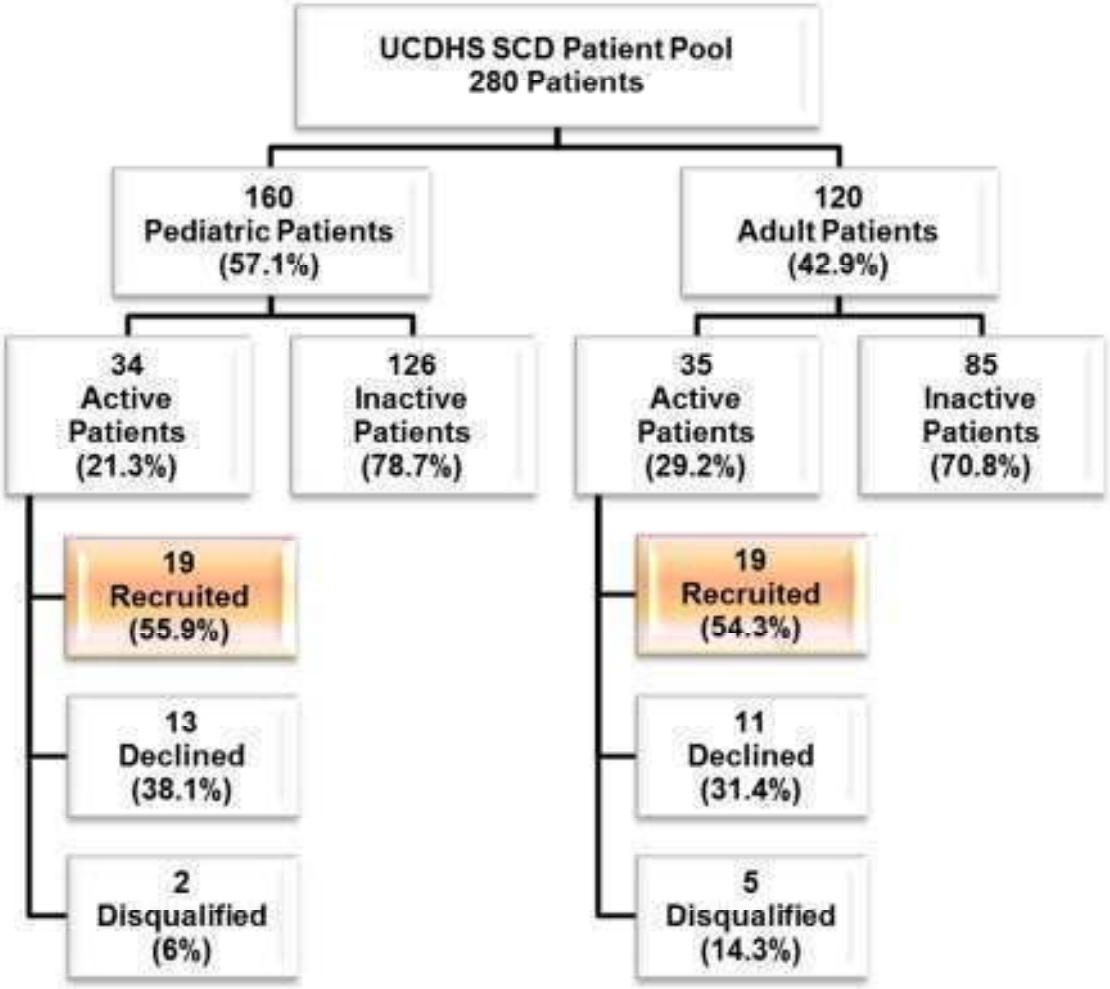
C. Results:

1. Patient Screening and Enrollment

At the University of California Davis Medical Center (UCDMC) there was a pool of 280 (120 adults, 160 pediatric) registered patients within the medical records database identified with sickle cell disease over the 4 years of study recruitment (2007 –

2011) (**Figure 3-2**). Of the 280 patients, 34 pediatric (21% of patient pool) and 35 adults (29% of patient pool) were classified as active patients ("active" defined as those who had been seen by a hematologist within the previous 12 months). Among the active patients 19 pediatric (56% of active pediatric patients) and 19 adults (54% of active adult patients) consented to participate in the study, 13 pediatric (38%) and 11 adults (31%) declined, and 2 (6%) pediatric and 5 adults (14%) were disqualified based on exclusion criteria.

Figure 3-2: Patient Recruitment Efforts



2. Patient Demographics

The study's patient demographics are presented in **Table 3-1.** Specific demographics and assay results for each patient are presented in **Appendix A**. Thirty-eight SCA patients were recruited for the study. The patients were equally split between pediatric (age ≤18 years, n = 19) and adult patients (age >18 years, n = 19). The adult gender distribution was heavily weighted toward female patients (79% female, 21% male); pediatric patients were 58% females and 42% males. Of the pediatric patients, 6 (32%) were 4 – 8 years of age, 4 (21%) were 9 – 13 years of age, and 9 (47.5%) were 14 – 18 years of age. The adult patients ranged from 19 to 61 years of age, with 7 (37%) patients age 20 – 29 years, 8 (42%) age 30 – 39 years and 4 (21%) age 48 – 61 years.

Nine (47.4%) of the adult patients and 2 (10.5%) of pediatric patients were on a stable dose of hydroxyurea treatment.

Transfusion frequency differed between the pediatric and adult patients. Pediatric patients receiving transfusions were on regularly scheduled transfusion cycles (approximately monthly). Adult patients were transfused as clinically indicated, and cycles 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 admitted to not following the recommended regimen—80% non-adherence. Data for folic acid supplement use represents only those who were adherent with their hematologists' prescription (defined as less than 2 out of 7 per week missed doses). Intake of multi-vitamin supplements was 16% among the pediatric patients and 37% among the adult patients.

Two patients (SCD-B6-24 and SCD-B6-25) consented to the study, provided demographic data, but withdrew prior to the first blood draw and CAIM procedures. These 2 patients were not analyzed.

Table 3-1: Patient Demographics

Data Reported:
Count (n)
or
Mean ± SD
Median (Range)

	Pediatric	Adult	All
Participants (n)	*19*	*19*	*38*
Male (n)	*7*	*4*	*11*
Female (n)	*12*	*15*	*27*
Age (y)	12.5 ± 4.9 13 (4 – 18)	34.8 ± 11.7 31 (22 – 61)	23.7 ± 14.3 20 (4 – 61)
Transfused (n)	*17*	*8*	*25*
Hydroxyurea (n)	*2*	*9*	*11*
Folic Acid Supplement (n)	*3*	*3*	*6*
Multi–vitamin Supplement (n)	*3*	*7*	*10*

3. Analyte Results

In the following sections the analytes and microvascular characteristics are reviewed and presented. The analytes are grouped by B-vitamins and related analytes, vascular and inflammatory markers, iron status indicators, and other related analytes. The last table summarizes microvascular characteristics as determined by CAIM. Sample numbers for analytes are listed; for sample numbers less than 19 each for the pediatric and adult patient groups (38 total), there was insufficient volume to measure.

When comparing the medians between pediatric and adult patients, the non-parametric Mann-Whitney test was utilized. This method was used both because the sample size was small and to reduce the influence that large outliers have on the comparisons. Additionally, medians were compared between males and females by the Mann-Whitney test for each of the variables reviewed below. No significant differences between males and females were observed except where noted.

A. B-Vitamins and Related Analytes

Table 3-2 summarizes the mean + SD, median (range) and the number of samples measured for the B-vitamins and related analytes measured in our SCA patients.

The reference range for plasma PLP is 20 – 125 nmol/L; values > 20 nmol/L are classified as deficient, and values 20 – 30 nmol/L are classified borderline deficient. One pediatric patient (age 4 years) had a high plasma PLP level (162 nmol/L); no adult patients had PLP values above range. No pediatric patients had deficient PLP levels; 3 adults (18% of adults) had deficient plasma PLP levels. Six pediatric and 3 adult patients had marginal (borderline deficient) status. The medians between pediatric and

adult patients were not significantly different (P = 0.26), 40.3 nmol/L (22.6 – 162.0 nmol/L) and 36.6 nmol/L (11.6 – 102.6 nmol/L), respectively.

There is no established reference range set for plasma pyridoxal (PL). The median values and ranges for pediatric and adult patients were 12.5 nmol/L (4.8 – 189.9 nmol/L) and 7.5 nmol/L (0.0 – 29.4 nmol/L), respectively (P = 0.07).

There is no established reference range set for plasma 4-pyridoxic acid (4-PA). There were 2 adult females (B6-09 and B6-31) with high 4-PA values (compared with the other SCD patients with values ranging from 4.6 – 236.1 nmol/L); a 29-year-old with a value of 29920 nmol/L and a 61-year-old with a value of 1418 nmol/L. Median values were not different between the pediatric [28.3 nmol/L (4.6 – 174.9 nmol/L)] and adult [27.0 nmol/L (6.8 – 29920 nmol/L)] patients (P = 0.25).

There are no reference ranges established for RBC PLP and PL (13). The median RBC PLP values were lower in the pediatric patients [232.7 nmol/L (121.7 – 691.5 nmol/L)] compared with the adult patients [346.2 nmol/L (246.8 – 661.5 nmol/L)]. This difference was, however, not statistically significant (P = 0.09). No difference was observed in median RBC PL values between pediatric patients [6.5 nmol/L (0.0 – 485.2 nmol/L)] and adult patients [9.0 nmol/L (0.0 – 41.2 nmol/L)] (P = 0.82).

Folate deficiency is defined as plasma folate <5.3 ng/mL (UCDMC Clinical Laboratory); values greater than 20 ng/mL are reported as >20 ng/mL only. Eight (42%) of the pediatric patients had plasma folate >20 ng/mL, 9 pediatric patients (47%) were within the reference range, and 1 female, a 17-year-old, was deficient (4.1 ng/mL). There were 6 adult patients with plasma folate values >20 ng/mL, 9 within the reference range and 2 below the reference range, both females: a 27-year-old (5.0 ng/mL) and a

33-year-old (4.9 ng/mL). The median (range) for pediatric patients was 19.2 ng/mL (4.1 – >20 ng/mL), and was 12.8 ng/mL (4.9 – >20 ng/mL) for adult patients (P = 0.32). Two adult patients had insufficient volume to measure plasma folate.

All but 1 patient had vitamin B12 values within the reference range of 211 – 911 pg/mL (UCDMC Clinical Laboratory); one adult had an out-of-range value of 1744 pg/mL (nearly 2 times the upper limit of the reference range). There was no significant difference in median B12 concentration between adult patients [549 pg/mL (313.0 – 793.0 pg/mL)] and pediatric patients [460.0 pg/mL (321.0 – 1744.0 pg/mL)] (P = 0.57).

The homocysteine reference ranges for adult males and females are 5.0 – 11.7 μmol/L and 3.8 – 11.0 μmol/L, respectively (UCDMC Clinical Laboratory). Two adults had elevated homocysteine levels, a 39-year-old female (17.0 μmol/L) and a 58-year-old male (18.9 μmol/L); there is no reference range for children, but all pediatric patient values were below 7.8 μmol/L. There was a significant difference in median homocysteine between pediatric and adult patients, 5.5 μmol/L (2.6 - 9.6 μmol/L) vs. 7.0 μmol/L (3.6 - 18.9 μmol/L), respectively (P = 0.01). The median value for male patients was higher than for female patients, 7.8 μmol/L (4.3 – 17.0 μmol/L) vs. 6.4 μmol/L (2.6 – 18.9 μmol/L) (P = 0.04).

No clinical reference range has been established for cysteine. The difference between pediatric and adult patient medians was significant, 253.0 ng/mL (166.9 – 346.8 ng/mL) vs. 281.0 ng/mL (207.3 – 393.0 ng/mL), respectively (P = 0.03).

There is no significant difference between pediatric and adult patient medians for tryptophan [60.9 μmol/L (37.8 – 98.2 μmol/L) vs. 53.0 μmol/L (21.2 – 78.3 μmol/L)] (P = 0.24), and kynurenine [2.5 μmol/L (1.4 – 4.0 μmol/L) vs. 2.2 μmol/L (0.9 – 4.9

μmol/L)] (P = 0.79), as well as for the tryptophan/kynurenine ratio [29.1 (17.1 – 38.7) vs. 24.4 (7.2 – 45.3)] (P = 0.62), respectively. The median kynurenine concentration is higher in males than females [2.6 μmol/L (1.7 – 4.9 μmol/L) vs. 2.2 μmol/L (0.9 – 4.0 μmol/L), respectively], but the difference did not reach statistical significance (P = 0.09).

Table 3-2: B-Vitamins and Related Analytes

Data Reported:
Mean ± SD
Median (Range)
Count (n)

	Pediatric	Adult	All	p-value*
PLP, plasma (nmol/L)	54.4 ± 39.4 40.3 (22.6 – 162.0) *18*	39.0 ± 22.9 36.6 (11.6 – 102.6) *17*	46.9 ± 32.9 39.3 (11.6 – 162.0) *35*	0.26
PLP, RBC (nmol/L)	312.7 ± 193.9 232.7 (121.7 – 691.5) *12*	377.3 ± 151.3 346.2 (246.8 – 661.5) *6*	334.3 ± 179.0 251.6 (121.7 – 691.5) *18*	0.09
PL, plasma (nmol/L)	33.2 ± 51.5 12.5 (4.8 – 189.9) *17*	8.9 ± 7.9 7.5 (0.0 – 29.4) *16*	21.4 ± 38.8 9.1 (0 – 189.9) *33*	0.07
PL, RBC (nmol/L)	59.8 ± 138.4 6.5 (0.0 – 485.2) *12*	10.5 ± 12.3 9.0 (0.0 – 41.2) *10*	39.4 ± 104.1 7.4 (0.0 – 485.2) *22*	0.82
4-PA, plasma (nmol/L)	38.9 ± 43.7 28.3 (4.6 – 174.9) *16*	2673.0 ± 8589.7 27.0 (6.8 – 29920) *12*	1167.8 ± 5641.2 28.3 (4.6 – 29920) *28*	0.25
Folate, plasma (ng/mL)	N/A 19.2 (4.1 – >20) *18*	N/A 12.8 (4.9 – >20) *18*	N/A 16.0 (4.1 – >20) *36*	0.32
Total B12, plasma (pg/mL)	536.2 ± 170.1 549 (313.0 – 793.0) *18*	544.8 ± 345.9 460.0 (321.0 – 1744.0) *17*	540.4 ± 268.8 478.0 (313.0 – 1744.0) *35*	0.57
Total Hcy, plasma (µmol/L)	5.7 ± 1.8 5.5 (2.6 - 9.6) *18*	8.1 ± 4.3 7.0 (3.6 - 18.9) *18*	6.8 ± 3.4 6.5 (2.6 - 18.9) *36*	**0.01**
Total Cys, plasma (µmol/L)	254.6 ± 44.4 253.0 (166.9 – 346.8) *18*	285.8 ± 42.4 281.0 (207.3 – 393.0) *18*	270.2 ± 45.6 269.9 (166.9 – 393.0) *36*	**0.03**
Tryptophan (µmol/L)	61.1 ± 28.7 60.9 (37.8 – 98.2) *11*	51.8 ± 15.7 53.0 (21.2 – 78.3) *13*	56.0 ± 22.6 55.4 (21.2 – 98.2) *24*	0.24
Kynurenine (µmol/L)	2.3 ± 1.1 2.5 (1.4 – 4.0) *11*	2.3 ± 1.1 2.2 (0.9 – 4.9) *13*	2.3 ± 1.1 2.4 (0.9 – 4.9) *24*	0.79
Tryptophan / Kynurenine Ratio	27.9 ± 13.0 29.1 (17.1 – 38.7) *11*	26.8 ± 11.9 24.4 (7.2 – 45.3) *13*	27.3 ± 12.3 26.8 (7.2 – 45.3) *24*	0.62

* Pediatric vs, adult p-values were determined by the Mann-Whitney test.

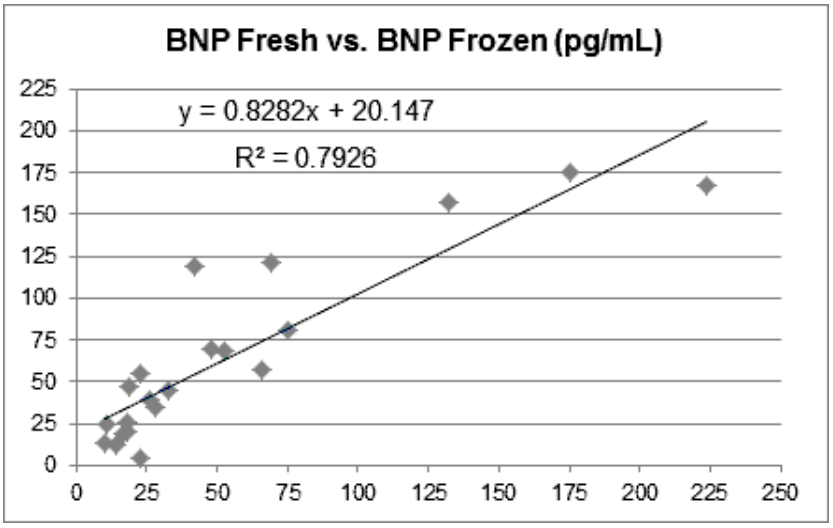
B. Vascular Markers and Inflammatory Cytokine Analytes

1. Vascular Markers

The vascular markers and inflammatory cytokine analytes are presented in **Table 3-3.**

As we were only able to measure 70% of the BNP concentrations in fresh samples, we measured BNP concentrations in frozen samples (where we had 90% of the samples). BNP values obtained for the same patients in fresh and frozen samples were strongly correlated ($R^2 = 0.793$, $P < 0.001$) (**Figure 3-3**). Therefore, frozen plasma BNP samples were utilized for statistical analysis in this study.

Figure 3-3: BNP Correlation of Frozen vs. Fresh Samples



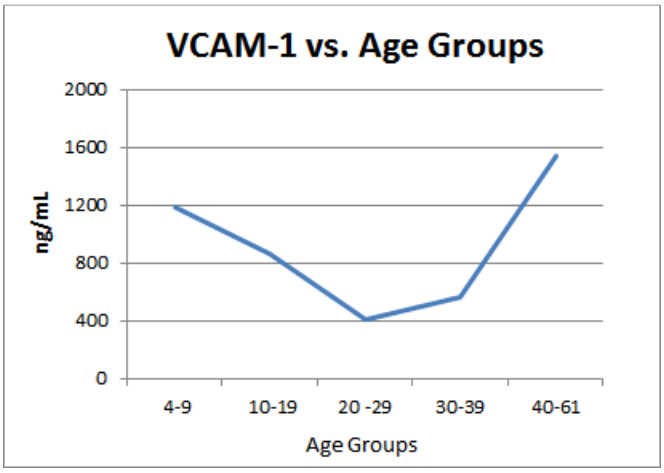
Acute increases in pulmonary pressures, cardiac failure and other pathologies may be associated with increased BNP levels ≥100 pg/mL. Three pediatric patients (25%) and 6 adult patients (40%) had BNP values >100 pg/mL. One male adult (58-year-old) who was in clinical congestive heart failure had a BNP value of 901 pg/mL, more than 4.5-fold higher than any other patient. There was no significant difference in median BNP between pediatric [47.0 pg/mL (16.0 – 126.0 pg/mL)] and adult [68.5 pg/mL (4.0 – 901.0 pg/mL)] patients (P = 0.53). The median BNP value (excluding the 1 outlier) for males [115.8 pg/mL (91.5 – 148.0 pg/mL)] was higher than for females [49.5 pg/mL (11.5 – 164.5 pg/mL)], although this was not significantly different (P = 0.09).

Clinical reference ranges for the vascular adhesion molecules have not been established. Ponthieux et al, and others have established reference ranges for ICAM-1, E-selectin and P-selectin (45), which were used as points of reference. The ICAM-1 reference ranges for children are 192 – 387 mg/L for males and 155 – 144 mg/L for females; for adults the reference ranges are 152 – 379 mg/L for males and 152 – 374 mg/L for females. The E-selectin reference ranges for children are 26.6 – 131.5 mg/L for males and 19.7 – 117.1 mg/L for females; for adults the reference ranges for adults are 19.1 – 105.5 mg/L for males and 9.3 – 94.2 mg/L for females. The P-selectin reference ranges for children are 67 - 272 mg/L for males and 31 - 200 mg/L for females; for adults the reference ranges are 84 - 215 mg/L for males and 46 - 181 mg/L for females.

There appeared to be a parabolic (open upward) relationship between age and sVCAM-1 plasma levels (**Figure 3-4**). There was no significant difference between adult [873.9 ng/mL (421.0 – 3533.2 ng/mL)] and pediatric [921.6 ng/mL (498.8 – 1688.6 ng/mL)] median VCAM-1 concentrations (*P*=0.79). However, the median value for

females was lower than males, 869.0 ng/mL (421.0 – 2209.7 ng/mL) and 1216.1 ng/mL (734.0 – 3533.2 ng/mL), respectively (P = 0.05).

Figure 3-4: Median sVCAM-1 values vs. Age Groups



sICAM-1 levels were higher in the pediatric patients, but there was no significant difference between pediatric and adult patients [313.2 mg/L (198.5 – 416.7 mg/L)] vs. [268.4 mg/L (151.9 – 416.0 mg/L)] (P = 0.10).

The median values for sE-selectin [122.8 mg/L (30.4 – 414.7 mg/L) vs. 67.1 mg/L (18.7 – 122.2 mg/L)] and sP-selectin [92.3 mg/L (55.1 – 229.3 mg/L) vs. 65.2 mg/L (45.0 – 92.4 mg/L)] were significantly higher in the pediatric group than the adult group, P = 0.01 and P = 0.001, respectively.

2. Cytokines and Inflammatory Markers

IL-2, IL-6 and VEGF cytokines had many samples that were below the detection threshold of the assay. These samples were assigned a value of "0" for analysis. In addition, no reference ranges exist for the cytokines IL-1b, IL-2, IL-8, MCP-1 and VEGF.

Twenty-nine of 38 (73%) had VEGF values below the level of detection of the multiplex assay. For the 9 samples that had measurable values, 4 samples were from pediatric patients (all female) and 5 from adult patients (4 females). There was no significant difference in median VEGF values between pediatric [0 ng/L (0 – 47.0 ng/L)] and adult [0 ng/L (0 – 41.0)] patients (P = 0.46).

All CRP values were below the cut-point marker for disease (<3.0 mg/L). There was no difference in median CRP values between the pediatric [0.2 mg/L (<0.1 – 2.5 mg/L)] and adult [0.4 mg/L (<0.1 – 1.4 mg/L)] patients (P = 0.20).

The normal cut-point for IL-1β is <5.0 pg/mL (46). There were 5 patients whose values were above this cut-point. Two adult female patients had considerably high IL-1β values, 20 - 43-fold above the cut-point value. Both were 31-year-old females with values of 58 pg/mL and 414 pg/mL. There was no significant difference in median values between pediatric [0.5 pg/mL (0 – 6.9 pg/mL)] and adult [0.7 pg/mL (0 – 414.0 pg/mL)] patients (P = 0.56).

There was no significant difference between adult [0 pg/mL (0 – 243.0 pg/mL)] and pediatric [0 pg/mL (0 – 0 pg/mL)] IL-2 median plasma concentrations (P = 0.47). Of the 18 pediatric samples measured, all values were 0, or below the sensitivity for the assay. Only 2 of the 13 adult patient (both female) samples had measurable concentrations within the sensitivity of the assay.

As established by the UCDMC Clinical Laboratory, the reference range for IL-6 is 0 – 14 pg/mL. Thirty-one samples were assayed. One female pediatric patient (18-year-old) had a value above the reference range (18 pg/mL), and 2 female adult patients (both 31-years-old) had a value above the reference range (values of 88 and 119 pg/mL). There was no significant difference between adult [0 pg/mL (0 – 18.0 pg/mL)] and pediatric [0 pg/mL (0 – 119.0 pg/mL)] patient medians (P = 0.20).

Although the UCDMC Clinical Laboratory has not established a reference range for IL-8, Berrahmoune et al (47), from a cohort study of biological determinants and reference values, determined reference ranges of 0.57 – 5.54 pg/mL for children and 0.56 – 7.52 pg/mL for adults. While there was no significant difference between adult [20.0 pg/mL (6.2 – 124.0 pg/mL)] and pediatric [15.5 pg/mL (6.5 – 86.0 pg/mL)] patients (P = 0.56), all pediatric and adult patients had IL-8 values above the reference ranges.

The reference ranges utilized for MCP-1 were also established by Berrahmoune et al (47). They are 32.7 – 146.6 pg/mL for children, 43.4 – 156.4 pg/mL for female adults and 29.2 – 138.5 pg/mL for male adults. No significant difference in median concentrations was seen between adult [531.0 pg/mL (198.0 – 1180 pg/mL)] and pediatric [322.5 pg/mL (122.0 – 855.0 pg/mL)] patients (P = 0.12). Ninety-four percent (17 out of 18) of the pediatric patients had values above the reference range, and 100% of the adults had values above the reference ranges.

The reference range for TNF-α is 0 – 22 pg/mL (UCDMC Clinical Laboratory). There was no significant difference between adult [0 pg/mL (0.0 – 97.0 pg/mL)] and pediatric [0.1 pg/mL (0.0 – 2.6 pg/mL)] patients (P = 0.73). All but 2 patients had values within the reference range.

Table 3-3: Vascular Markers and Inflammatory Cytokine Analytes

***Data Reported*:**
Mean ± SD
Median (Range)
Count (n)

	Pediatric	Adult	All	p-value*
BNP, plasma (pg/mL)	61.8 ± 37.4 47.0 (16.0 – 126.0) *15*	131.8 ± 215.1 68.5 (4.0 – 901.0) *16*	97.9 ± 158.3 57.0 (4.0 – 901.0) *31*	0.53
VCAM-1, serum (ng/mL)	988.2 ± 351.7 921.6 (498.8 – 1688.6) *18*	1192.8 ± 931.1 873.9 (421.0 – 3533.2) *11*	1065.8 ± 628.4 898.5 (421.0 – 3533.2) *29*	0.79
ICAM-1, serum (mg/L)	310.9 ± 56.6 313.2 (198.5 – 416.7) *18*	271.5 ± 72.4 268.4 (151.9 – 416.0) *12*	295.1 ± 65.2 302.1 (151.9 – 416.7) *30*	0.10
E-Selectin, serum (mg/L)	133.7 ± 87.3 122.8 (30.4 – 414.7) *18*	68.6 ± 29.5 67.1 (18.7 – 122.2) *11*	109.0 ± 77.3 97.7 (18.7 – 414.7) *29*	**0.01**
P-Selectin, serum (mg/L)	104.3 ± 41.0 92.3 (55.1 – 229.3) *18*	68.3 ± 13.2 65.2 (45.0 – 92.4) *11*	90.7 ± 37.4 81.7 (45.0 – 229.3) *29*	**0.001**
VEGF, serum (ng/L)	5.6 ± 12.9 0 (0 – 47.0) *18*	8.9 ± 13.5 0 (0 – 41.0) *13*	7.0 ± 13.0 0 (0 – 47.0) *31*	0.46
CRP (mg/dL)	N/A 0.2 (<0.1 – 2.5) *17*	N/A 0.4 (<0.1 – 1.4) *18*	N/A 0.2 (<0.1 – 2.5) *35*	0.20
IL-1β (pg/mL)	1.2 ± 1.8 0.5 (0 – 6.9) *18*	37.4 ± 114.2 0.7 (0 – 414.0) *13*	16.4 ± 74.5 0.6 (0 – 414.0) *31*	0.56
IL-2 (pg/mL)	0 ± 0 0 (0 – 0) *18*	19.4 ± 67.2 0 (0 – 243.0) *13*	8.2 ± 43.6 0 (0 – 243.0) *31*	0.47
IL-6 (pg/mL)	1.4 ± 4.3 0 (0 – 119.0) *18*	17.1 ± 38.9 0 (0 – 119.0) *13*	8.0 ± 26.0 0 (0 – 119.0) *31*	0.20
IL-8 (pg/mL)	23.4 ± 20.9 15.5 (6.5 – 86.0) *18*	32.4 ± 32.7 20.0 (6.2 – 124.0) *13*	27.2 ± 26.4 17.0 (6.2 – 124.0) *31*	0.28
MCP-1 (pg/mL)	373.3 ± 191.6 322.5 (122.0 – 855.0) *18*	543.7 ± 300.6 531.0 (198.0 – 1180.0) *13*	444.7 ± 253.5 361.0 (122.0 – 1180.0) *31*	0.12
TNF-α (pg/mL)	0.6 ± 0.9 0.1 (0.0 – 2.6) *18*	15.3 ± 35.2 0 (0 – 97.0) *13*	6.8 ± 23.5 0.1 (0 – 97.0) *31*	0.73

* Pediatric vs, adult p-values were determined by the Mann-Whitney test.

C. Iron Status Measures

Total serum iron, transferrin saturation, and ferritin were lower in the adult patients, whereas total iron binding capacity was higher in adults, compared with pediatric patients—values are presented in **Table 3-4**.

Median total iron concentration was significantly lower in the adult patients [78.0 mcg/dL (51.0 – 216.0 mcg/dL)] compared with pediatric patients [110.0 mcg/dL (47.0 – 249.0 mcg/dL)] (P = 0.04). Seven pediatric (37%) and 3 adult (16%) patients had total iron concentrations above the reference ranges (pediatric 42-135 mcg/dL, adult male 65-198 mcg/dL, adult female 26-170 mcg/dL); no patient had a low total iron concentration. The total iron concentrations in SCA females were higher than the general population where the reference range for females is 26 – 170 mcg/dL (UCDMC Clinical Laboratory).

The reference range for transferrin saturation is 20 – 50% (UCDMC Clinical Laboratory). There was limited data for pediatric transferrin saturation (2/19 patients reported). One pediatric patient had a value well above the reference range (female, 15-years-old receiving transfusions, 96.6%). Ten of 12 adult patients with reported concentrations were within range, and 2 had low values (females, 22- and 23-years-old).

The total iron binding capacity medians were not significantly different between pediatric and adult patients [231.0 mcg/dL (192.0 – 367.0 mcg/dL) vs. 278.0 mcg/dL (197.0 – 416.0 mcg/dL)], (P = 0.1).

The reference ranges for ferritin are 10 - 291 ng/mL for adult females, and 22 – 322 ng/mL for adult males; there are no reference ranges for children (UCDMC Clinical Laboratory). Thirteen of the pediatric patients (72%) had high ferritin values, as high as 13-fold higher than the upper limit of the reference range. A similar trend is seen in the

adults, with 9 out of 16 (56%) above the reference range. The ferritin median values were not significantly different between pediatric [1099.5 ng/mL (54.0 – 4784.0 ng/mL)] and adult [481.5 ng/mL (18.0 – 5825.0 ng/mL)] patients (P = 0.41).

The reference range for transferrin is 192-382 mg/dL (UCDMC Clinical Laboratory) for both adults and children. Fifty-nine percent (59%) of the pediatric patients and 41.2% of the adults had low transferrin concentrations. No patients had values above the reference range. The transferrin medians were also not significantly different between pediatric and adult patients [166.0 mg/dL (138.0 – 264.0 mg/dL) vs. 200.0 mg/dL (142.0 – 299.0 mg/dL)], respectively (P = 0.10).

Table 3-4: Serum Iron Analytes

***Data Reported*:**
Mean ± SD
Median (Range)
Count (n)

	Pediatric	Adult	All	p-value*
Total Iron (mcg/dL)	129.1 ± 54.2 110.0 (47.0 – 249.0) *17*	99.5 ± 52.8 78.0 (51.0 – 216.0) *17*	114.3 54.8 92.0 (47.0 – 249.0) *34*	**0.04**
Total Iron Binding Capacity (mcg/dL)	258.8 ± 54.5 231.0 (192.0 – 367.0) *17*	290.4 ± 92.1 278.0 (197.0 – 416.0) *17*	274.6 ± 75.5 266.5 (192.0 – 416.0) *34*	0.10
Iron Saturation (%)	68.4 ± 28.3 68.4 (40.0 – 96.6) *2*	34.0 ± 24.0 25.9 (13.9 – 97.5) *10*	39.7 ± 28.2 29.5 (13.9 – 97.5) *12*	0.13
Ferritin (ng/mL)	1406.2 ± 1405.7 1099.5 (54.0 – 4784.0) *18*	1540.6 ± 2061.9 481.5 (18.0 – 5825.0) *16*	1469.5 ± 1719.0 861.5 (18.0 – 5825.0) *34*	0.41
Transferrin (mg/dL)	186.2 ± 39.2 166.0 (138.0 – 264.0) *17*	208.9 ± 66.2 200.0 (142.0 – 299.0) *17*	197.5 ± 54.3 191.5 (138.0 – 299.0) *34*	0.10

* Pediatric vs, adult p-values are non-parametric Mann-Whitney test.

D. Other Analytes

The reference range for albumin is 3.5 – 4.8 g/dL (UCDMC Clinical Laboratory). All patients had concentrations within the reference range. The median albumin values were not different between pediatric and adult patients [4.3 g/dL (3.8 – 4.6 g/dL) vs. 4.1 g/dL (3.7 – 4.6 g/dL)], (P = 0.23).

The creatinine reference range is 0.44 – 1.27 mg/dL (UCDMC Clinical Laboratory). The median creatinine levels were significantly different in pediatric [0.4 mg/dL (0.2 – 0.9 mg/dL)] and adult [0.6 mg/dL (0.3 – 1.4 mg/dL)] patients (P = 0.001). Additionally, 33% of pediatric patients and 12% of adult patients had creatinine concentrations below the reference range. The low creatinine concentrations may be attributed to the hyperfiltration typically seen in SCD (48-50). One male adult (58-year-old) had a high creatinine concentration (1.38 mg/dL).

The alkaline-phosphatase (ALP) reference range is 70 – 160 U/L (UCDMC Clinical Laboratory). The median ALP levels were significantly higher in pediatric patients [131.0 U/L (45.0 – 278.0 U/L)] compared with adult patients [73.0 U/L (37.0 – 135.0 U/L)] (P = 0.005). ALP is elevated in children as a whole due to active bone formation, and may account for the higher values in this pediatric group.

Table 3-5: Other Analytes

***Data Reported*:**
Mean ± SD
Median (Range)
Count (n)

	Pediatric	Adult	All	p-value
Albumin (g/dL)	4.2 ± 0.3 4.3 (3.8 – 4.6) *17*	4.1 ± 0.3 4.1 (3.7 – 4.6) *18*	4.2 ± 0.3 4.2 (3.7 – 4.6) *35*	0.23
Creatinine (mg/dL)	0.4 ± 0.2 0.4 (0.2 – 0.9) *18*	0.7 ± 0.3 0.6 (0.3 – 1.4) *17*	0.5 ± 0.2 0.5 (0.2 – 1.4) *35*	**0.001**
Alkaline–Phosphatase (U/L)	141.8 ± 75.4 131.0 (45.0 – 278.0) *17*	75.1 ± 23.9 73.0 (37.0 – 135.0) *17*	108.5 ± 64.7 78.5 (37.0 – 278.0) *35*	**0.005**

* Pediatric vs, adult p-values are non-parametric Mann-Whitney test.

E. CAIM Vascular Markers

Median severity index values were higher in adult patients [7 (1 – 9)] compared with pediatric patients [4 (0 – 7)] ($P < 0.001$), indicating greater severity of morphological damage to the microvasculature of SCA patients Of note, the parameters that demonstrated higher prevalence in adults compared with pediatric patients were diameter, distribution, morphology, sludging, tortuosity, AV ratio, boxcar phenomena, and hemosiderin. The number of comma signs and abnormal velocity were lower in adult than pediatric patients. (Refer to Chapter 5 for a more detailed analysis of the association between age and microvasculopathy in this cohort as well as explanation of the individual microvascular abnormalities.)

Table 3-6: CAIM Vascular Markers

***Data Reported*:**
Mean ± SD
Median (Range)
Count (n)

	Pediatric	Adult	All	p-value
Severity Index	3.8 ± 1.9 4.0 (0.0 – 7.0) *18*	6.5 ± 2.5 7.0 (1.0 – 9.0) *17*	5.1 ± 2.5 6.0 (0.0 – 9.0) *35*	**<0.001**
Number of patients scoring in each of the abnormality categories:				
Abnormal Diameter	*9*	*13*	*22*	
Abnormal Distribution	*12*	*15*	*27*	
Uneven Thickness	*0*	*1*	*1*	
Abnormal Morphology	*2*	*7*	*9*	
Distended Vessels	*0*	*0*	*0*	
Damaged Vessels	*0*	*1*	*1*	
Sludging	*11*	*14*	*25*	
Tortuosity	*5*	*12*	*17*	
Ischemia	*1*	*3*	*4*	
Abnormal AV Ratio	*11*	*16*	*27*	
Micro Aneurysm	*0*	*2*	*2*	
Boxcar Phenomena	*9*	*13*	*22*	
Hemosiderin	*5*	*12*	*17*	
Comma Sign	*1*	*0*	*1*	
Abnormal Velocity	*3*	*1*	*4*	

* Pediatric vs, adult p-values are non-parametric Mann-Whitney test.

D. Discussion:

1. B-Vitamins and Related Analytes

It was determined that there was a significant difference in the homocysteine (P = 0.01) and cysteine (P = 0.03) median values between the pediatric and adult patients. It has been well documented that individuals with vascular disease among the general population, specifically adults, have elevated homocysteine values. Therefore, as much of the morbidity and mortality in SCD is vascular in nature, significantly higher homocysteine values in the adult group, compared with the pediatric group, might be anticipated. As cysteine is one of the products of 1-carbon metabolism with homocysteine as an intermediate analyte, the significant difference demonstrated in elevated homocysteine values in adults could also explain the higher levels of cysteine values in the adult group.

As for plasma PLP, 1 pediatric patient (4-years-old) had a high level (162 nmol/L); no adult patients were above range. No pediatric patients had deficient PLP levels; 3 adults (18% of adults) had a deficient level. Six pediatric and 3 adult patients had marginal status. These percentages of low and marginal status findings are lower than reported in other studies (27), which may be due to a small sample size. RBC PLP and plasma PL were not significantly different between the pediatric and adult patients, (P = 0.09) and (P = 0.07), respectively.

All but 1 patient had vitamin B12 levels within the reference range of 211 – 911 pg/mL (UCDMC Clinical Laboratory); one adult had an out-of-range value of 1744 pg/mL (nearly 2 times the upper range). This may be due to unreported supplement use.

2. Vascular Markers and Inflammatory Cytokine Analytes

Of the circulating vascular adhesion molecules, sE-selectin and sP-selectin median values were significantly different between pediatric and adult patients, P = 0.01 and P = 0.001, respectively. The levels of vascular adhesion molecules, sVCAM-1 and sICAM-1, were not significantly different; however, sVCAM-1 levels were significantly different between the genders (P = 0.05).

Lower sVCAM-1 and sICAM-1 have been reported in SCD patients receiving hydroxyurea treatment (51-53). In this cohort, 47.4% adult patients (9 out of 19), but only 10% pediatric patients (2 out of 19), were receiving hydroxyurea treatment. Nonetheless, no significant differences in sVCAM-1 and sICAM-1 were seen between pediatric and adult patients. The higher percentage of hydroxyurea treatment in adult patients, may explain why we do not see higher sVCAM-1 and sICAM-1 levels compared with the pediatric patients.

sE-selectin has been shown to be elevated in SCD patients and its levels are correlated with the severity of the condition (vascular inflammation) (53). However differences in sE-selectin levels in adults and children with SCD have not previously been reported. Interestingly, as age progresses, the median value for SCD adult patients approaches that of the healthy population.

As with sE-selectin, sP-selectin levels are elevated with endothelial and platelet activation, and vascular damage. This has been reported in the general population and in SCD patients. In this study's SCA cohort, sP-selectin levels were significantly different between the pediatric patients and the adult patients (P = 0.001). It has also been demonstrated that elevated sP-selectin levels in SCD are correlated with elevated VCAM-1 levels (54) and elevated VEGF levels (55). Interestingly, the sP-selectin levels in these adult patients were lower than those in the pediatric patients.

Following endothelial activation, MCP-1 is upregulated to recruit monocytes to damaged tissues. In addition to this study, elevated levels of MCP-1 have been observed in SCD patients (56, 57), and in patients with metabolic syndrome (58), hemodialysis patients (59) and in atherosclerosis (60). MCP-1 median values were higher in adult patients than in pediatric patients, though not significantly (P = 0.12).

Although not significant, the tendency toward higher values of MCP-1 in the adult patients could be correlated with disease progression of SCD and accumulated vascular damage, and may attain significance with larger sample sizes. The median and reference ranges for MCP-1 established by Berrahmoune et al (47) for males <18 years are 78.9 pg/mL (53.7–115.8 pg/mL), for females <18 years are 71.8 pg/mL (50.7–101.6 pg/mL), for adult males are 95.7 pg/mL (68.2–134.2 pg/mL), and for adult females are 77.5 pg/mL (52.7–113.9 pg/mL). Our patient medians, both pediatric and adult, were higher than the established reference ranges.

The growth factor VEGF was not significantly different between the pediatric patients and adult patients in this study. Qari et al (56) demonstrated that SCD patients

have a sustained increase of 2- to 3-fold during painful crisis and steady-state compared with normal controls.

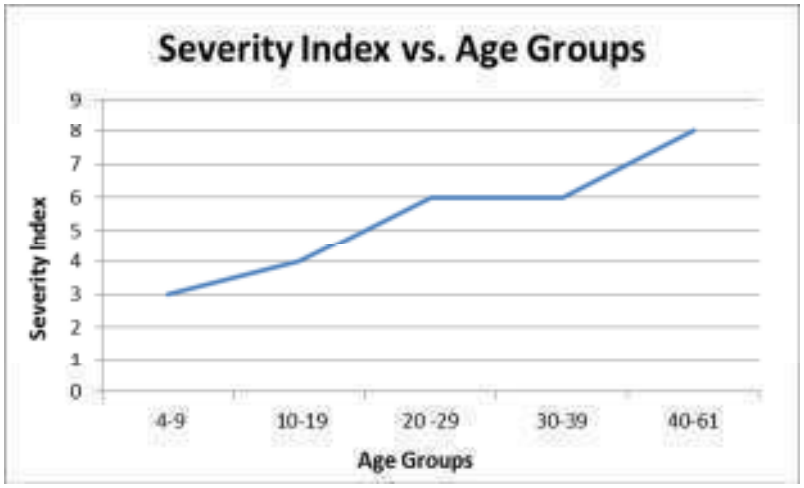
3. Iron analytes

Among the iron analytes, pediatric total iron levels were greater than the adults. The significantly higher pediatric patient values (P = 0.04) are potentially due to the greater frequency of blood transfusion within the pediatric group compared with the adult group. Total iron binding capacity (P = 0.10), iron saturation (P = 0.13) and transferrin values (P = 0.10) were not significantly different between the pediatric and adult groups.

4. Severity Index

As in the general population, and specifically those with SCD, the severity index was significantly higher in the adult patients (P <0.001) (**Figure 3-5**). This indicates that morphological damage to the microvasculature increases with age in SCD. (See Chapter 5 for further discussion on this point.)

Figure 3-5: Median Severity Index vs. Age Groups



5. Other analytes

Of the other analytes, a significant difference was observed between pediatric and adult medians for creatinine (P = 0.001) and ALP (P = 0.005). Creatinine values are reflective of renal function, and it has been well documented that SCD patients experience accumulated renal damage with age. Lower ALP levels in the adult compared with the pediatric patients has been observed in other SCD cohorts and may in part reflect the cessation of bone growth in adulthood (61, 62).

In this cohort and other studies, patients with SCD develop vascular complications at an early age (63, 64). However, it has not been previously demonstrated if there are differences between pediatric and adult SCD patients. In the following chapter, associations between the blood biomarkers and real-time microvascular abnormalities are assessed in the SCD patients to determine whether there are biomarkers which could give insight into the development of vascular complications, disease severity and progress. The identification of such biomarkers could potentially identify treatment targets for early intervention.

E. References

1. Swerlick RA EJ, Kumar A, Jeitler M, Wick TM. *Alpha 4 beta 1-integrin expression on sickle reticulocytes: vascular cell adhesion molecule-1-dependent binding to endothelium*. Blood 1993;82(6):1891-9.
2. Kumar A, Eckmam JR, Swerlick RA, Wick TM. *Phorbol ester stimulation increases sickle erythrocyte adherence to endothelium: a novel pathway involving alpha 4 beta 1 integrin receptors on sickle reticulocytes and fibronectin*. Blood 1996;88(11):4348-58.
3. Hebbel RP, Osarogiagbon R, Kaul D. *The Endothelial Biology of Sickle Cell Disease: Inflammation and a Chronic Vasculopathy*. Microcirculation 2004;11(2):129-51.
4. Bunn HF. *Pathogenesis and Treatment of Sickle Cell Disease*. New England Journal of Medicine 1997;337(11):762-9.
5. Natarajan M, Udden MM, McIntire LV. *Adhesion of sickle red blood cells and damage to interleukin-1 beta stimulated endothelial cells under flow in vitro*. Blood 1996;87(11):4845-52.
6. Claster S, Wood JC, Noetzli L, Carson SM, Hofstra TC, Khanna R, Coates TD. *Nutritional deficiencies in iron overloaded patients with hemoglobinopathies*. Am J Hematol 2009;84(6):344-8.
7. Segal JB, Miller ER, 3rd, Brereton NH, Resar LM. *Concentrations of B vitamins and homocysteine in children with sickle cell anemia*. South Med J 2004;97(2):149-55.
8. al-Momen A. *Diminished vitamin B12 levels in patients with severe sickle cell disease*. J Intern Med 1995;237(6):551-5.
9. Carmel R, Bellevue R, Kelman Z. *Low cobalamin levels associated with sickle cell disease: Contrasting origins and clinical meanings in two instructive patients*. Am J Hematol 2010;85(6):436-9.
10. Charney E, Miller G. *Reticulocytopenia In Sickle Cell Disease. Aplastic Episodes In The Course Of Sickle Cell Disease In Children*. Am J Dis Child 1964;107:450-5.
11. Harrison KA. *Anaemia, malaria and sickle cell disease*. Clin Obstet Gynaecol 1982;9(3):445-77.
12. Nkrumah FK, Neequaye JE, Ankra-Badu G. *Bone marrow in sickle cell anaemia at time of anaemic crisis*. Arch Dis Child 1984;59(6):561-5.
13. Brody T, Shane B. *Handbook of Vitamins*. Third ed, 2001; 427-462.
14. Fox JT, Stover PJ. *Folate-mediated one-carbon metabolism*. Vitam Horm 2008;79:1-44.
15. Lowenthal EA, Mayo MS, Cornwell PE, Thornley-Brown D. *Homocysteine elevation in sickle cell disease*. J Am Coll Nutr 2000;19(5):608-12.
16. Houston PE, Rana S, Sekhsaria S, Perlin E, Kim KS, Castro OL. *Homocysteine in sickle cell disease: relationship to stroke*. Am J Med 1997;103(3):192-6.

17. Rana S, Houston P, Castro OL. *Homocysteine levels and sickle cell anemia*. J Pediatr Hematol Oncol 2000;22(2):185; author reply -6.

18. Collaboration HS. *Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis*. JAMA 2002;288(16):2015-22.

19. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. *A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes*. JAMA 1995;274(13):1049-57.

20. Choy PC, Mymin D, Zhu Q, Dakshinamurti K, O K. *Atherosclerosis risk factors: the possible role of homocysteine*. Mol Cell Biochem 2000;207(1-2):143-8.

21. Miller J, Green R, Mungas D, Reed B, Jagust W. *Homocysteine, vitamin B6, and vascular disease in AD patients*. Neurology 2002;58:1471-5.

22. Refsum H, Ueland PM, Nygard O, Vollset SE. *Homocysteine and cardiovascular disease*. Annu Rev Med 1998;49:31-62.

23. Robinson K, Arheart K, Refsum H, Brattstrom L, Boers G, Ueland P, Rubba P, Palma-Reis R, Meleady R, Daly L, Witteman J, Graham I. *Low circulating folate and vitamin B6 concentrations: risk factors for stroke, peripheral vascular disease, and coronary artery disease. European COMAC Group*. Circulation 1998;97(5):437-43.

24. van der Dijs FP, Schnog JJ, Brouwer DA, Velvis HJ, van den Berg GA, Bakker AJ, Duits AJ, Muskiet FD, Muskiet FA. *Elevated homocysteine levels indicate suboptimal folate status in pediatric sickle cell patients*. Am J Hematol 1998;59(3):192-8.

25. Balasa BV, Kalinyak KA, Bean JA SD, Gruppo RA. *Hyperhomocysteinemia is associated with low plasma pyridoxine levels in children with sickle cell disease*. J Pediatr Hematol Oncol 2002;24:374-9.

26. Natta C, Reynolds R. *Apparent vitamin B6 deficiency in sickle cell anemia.* Am J Clin Nutr 1984;40:235-9.

27. Nelson MC ZB, Kawchak DA, Barden EM, Frongillo EA Jr, Coburn SP, Ohene-Frempong K, Stallings VA. *Vitamin B6 status of children with sickle cell disease.* J Pediatr Hematol Oncol 2002;26:24(6):463-9.

28. Miller JW, Medina MV, Wun T, Green R. *Plasma pyridoxal-5'-phosphate is inversely correlated with VCAM levels in adult sickle cell disease patients*. FASEB J, 2004.

29. Friso S, Girelli D, Martinelli N, Olivieri O, Lotto V, Bozzini C, Pizzolo F, Faccini G, Beltrame F, Corrocher R. *Low plasma vitamin B-6 concentrations and modulation of coronary artery disease risk*. The American journal of clinical nutrition 2004;79(6):992-8.

30. Friso S, Jacques PF, Wilson PW, Rosenberg IH, Selhub J. *Low circulating vitamin B(6) is associated with elevation of the inflammation marker C-reactive protein independently of plasma homocysteine levels*. Circulation 2001;103(23):2788-91.

31. Cheung AT, Harmatz P, Wun T, Chen PC, Larkin EC, Adams RJ, Vichinsky EP. *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 2001;97(11):3401-4.

32. Cheung AT, Chen PC, Larkin EC, Duong PL, Ramanujam S, Tablin F, Wun T. *Microvascular abnormalities in sickle cell disease: a computer-assisted intravital microscopy study*. Blood 2002;99(11):3999-4005.

33. Cheung AT, Price AR, Duong PL, Ramanujam S, Gut J, Larkin EC, Chen PC, Wilson DM. *Microvascular abnormalities in pediatric diabetic patients*. Microvasc Res 2002;63(3):252-8.

34. Cheung AT, Chan MS, Ramanujam S, Rangaswami A, Curl K, Franklin P, Wun T. *Effects of poloxamer 188 treatment on sickle cell vaso-occlusive crisis: computer-assisted intravital microscopy study*. J Investig Med 2004;52(6): 402-6.

35. Cheung AT MJ, Craig SM, To PL, Lin X, Samarron SL, Chen PC, Zwerdling T, Wun T, Li CS, Green R. *Comparison of real-time microvascular abnormalities in pediatric and adult sickle cell anemia patients*. Am J Hematol 2010;85(11):899-901.

36. Marszałł ML LA, Czarnowski W, Makarowski R, Kłos M, Szefer P. *Application of the high-performance liquid chromatography method with coulometric detection for determination of vitamin B(6) in human plasma and serum.* J Chromatogr B Analyt Technol Biomed Life Sci 2009;877(27):3151-8.

37. Talwar D, Quasim T, McMillan DC, Kinsella J, Williamson C, O'Reilly DS. *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*. J Chromatogr B Analyt Technol Biomed Life Sci 2003;792(2):333-43.

38. Gregory JF, 3rd, Kirk JR. *Determination of urinary 4-pyridoxic acid using high performance liquid chromatography*. The American journal of clinical nutrition 1979;32(4):879-83.

39. Zhang X, He Y, Ding M. *Simultaneous determination of tryptophan and kynurenine in plasma samples of children patients with Kawasaki disease by high-performance liquid chromatography with programmed wavelength ultraviolet detection*. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877(16-17):1678-82.

40. Berthoud TK, Manaca MN, Quelhas D, Aguilar R, Guinovart C, Puyol L, Barbosa A, Alonso PL, Dobano C. *Comparison of commercial kits to measure cytokine responses to Plasmodium falciparum by multiplex microsphere suspension array technology*. Malar J 2011;10:115.

41. Bunger S, Haug U, Kelly FM, Klempt-Giessing K, Cartwright A, Posorski N, Dibbelt L, Fitzgerald SP, Bruch HP, Roblick UJ, von Eggeling F, Brenner H, Habermann JK. *Toward standardized high-throughput serum diagnostics: multiplex-protein array identifies IL-8 and VEGF as serum markers for colon cancer*. J Biomol Screen 2011;16(9):1018-26.

42. Devaraj S, Cheung AT, Jialal I, Griffen SC, Nguyen D, Glaser N, Aoki T. *Evidence of increased inflammation and microcirculatory abnormalities in patients with type 1 diabetes and their role in microvascular complications*. Diabetes 2007;56(11):2790-6.

43. Cheung A, Tomic M, Chen P, Miguelino E, Li C, Devaraj S. *Correlation of microvascular abnormalities and endothelial dysfunction in Type-1 Diabetes Mellitus (T1DM): a real-time intravital microscopy study*. Clin Hemorheol Microcirc 2009;42(4):285-95.

44. Smith MM, Chen PC, Li CS, Ramanujam S, Cheung AT. *Whole blood viscosity and microvascular abnormalities in Alzheimer's Disease*. Clin Hemorheol Microcirc 2009;41(4):229-39.

45. Ponthieux A, Herbeth B, Droesch S, Lambert D, Visvikis S. *Age- and Sex-related Reference Values for Serum Adhesion Molecule Concentrations in Healthy Individuals: Intercellular Adhesion Molecule-1 and E-, P-, and L-Selectin*. Clinical Chemistry 2003;49(9):1544-46.

46. *ARUP Laboratories User's Guide*. 2004 - 2005 ed: Associated Regional & Univristy Pathologists, Inc., Salt Lake City, Utah, 2004.

47. Berrahmoune H, Lamont JV, Herbeth B, FitzGerald PS, Visvikis-Siest S. *Biological determinants of and reference values for plasma interleukin-8, monocyte chemoattractant protein-1, epidermal growth factor, and vascular endothelial growth factor: Results from the STANISLAS cohort*. Clin Chem 2006;52(3):504-10.

48. Arlet J-B, Ribeil J-A, Chatellier G, Eladari D, De Seigneux S, Souberbielle J-C, Friedlander G, de Montalembert M, Pouchot J, Prie D, Courbebaisse M. *Determination of the best method to estimate glomerular filtration rate from serum creatinine in adult patients with sickle cell disease: a prospective observational cohort study*. BMC Nephrology 2012;13(1):83.

49. Becker AM. *Sickle cell nephropathy: challenging the conventional wisdom*. Pediatr Nephrol 2011;26(12):2099-109.

50. Wigfall DR, Ware RE, Burchinal MR, Kinney TR, Foreman JW. *Prevalence and clinical correlates of glomerulopathy in children with sickle cell disease*. J Pediatr 2000;136(6):749-53.

51. Gladwin MT, Shelhamer JH, Ognibene FP, Pease-Fye ME, Nichols JS, Link B, Patel DB, Jankowski MA, Pannell LK, Schechter AN, Rodgers GP. *Nitric oxide donor properties of hydroxyurea in patients with sickle cell disease*. Br J Haematol 2002;116(2):436-44.

52. Conran N, Fattori A, Saad ST, Costa FF. *Increased levels of soluble ICAM-1 in the plasma of sickle cell patients are reversed by hydroxyurea*. Am J Hematol 2004;76(4):343-7.

53. Kato GJ, Martyr S, Blackwelder WC, Nichols JS, Coles WA, Hunter LA, Brennan ML, Hazen SL, Gladwin MT. *Levels of soluble endothelium-derived adhesion molecules in patients with sickle cell disease are associated with pulmonary hypertension, organ dysfunction, and mortality*. Br J Haematol 2005;130(6):943-53.

54. Klings ES, Anton Bland D, Rosenman D, Princeton S, Odhiambo A, Li G, Bernard SA, Steinberg MH, Farber HW. *Pulmonary arterial hypertension and left-sided heart disease in sickle cell disease: clinical characteristics and association with soluble adhesion molecule expression*. Am J Hematol 2008;83(7):547-53.

55. Blann AD, Mohan JS, Bareford D, Lip GY. *Soluble P-selectin and vascular endothelial growth factor in steady state sickle cell disease: relationship to genotype*. J Thromb Thrombolysis 2008;25(2):185-9.

56. Qari MH, Dier U, Mousa SA. *Biomarkers of inflammation, growth factor, and coagulation activation in patients with sickle cell disease*. Clin Appl Thromb Hemost 2012;18(2):195-200.

57. Selvaraj SK, Giri RK, Perelman N, Johnson C, Malik P, Kalra VK. *Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor*. Blood 2003;102(4):1515-24.

58. Valenti L, Dongiovanni P, Motta BM, Swinkels DW, Bonara P, Rametta R, Burdick L, Frugoni C, Fracanzani AL, Fargion S. *Serum hepcidin and macrophage iron correlate with MCP-1 release and vascular damage in patients with metabolic syndrome alterations*. Arterioscler Thromb Vasc Biol 2011;31(3):683-90.

59. Papayianni A, Alexopoulos E, Giamalis P, Gionanlis L, Belechri A-M, Koukoudis P, Memmos D. *Circulating levels of ICAM-1, VCAM-1, and MCP-1 are increased in haemodialysis patients: association with inflammation, dyslipidaemia, and vascular events*. Nephrol Dial Transplant 2002;17:432-41.

60. Schwartz CJ, Valente AJ, Sprague EA, Kelley JL, Nerem RM. *The pathogenesis of atherosclerosis: an overview*. Clin Cardiol 1991;14(2 Suppl 1):I1-16.

61. Shastri S, Tangri N, Tighiouart H, Beck GJ, Vlagopoulos P, Ornt D, Eknoyan G, Kusek JW, Herzog C, Cheung AK, Sarnak MJ. *Predictors of sudden cardiac death: a competing risk approach in the hemodialysis study*. Clin J Am Soc Nephrol 2012;7(1):123-30.

62. Mohammed SM, Suleiman SA, Addae SK, Annobil SH, Adzaku FK, Kadoummi OF, Richards JT. *Urinary hydroxyproline and serum alkaline phosphatase in sickle cell disease*. Clin Chim Acta 1991;203(2-3):285-94.

63. Ranthe MF, Winkel BG, Andersen EW, Risgaard B, Wohlfahrt J, Bundgaard H, Haunso S, Melbye M, Tfelt-Hansen J, Boyd HA. *Risk of cardiovascular disease in family members of young sudden cardiac death victims*. Eur Heart J 2012.

64. Gladwin MT, Sachdev V. *Cardiovascular abnormalities in sickle cell disease*. J Am Coll Cardiol 2012;59(13):1123-33.

Chapter 4: Cross-Sectional Relationships Between Biomarkers of B Vitamin Status, Inflammation, Vascular Adhesion, and Microvascular Characteristics in Sickle Cell Anemia Patients Under Steady-State Conditions

A. Background

Sickle cell disease results from a single DNA base mutation and is characterized by a myriad of clinical consequences (1). Acute clinical manifestations of SCD include painful crisis, acute chest syndrome, priapism, and stroke. The 1998 Cooperative Study of Sickle Cell Disease (CSSCD) reported that patients who were homozygous for S genotype (HbSS) had the chances of having a first cerebrovascular accident by 20, 30, and 45 years of age of 11%, 15%, and 24%, respectively (2). However, in the CSSCD post-trial follow-up study on their low-risk group who had experienced a cerebrovascular event, they could not identify any predictive cerebrovascular biomarkers (3). Additionally, a significant proportion of children with HbSS who have not had a clinically apparent neurologic event have had silent cerebral infarctions based on magnetic resonance imaging (4). There have been very few, if any, clinical biomarkers identified in SCD patients that provide specific prognostic or clinical information (5).

With significantly improved treatment, the life expectancy and quality of life of SCD patients has improved considerably since 1960 (6, 7). Nonetheless, SCD patients have significantly lower life expectancy compared with age-, sex-, and ethnic-matched control groups (6, 7). It is well understood that the prevalence and severity in phenotypic expression of SCD varies greatly; however, the determinants are still not well defined (8-13).

Cerebrovascular disease significantly affects cognitive functioning and academic attainment in SCD children (14). Irreversible major organ damage to the heart, lungs, kidneys, eyes, spleen, and femoral heads also occurs (12, 15). While anemia accounts for some of the morbidity in SCD, microvascular abnormalities underlie many of the serious acute and chronic complications (1, 16-20).

Current experimental evidence suggests that adherence of sickle erythrocytes (sRBC) to the microvascular endothelium, mediated by vascular adhesion molecules and other cytokines, is the initiating event in sickle cell vaso-occlusion (21-24). The resulting decreased blood flow leads to local hypoxemia, hemoglobin polymerization, and subsequent further red cell sickling (25). Persistent adherence of sRBC to activated endothelium, with recurrent episodes of ischemia/reperfusion injury, eventually results in occlusion and permanent vascular damage (26).

Several investigative teams have found a high prevalence of vitamin B6 deficiency (exceeding 50%) in SCD patients (27-30). It has also been observed that vitamin B6 status in SCD patients correlates with circulating levels of vascular adhesion molecules (indicative of endothelial activation) (30), and hyperhomocysteinemia, defined as fasting homocysteine levels greater than the 95th percentile of similarly aged controls (27). Thus, vitamin B6 deficiency may be a potential factor contributing to sRBC and leukocyte adhesion with consequent vaso-occlusion and vasculopathy (30).

Dhar et al (31) found that mild hyperhomocysteinemia (>13.8 mmol/L in men and >12.5 mmol/L in women) in adult patients with SCD was a common finding and unrelated to folate and cobalamin status. Furthermore, they noted that SCD patients had homocysteine levels that correlated directly with serum creatinine ($P < 0.0001$), and

age (P = 0.02). Their logistic regression analysis only showed creatinine as an independent predictor of homocysteine levels.

In contrast to the above, Houston et al (32) showed conflicting results as they found homocysteine levels and folate levels were inversely correlated (P < 0.00005), and that high homocysteine (>10.1 μmol/L) levels may be a risk factor for development of stroke in SCD patients. Furthermore, Kennedy et al (33) found that more than half of their SCD subjects had inadequate folate intake, in spite of daily folate supplementation, and 15% had low RBC folate levels.

As for vitamin B12, Kamineni et al (34) found 68% of SCD patients had B12 levels below 200 pg/mL (mean and SD: 496 ± 352 pg/mL) with a reference range of 200-950 pg/ml, compared with non-SCD patients (36%) (mean and SD: 869 ± 660 pg/ml, P < 0.0001), and that SCD patients with low B12 were younger. To the contrary, Kennedy et al (33) demonstrated in SCD children that low serum B12 levels were rare, and dietary B12 intake was adequate.

In a study on B-vitamin supplementation and correlation with homocysteine levels in SCD pediatric patients, van der Dijs et al (35) determined that folate supplementation was associated with a 53% reduction in homocysteine levels, but 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 (36) utilized homocysteine as a biomarker to determine optimal folate, B12 and B6 doses. They determined an optimal daily combination of 1 mg of folic acid, 6 μg of B12 and 6 mg of B6 induced a significant 12.2% homocysteine decrease in 43% of their patients.

B. Objectives

The objective of this study was to determine the cross-sectional associations between biomarkers of B vitamin status (including vitamin B6, folate, and vitamin B12), inflammation, vascular adhesion, and microvascular characteristics in SCD subjects under steady-state conditions. The overall goal was to identify potentially modifiable biomarkers that could assist in evaluating future risk of microvasculopathy and could be used to monitor therapy aimed at ameliorating vascular damage.

C. Methods:

1. Subjects Recruitment

Subject recruitment and procedures for the study were approved by the Office of Research Institutional Review Board (IRB) Administration at the University of California, Davis, and written informed consent was obtained from all study participants. Subjects diagnosed with SCD (HbSS and HbSC genotype) and greater than 4 years of age were recruited from the Pediatric and Adult Sickle Cell Clinics at the University of California, Davis Medical Center (UCDMC) during outpatient clinic visits, or local SCD symposiums. The methods and procedures for subject recruitment are presented in detail in Chapter 3 of this thesis.

2. Sample Processing and Analysis

Fasting blood of ~15mL was collected while the patient was free of acute complications into plasma (EDTA) and serum (SST) tubes from each subject. Immediately (within 30 minutes) following blood collection, subjects had their bulbar conjunctiva visualized and recorded via Computer-Assisted Intravital Microscopy (CAIM)

as previously described (19). A severity index (SI) was computed to quantify the degree of microvasculopathy in each subject (19). The sample processing and analysis procedures are presented in detail in Chapter 3 of this thesis.

The UCDMC Department of Pathology and Laboratory Medicine Clinical Laboratory processed and measured the following serum and plasma analytes by standardized clinical procedures: beta-human chorionic gonadotropin (serum β-hCG) by chemiluminescent immunoassay; plasma complete blood count (CBC) and automated differential by automated cell count with flow cell differential; creatinine and albumin by spectrophotometry; alkaline phosphatase by kinetic rate reaction (p-nitrophenyl phosphate); total iron, total iron binding capacity (TIBC), and transferrin by turbidimetric assay; iron saturation by spectrophotometry; ferritin by chemiluminometric immunoassay; C-reactive protein by immunoassay (near infrared particle immunoassay rate); serum folate and vitamin B12 by SIEMENS ADVIA Centaur Competitive Chemiluminescent Immunoassay; and plasma BNP by immunoenzymatic assay.

The remainder of the blood was transported to the UCDMC Pathology Research laboratory for further processing. A portion of the whole blood was aliquoted from plasma EDTA tubes and stored frozen at –80 °C for later analysis. The remainder of the EDTA whole blood and additional tubes of whole blood collected without anticoagulant were centrifuged at 2,200 rpm for 10 minutes. The plasma and serum supernatants were then aliquoted and stored at –80 °C for later analysis. Plasma concentrations of VCAM-1 were measured by commercial ELISA assays (R & D, Minneapolis, MN). Plasma homocysteine was determined by high-performance liquid chromatography (HPLC) with post-column fluorescence detection by the method of Gilfix et al (37) Plasma and RBC PLP and PL concentrations were determined by high-performance

liquid chromatography (HPLC) by the method of Talwar et al (2003) (38, 39), plasma 4-PA by the HPLC method described by Gregory et al (1979) (40), and plasma kynurenine and tryptophan by the HPLC method described by Zheng et al (2009) (41).

Cytokines (MCP-1, VEGF, IL-1β, IL-2, IL-6, IL-8 and TNF-α) and adhesion molecules (sICAM, sP-selectin and sE-selectin) were measured by Invitrogen human multiplex assay.

3. Statistical Analysis

Associations between independent variables and severity index were assessed by multiple linear regression. The baseline model included only age and sex. Subsequent models assessed the association of each independent variable with severity index 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. Some independent variables that were not normally distributed were natural log transformed before inclusion in the model. Nominal variables, including transfusions and hydroxyurea treatment, were assigned values of 0 (no transfusions or hydroxyurea) or 1 (yes transfusions or hydroxyurea) and then treated as continuous variables in the multiple regression analyses. Statistical significance was defined as $P < 0.05$.

D. Results:

1. Age and Sex

Age was a strong determinant of severity index, with severity index increasing about 0.1 unit per 1 year increase in age (**Table 4-1**). Sex was not significantly

associated with severity index. Together, age and sex explained 43.8% (R^2=0.438) of the variance in severity index among all SCA patients in the cohort. (Refer to Chapter 5 of this thesis for a more detailed analysis of the association between age and microvasculopathy in this cohort.)

Table 4-1: Predictors of Severity Index in SCA Patients: Age and Sex*

Independent Variable	N	Coeff.	P-value
Age	35	0.104	**<0.001**
Sex	35	-0.544	0.420

* R^2 for regression model including age and sex is 0.438

2. B Vitamins, Homocysteine, Cysteine, and Creatinine

Among the B vitamins and related analytes, the only variable that was significantly associated with severity index after controlling for age and sex was ln homocysteine (**Table 4-2).** Addition of ln homocysteine to the regression model explained an additional 6.8% of the variance in severity index over the model containing only age and sex (**Figure 4-1**). Also, addition of determinants of homocysteine (B vitamins, cysteine, or creatinine) to the model containing ln homocysteine did not change the ln homocysteine coefficient by more than 10%, thus indicating that the ln homocysteine association was independent of known determinants of homocysteine.

The distribution of homocysteine values was skewed to the right. The distribution was normalized by natural log transformation. This is typical for homocysteine in such studies. Some of the other independent variables had skewed distributions, but natural log transformation did not change the strength of the association with severity index (**Table 4-2**).

Figure 4-1: Homocysteine vs. Severity Index

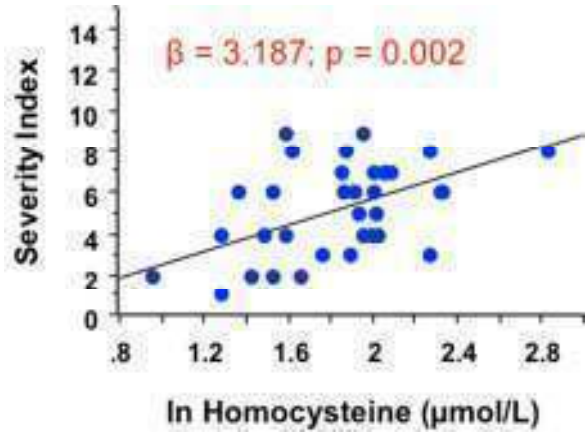


Table 4-2: Predictors of Severity Index in SCA Patients: B Vitamins, Homocysteine, Cysteine, and Creatinine*

Independent Variable	N	Coeff.	P-value	ΔR^{2}**
PLP	34	-0.013	0.180	0.033
Pyridoxal	31	-0.002	0.795	0.002
Folate	34	-0.047	0.443	0.009
B12	34	-0.001	0.548	0.007
Homocysteine	35	0.242	0.180	0.032
ln Homocysteine	35	2.376	**0.047**	**0.068**
Cysteine	35	0.007	0.403	0.012
Creatinine	34	0.848	0.702	0.003

*All models 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.

3. Vascular and Inflammation Biomarkers

The only vascular and inflammatory biomarker variable that was significantly associated with severity index after controlling for age and sex was ln MCP-1 (**Table 4-3**) (**Figure 4-2).** Addition of ln MCP-1 to the regression model explained an additional 9.6% of the variance in severity index over the model containing only age and sex.

The distribution of MCP-1 values was skewed to the right. The distribution was normalized by natural log transformation. Some of the other independent variables had skewed distributions, but natural log transformation did not change the strength of their associations with severity index.

Figure 4-2: MCP-1 vs. Severity Index

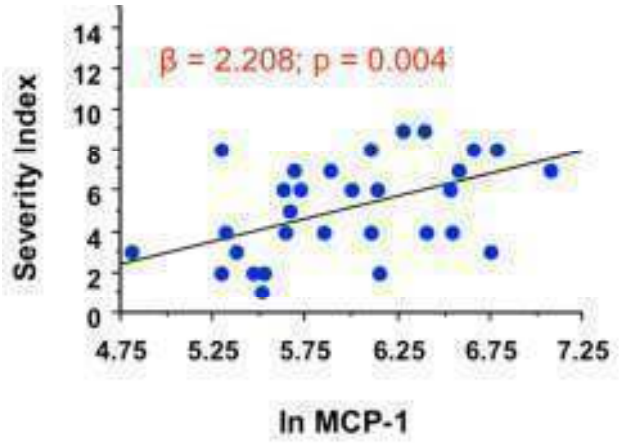


Table 4-3: Predictors of Severity Index in SCA Patients: Vascular and Inflammation Biomarkers*

Independent Variable	N	Coeff.	P-value	ΔR^{2}**
BNP	30	0.002	0.571	0.007
VCAM	29	-0.0001	0.902	0
ICAM	30	-0.008	0.207	0.035
E-Selectin	29	0.001	0.789	0.002
P-Selectin	29	-0.003	0.800	0.001
CRP	34	0.046	0.946	0
IL-8	31	0.010	0.480	0.010
MCP-1	31	0.003	0.059	0.070
ln MCP-1	31	1.456	**0.026**	**0.096**

*All models 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.

4. Iron Status

None of the iron status biomarkers was significantly associated with severity index after controlling for age and sex (**Table 4-4**). Some of the iron status biomarkers had skewed distributions, but natural log transformation did not change the significance of their associations with severity index.

Table 4-4: Predictors of Severity Index in SCA Patients: Iron Status*

Independent Variable	N	Coeff.	P-value	ΔR^{2**}
Total Iron	33	-0.001	0.898	0
Transferrin	33	-0.002	0.810	0.001
TIBC	33	-0.001	0.816	0.001
Ferritin	33	0.0003	0.139	0.042

*All models 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.

5. Other Variables

Albumin, alkaline phosphatase, regular transfusions (approximately monthly), and treatment with hydroxyurea were not significantly associated with severity index after controlling for age and sex (**Table 4-5**). The distribution of alkaline phosphatase was skewed to the right. Natural log transformation did not change the significance of its associations with severity index.

Table 4-5: Predictors of Severity Index in SCA Patients: Other Variables*

Independent Variable	N	Coeff.	P-value	ΔR^{2**}
Albumin	34	-0.502	0.702	0.003
Alkaline Phosphatase	33	0.003	0.624	0.004
Transfusions[a]	35	1.304	0.135	0.039
Hydroxyurea[b]	35	-0.043	0.956	0

*All models 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.

[a]Subjects transfused approximately monthly (yes or no)

[b]Subjects on hydroxyurea treatment (yes or no)

As indicated in chapter 3 of this thesis, several other analytes have been measured within this cohort. However, none of these variables have been analyzed in multiple regression analyses because of low sample sizes and/or large numbers of subjects with zero values. These variables include: RBC pyridoxal-5'-phosphate, RBC pyridoxal, 4-pyridoxic acid, kynurenine, tryptophan, IL-1, IL-2, IL-6, TNF-alpha, and VEGF.

E. Discussion

The multiple regression analyses revealed three predictive factors for microvasculopathy, as indicated by the CAIM severity index scores, in the SCA patients. These include age, homocysteine, and MCP-1. Homocysteine and MCP-1 are reviewed below, and a detailed analysis and interpretation of the association with age and severity index are presented in Chapter 5.

1. Homocysteine Association

It is well documented and recognized that hyperhomocysteinemia is an independent risk factor for vascular disease in the general population (42-46). There is a consensus within the literature that plasma homocysteine >15.0 μmol/L is associated with increased risk for cardiovascular events (44, 47-50). Additionally, a 5 μmol/L increase in homocysteine is associated with a 60% increase in cardiovascular mortality in the general population (51).

At present, the mechanistic role of homocysteine in the pathogenesis of CVD, if any, is poorly understood and is still to be determined in the general population, as well as the SCD population. It has been proposed that homocysteine binds to LDL, inducing a high level of reactive oxygen species, resulting in reduced endothelial cell proliferation and viability (52). An additional hypothesis is that homocysteine has a thrombogenic effect on clotting factors and endothelial cells, or their interactions (32).

There are few studies on hyperhomocysteinemia in SCD patients. Dhar et al (31) 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 control subjects (20% vs. 3%, respectively, $P < 0.001$). Moreover, Lowenthal et al (53) found

similar results, with ~1.5-fold higher homocysteine levels (>13.5 μmol/L in adults) than that of controls ($P < 0.001$). In addition, Lowenthal et al found SCD patients had 1.5-fold higher folate levels ($P < 0.05$), but no difference in B12, compared with controls.

In a study of SCD children by Balasa et al (27), they found 38% of SCD 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 (27, 54, 55) and inadequate dietary intake of folate (33). Balasa et al (27) additionally found that hyperhomocysteinemia was more common in those with B6 deficiency (62%) than those with normal B6 levels (30%).

In the present study, we found folate status, creatinine levels, age and sex, but not B12 or B6, were determinants of homocysteine levels. Studies of B vitamin supplementation, as a therapeutic intervention for lowering homocysteine levels to reduce vascular disease risk, have been conducted in the general population (56-59). There is a consensus among the studies that B vitamin supplementation lowers homocysteine levels, but does not reduce or improve cardiovascular outcomes (48, 56, 60-64). Interestingly, studies thus far have been conducted on subjects who had previously presented with vascular events (48, 57, 60). At present, no intervention studies have been carried out with the goal of maintaining low homocysteine levels for primary prevention of vascular events.

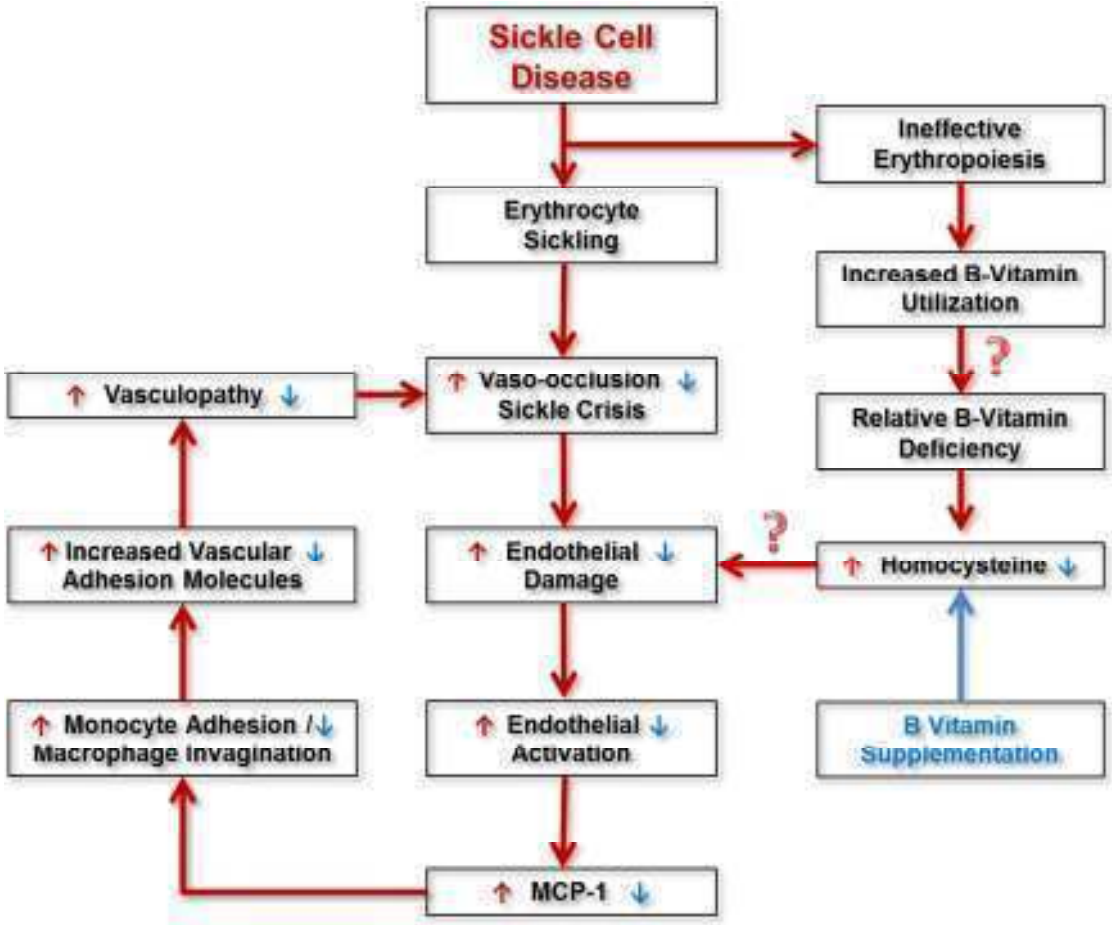
An extensive literature search did not reveal previous studies on the association of homocysteine levels and vascular disease in SCD patients. Our cross-sectional

cohort study in SCA patients is the first to demonstrate that elevated homocysteine is directly associated with the severity of microvasculopathy, as indicated by the severity index determined by the CAIM procedure.

Current recommendations on the prevention of stroke, as outlined in the Guidelines for the Primary Prevention of Stroke set forth by Goldstein et al (2011) (65), are to screen children with SCD with transcranial Doppler ultrasound (TCD) for development of vasculopathy, and if increased velocity in the middle cerebral circulation is found, institute primary prophylaxis with red cell transfusion. There are no other proven therapies to prevent vascular events in SCD. Additionally, there are no biomarkers to identify SCD patients who are at increased risk of vascular events.

Elevated homocysteine levels are strongly correlated with vascular events in the general population. However, the mechanism of vascular disease in SCD is different than the general population. Though unclear at this time, preemptive protection of the vasculature through homocysteine lowering therapy might be beneficial in SCD patients, if begun early in life. Additionally, with aggressive B vitamin therapy there may be the added benefit of preventing neurological damage by preventing stroke, and improving cognitive abilities (**Figure 4-3**).

Figure 4-3: Proposed B Vitamin Supplementation Impact on Vasculopathy in SCD



2. MCP-1 Association

Monocyte recruitment to the site of endothelial injury involves chemotactic signaling mediated in part by MCP-1. In SCD patients, circulating MCP-1 concentrations are elevated during both steady state and vaso-occlusive crisis (66). MCP-1 concentrations have been shown to be significantly higher (20- to 30-fold elevation) in

SCD patients, regardless of whether they were in painful crisis or in steady state, compared with healthy controls.

Our results indicate that MCP-1 is associated with and may directly contribute to the microvasculopathy observed in SCD subjects. Furthermore, MCP-1 levels are correlated with ferritin concentrations (**Figure 4-4**). Ferritin reflects body iron stores, and an increase of iron stores in SCD resulting from chronic blood transfusion as well as increased iron absorption associated with hemolysis and ineffective erythropoiesis may lead to monocyte recruitment to the vascular endothelium and contribute to vasculopathy. Measurements of MCP-1 and ferritin may also serve as surrogate biomarkers of microvasculopathy severity and inflammation.

Figure 4-4: ln Ferritin vs. ln MCP-1

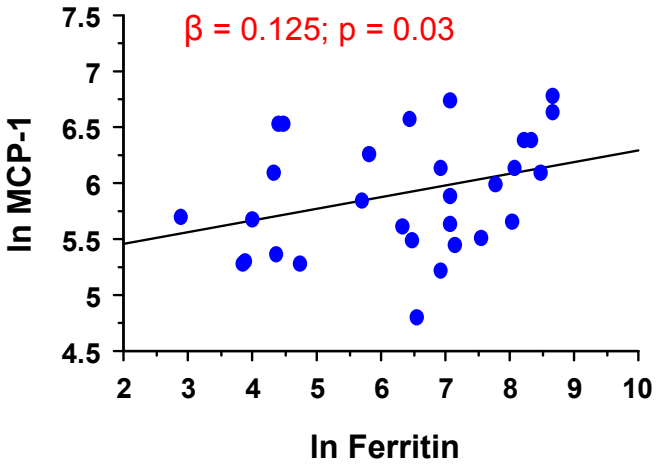
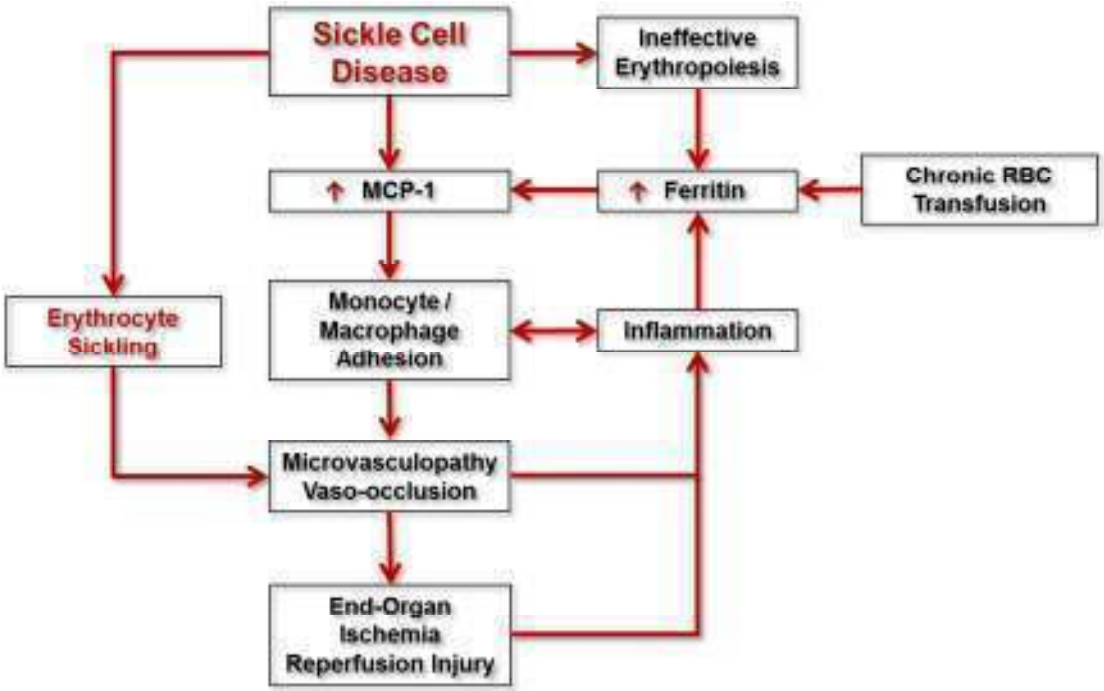


Figure 4-5: MCP-1, Ferritin, and End-Organ Injury



3. Varying Degrees of Severity Between Siblings

We encountered 2 sets of siblings in our study (a 4-year-old female and a 5-year-old male, and 2 females, 17--year-old and 22-year-old). Although each of these sets of siblings were homozygous for sickle cell (HbSS), they demonstrated markedly different phenotypes as reflected by the severity of their clinical complications. While lacking statistical power, these observations are illustrative of the potential usefulness of some candidate biomarkers for severity of microvasculopathy. In the younger set, the 4-year-old had pain crisis (including chest syndrome and spinal pain) and blood transfusions twice as frequently of her 5-year-old male sibling; the 17-year-old sibling had frequent

transfusions and hospitalization due to pain, while the 22-year-old rarely had pain crisis (less than 1 per year). **Table 4-6** compares the severity index and biomarkers for the sibling sets of this study (shaded columns are the siblings with reported higher frequency of incidents or symptoms). The 17-year-old had a higher severity index, MCP-1 level and homocysteine level than her 22-year-old sibling; likewise, this pattern is also seen in the 4-year-old and 5-year-old siblings.

Table 4-6: Comparison of SCA Sibling Severity Index and Biomarkers

	Sibling Set 1		Sibling Set 2	
	B6-28 * (F, 4)	B6-27 (M, 5)	B6-10 * (F, 17)	B6-13 (F, 22)
SI (0-15)	2	0	7	4
Hcy (µmol/L)	4.6	4.3	6.4	4.4
MCP-1 (pg/mL)	236	185	361	204
Ferritin (ng/mL)	1290	1015	1184	49

* Sibling with reported more frequent incidents or symptoms (greater frequency of blood transfusions, pain crisis, and duration of pain crisis).

4. Summary

This cross-sectional study on the relationships between biomarkers of B vitamin status, inflammation, vascular adhesion and microvascular characteristics in SCA patients under steady-state conditions had several key strengths in design and outcomes. The age range spanned from very young (age 4 years) to late middle age (age 61 years). The current known analytes associated with vascular inflammation and metabolites of B vitamin status were measured in these groups, and the study utilized innovative technology, including CAIM and multiplex analyte profiling. One limitation of the study was the relatively small sample size, which limited the power to detect significant associations. Nonetheless, the multiple regression analyses revealed that age, homocysteine, and MCP-1 are predictive factors for microvasculopathy in SCA patients, as indicated by severity index score. These biomarkers may therefore identify those at a high risk of vascular damage and who may benefit from intervention therapies. Specifically, B vitamin supplementation should be considered to determine if maintaining low circulating homocysteine concentrations minimizes vasculopathy and morbidity in SCD patients.

F. References

1. Francis RB JC. *Vascular Occlusion in Sickle Cell Disease: Current Concepts and Unanswered Questions*. Blood 1991;77(7):1405-14.
2. Ohene-Frempong K WS, Sleeper LA, et al. *Cerebrovascular accidents in sickle cell disease: rates and risk factors.* . Blood 1998;91:288-94.
3. Lee MT, Piomelli S, Granger S, Miller ST, Harkness S, Brambilla DJ, Adams RJ. *Stroke Prevention Trial in Sickle Cell Anemia (STOP): extended follow-up and final results*. Blood 2006;108(3):847-52.
4. Adams RJ BD, McKie VC, et al., (Abstract). *Stroke prevention in sickle cell disease (STOP): Baseline characterisitics of trial patients.* . Blood 1997;90(123a).
5. Rees DC, Gibson JS. *Biomarkers in sickle cell disease*. Br J Haematol 2012;156(4):433-45.
6. Platt OS. *Easing the Suffering Caused by Sickle Cell Disease*. New England Journal of Medicine 1994;330(11):783-4.
7. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, Klug PP. *Mortality In Sickle Cell Disease -- Life Expectancy and Risk Factors for Early Death*. New England Journal of Medicine 1994;330(23):1639-44.
8. Bakare MO. *Severity in phenotypic expression of homozygous sickle cell disease (Hb.SS)--does hypermelanotic or hypomelanotic skin status of affected patients play a role?* Med Hypotheses 2011;76(5):673-5.
9. Ender KL, Lee MT, Sheth S, Licursi M, Crotty J, Barral S, Green NS. *Fetal hemoglobin levels in African American and Hispanic children with sickle cell disease at baseline and in response to hydroxyurea*. J Pediatr Hematol Oncol 2011;33(7):496-9.
10. Al-Saqladi AW, Delpisheh A, Bin-Gadeem H, Brabin BJ. *Clinical profile of sickle cell disease in Yemeni children*. Ann Trop Paediatr 2007;27(4):253-9.
11. Schneidereith TA. *Pediatric hemoglobinopathies: from the bench to the bedside*. Annu Rev Nurs Res 2011;29:353-77.
12. Bender MA, Hobbs W. *Sickle Cell Disease*. Edtion ed. In: Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP, eds. GeneReviews. Seattle WA: University of Washington, Seattle, 1993.
13. Ataga KI, Moore CG, Jones S, Olajide O, Strayhorn D, Hinderliter A, Orringer EP. *Pulmonary hypertension in patients with sickle cell disease: a longitudinal study*. Br J Haematol 2006;134(1):109-15.
14. Schatz J. *Brief Report: Academic Attainment in Children With Sickle Cell Disease*. Journal of Pediatric Psychology 2004;29(8):627-33.
15. Rees DC, Williams TN, Gladwin MT. *Sickle-cell disease*. CORD Conference Proceedings 2010;376(9757):2018-31.
16. Cheung AT, Chen PC, Larkin EC, Duong PL, Ramanujam S, Tablin F, Wun T. *Microvascular abnormalities in sickle cell disease: a computer-assisted intravital microscopy study*. Blood 2002;99(11):3999-4005.

17. Cheung AT, Harmatz P, Wun T, Chen PC, Larkin EC, Adams RJ, Vichinsky EP. *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 2001;97(11):3401-4.

18. Cheung AT, Price AR, Duong PL, Ramanujam S, Gut J, Larkin EC, Chen PC, Wilson DM. *Microvascular abnormalities in pediatric diabetic patients*. Microvasc Res 2002;63(3):252-8.

19. Cheung AT MJ, Craig SM, To PL, Lin X, Samarron SL, Chen PC, Zwerdling T, Wun T, Li CS, Green R. *Comparison of real-time microvascular abnormalities in pediatric and adult sickle cell anemia patients*. Am J Hematol 2010;85(11):899-901.

20. Wun T, Cordoba M, Rangaswami A, Cheung AW, Paglieroni T. *Activated monocytes and platelet-monocyte aggregates in patients with sickle cell disease*. Clin Lab Haem 2002;24:81-8.

21. Swerlick RA EJ, Kumar A, Jeitler M, Wick TM. *Alpha 4 beta 1-integrin expression on sickle reticulocytes: vascular cell adhesion molecule-1-dependent binding to endothelium*. Blood 1993;82(6):1891-9.

22. Kumar A, Eckmam JR, Swerlick RA, Wick TM. *Phorbol ester stimulation increases sickle erythrocyte adherence to endothelium: a novel pathway involving alpha 4 beta 1 integrin receptors on sickle reticulocytes and fibronectin*. Blood 1996;88(11):4348-58.

23. Hebbel RP. *Adhesive interactions of sickle erythrocytes with endothelium*. J Clin Invest 1997;100(11 Suppl):S83-6.

24. Frenette PS. *Sickle cell vaso-occlusion: multistep and multicellular paradigm*. Current Opinion in Hematology 2002;9(2):101-6.

25. Bunn HF. *Pathogenesis and Treatment of Sickle Cell Disease*. New England Journal of Medicine 1997;337(11):762-9.

26. Natarajan M, Udden MM, McIntire LV. *Adhesion of sickle red blood cells and damage to interleukin-1 beta stimulated endothelial cells under flow in vitro*. Blood 1996;87(11):4845-52.

27. Balasa BV, Kalinyak KA, Bean JA SD, Gruppo RA. *Hyperhomocysteinemia is associated with low plasma pyridoxine levels in children with sickle cell disease*. J Pediatr Hematol Oncol 2002;24:374-9.

28. Natta C, Reynolds R. *Apparent vitamin B6 deficiency in sickle cell anemia.* Am J Clin Nutr 1984;40:235-9.

29. Nelson MC ZB, Kawchak DA, Barden EM, Frongillo EA Jr, Coburn SP, Ohene-Frempong K, Stallings VA. *Vitamin B6 status of children with sickle cell disease.* J Pediatr Hematol Oncol 2002;26:24(6):463-9.

30. Miller JW, Medina MV, Wun T, Green R. *Plasma pyridoxal-5'-phosphate is inversely correlated with VCAM levels in adult sickle cell disease patients*. FASEB J, 2004.

31. Dhar M, Bellevue R, Brar S, Carmel R. *Mild hyperhomocysteinemia in adult patients with sickle cell disease: a common finding unrelated to folate and cobalamin status*. Am J Hematol 2004;76(2):114-20.

32. Houston PE, Rana S, Sekhsaria S, Perlin E, Kim KS, Castro OL. *Homocysteine in sickle cell disease: relationship to stroke*. Am J Med 1997;103(3):192-6.

33. Kennedy TS, Fung EB, Kawchak DA, Zemel BS, Ohene-Frempong K, Stallings VA. *Red blood cell folate and serum vitamin B12 status in children with sickle cell disease*. J Pediatr Hematol Oncol 2001;23(3):165-9.

34. Kamineni P, Chirla S, Dinh K, Hasan S, Nidhiry E, Kwagyan J, Naab T, Lombardo F, Castro O, Dawkins F. *Low cobalamin levels in African Americans with and without sickle cell disease*. J Natl Med Assoc 2006;98(3):352-6.

35. van der Dijs FP, Schnog JJ, Brouwer DA, Velvis HJ, van den Berg GA, Bakker AJ, Duits AJ, Muskiet FD, Muskiet FA. *Elevated homocysteine levels indicate suboptimal folate status in pediatric sickle cell patients*. Am J Hematol 1998;59(3):192-8.

36. van der Dijs FP, Fokkema M, Dijck-Brouwer D, Niessink B, Wal Tvd, Schnog J, Duits A, Muskiet F, Muskiet F. *Optimization of folic acid, vitamin B(12), and vitamin B(6) supplements in pediatric patients with sickle cell disease*. Am J Hematol 2002;69(4):239-46.

37. Gilfix BM, Blank DW, Rosenblatt DS. *Novel reductant for determination of total plasma homocysteine*. Clin Chem 1997;43(4):687-8.

38. Marszałł ML LA, Czarnowski W, Makarowski R, Kłos M, Szefer P. *Application of the high-performance liquid chromatography method with coulometric detection for determination of vitamin B(6) in human plasma and serum.* J Chromatogr B Analyt Technol Biomed Life Sci 2009;877(27):3151-8.

39. Talwar D, Quasim T, McMillan DC, Kinsella J, Williamson C, O'Reilly DS. *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*. J Chromatogr B Analyt Technol Biomed Life Sci 2003;792(2):333-43.

40. Gregory JF, 3rd, Kirk JR. *Determination of urinary 4-pyridoxic acid using high performance liquid chromatography*. The American journal of clinical nutrition 1979;32(4):879-83.

41. Zhang X, He Y, Ding M. *Simultaneous determination of tryptophan and kynurenine in plasma samples of children patients with Kawasaki disease by high-performance liquid chromatography with programmed wavelength ultraviolet detection*. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877(16-17):1678-82.

42. Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PWF, Belanger AJ, O'Leary DH, Wolf PA, Schaefer EJ, Rosenberg IH. *Association between Plasma Homocysteine Concentrations and Extracranial Carotid-Artery Stenosis*. New England Journal of Medicine 1995;332(5):286-91.

43. Choy PC, Mymin D, Zhu Q, Dakshinamurti K, O K. *Atherosclerosis risk factors: the possible role of homocysteine*. Mol Cell Biochem 2000;207(1-2):143-8.

44. Stubbs PJ, Al-Obaidi MK, Conroy RM, Collinson PO, Graham IM, Noble IM. *Effect of plasma homocysteine concentration on early and late events in patients with acute coronary syndromes*. Circulation 2000;102(6):605-10.

45. Collaboration HS. *Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis*. JAMA 2002;288(16):2015-22.

46. World Health Organization. *Genomic resource centre: Genes and human disease*. Internet: http://www.who.int/entity/genomics/public/Maphaemoglobin.pdf. (accessed April 2012).

47. Stauffenberg MT, Lange RA, Hillis LD, Cigarroa J, Hsu RM, Devaraj S, Jialal I. *Hyperhomocysteinemia measured by immunoassay: a valid measure of coronary artery atherosclerosis*. Arch Pathol Lab Med 2004;128(11):1263-6.

48. Toole JF, Malinow MR, Chambless LE, Spence JD, Pettigrew LC, Howard VJ, Sides EG, Wang CH, Stampfer M. *Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death: the Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial*. JAMA 2004;291(5):565-75.

49. Rauh M, Verwied S, Knerr I, Dorr HG, Sonnichsen A, Koletzko B. *Homocysteine concentrations in a German cohort of 500 individuals: reference ranges and determinants of plasma levels in healthy children and their parents*. Amino Acids 2001;20(4):409-18.

50. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. *A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes*. JAMA 1995;274(13):1049-57.

51. Hoogeveen EK, Kostense PJ, Jakobs C, Dekker JM, Nijpels G, Heine RJ, Bouter LM, Stehouwer CD. *Hyperhomocysteinemia increases risk of death, especially in type 2 diabetes : 5-year follow-up of the Hoorn Study*. Circulation 2000;101(13):1506-11.

52. Zinellu A, Sotgia S, Scanu B, Pintus G, Posadino AM, Cossu A, Deiana L, Sengupta S, Carru C. *S-homocysteinylated LDL apolipoprotein B adversely affects human endothelial cells in vitro*. Atherosclerosis 2009;206(1):40-6.

53. Lowenthal EA, Mayo MS, Cornwell PE, Thornley-Brown D. *Homocysteine elevation in sickle cell disease*. J Am Coll Nutr 2000;19(5):608-12.

54. Claster S, Wood JC, Noetzli L, Carson SM, Hofstra TC, Khanna R, Coates TD. *Nutritional deficiencies in iron overloaded patients with hemoglobinopathies*. Am J Hematol 2009;84(6):344-8.

55. Flores L, Pais R, Buchanan I, Arnelle D, Camp VM, Kutner M, Faraj BA, Eckman J, Ragab A. *Pyridoxal 5'-phosphate levels in children with sickle cell disease*. Am J Pediatr Hematol Oncol 1988;10(3):236-40.

56. Pan Y, Guo LL, Cai LL, Zhu XJ, Shu JL, Liu XL, Jin HM. *Homocysteine-lowering therapy does not lead to reduction in cardiovascular outcomes in chronic kidney disease patients: a meta-analysis of randomised, controlled trials*. Br J Nutr 2012;108(3):400-7.

57. Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, McQueen MJ, Probstfield J, Fodor G, Held C, Genest J, Jr. *Homocysteine lowering with folic acid and B vitamins in vascular disease*. N Engl J Med 2006;354(15):1567-77.

58. Marti-Carvajal AJ, Sola I, Lathyris D, Salanti G. *Homocysteine lowering interventions for preventing cardiovascular events*. Cochrane Database Syst Rev 2009(4):CD006612.

59. McNeil CJ, Beattie JH, Gordon MJ, Pirie LP, Duthie SJ. *Differential effects of nutritional folic acid deficiency and moderate hyperhomocysteinemia on aortic plaque formation and genome-wide DNA methylation in vascular tissue from ApoE-/- mice*. Clin Epigenetics 2011;2(2):361-8.

60. Ebbing M, Bonaa KH, Arnesen E, Ueland PM, Nordrehaug JE, Rasmussen K, Njolstad I, Nilsen DW, Refsum H, Tverdal A, Vollset SE, Schirmer H, Bleie O, Steigen T, Midttun O, Fredriksen A, Pedersen ER, Nygard O. *Combined analyses and extended follow-up of two randomized controlled homocysteine-lowering B-vitamin trials*. J Intern Med 2010;268(4):367-82.

61. Spence JD, Stampfer MJ. *Understanding the complexity of homocysteine lowering with vitamins: the potential role of subgroup analyses*. JAMA 2011;306(23):2610-1.

62. Clarke R, Halsey J, Bennett D, Lewington S. *Homocysteine and vascular disease: review of published results of the homocysteine-lowering trials*. J Inherit Metab Dis 2011;34(1):83-91.

63. Cheng X. *Updating the Relationship Between Hyperhomocysteinemia Lowering Therapy and Cardiovascular Events*. Cardiovasc Ther 2012.

64. Cavalieri M, Schmidt R, Chen C, Mok V, de Freitas GR, Song S, Yi Q, Ropele S, Grazer A, Homayoon N, Enzinger C, Loh K, Wong KS, Wong A, Xiong Y, Chang HM, Wong MC, Fazekas F, Eikelboom JW, Hankey GJ. *B Vitamins and Magnetic Resonance Imaging-Detected Ischemic Brain Lesions in Patients With Recent Transient Ischemic Attack or Stroke: The VITAmins TO Prevent Stroke (VITATOPS) MRI-Substudy*. Stroke 2012;43(12):3266-70.

65. Goldstein LB, Bushnell CD, Adams RJ, Appel LJ, Braun LT, Chaturvedi S, Creager MA, Culebras A, Eckel RH, Hart RG, Hinchey JA, Howard VJ, Jauch EC, Levine SR, Meschia JF, Moore WS, Nixon JV, Pearson TA. *Guidelines for the primary prevention of stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association*. Stroke 2011;42(2):517-84.

66. Qari MH, Dier U, Mousa SA. *Biomarkers of inflammation, growth factor, and coagulation activation in patients with sickle cell disease*. Clin Appl Thromb Hemost 2012;18(2):195-200.

Chapter 5: Comparison of Real-Time Microvascular Abnormalities in Pediatric and Adult Sickle Cell Anemia Patients

Cheung AT[1], Miller JW[1], Craig SM[1], To PL[1], Lin X[1], **Samarron SL**[1], Chen PC[2], Zwerdling T[3], Wun T[4,5], Li CS[6], Green R[1,4]

1 Department of Pathology and Laboratory Medicine, University of California Davis School of Medicine, Sacramento, California

2 Shiley Center for Orthopedic Research and Education, Scripps Clinic, La Jolla, California

3 Department of Pediatrics (Hematology-Oncology), University of California Davis School of Medicine, Sacramento, California

4 Department of Internal Medicine (HematologyOncology), University of California Davis School of Medicine, Sacramento, California

5 Section of Hematology and Oncology, VA Northern California Health Care System, Sacramento, California

6 Department of Public Health Sciences (Biostatistics), University of California Davis School of Medicine, Sacramento, California

Format modified from published article:

Cheung AT, Miller J, Craig SM, To PL, Lin X, Samarron SL, Chen PC, Zwerdling T, Wun T, Li CS, Green R., *Comparison of real-time microvascular abnormalities in pediatric and adult sickle cell anemia patients.* Am J Hematol., 2010. 85(11): p. 899-901.

A. Abstract

The conjunctival microcirculation in 14 pediatric and eight adult sickle cell anemia (SCA) patients was studied using computer-assisted intravital microscopy. The bulbar conjunctiva in SCA patients in both age groups exhibited a blanched/avascular appearance characterized by decreased vascularity. SCA patients from both age groups had many of the same abnormal morphometric (vessel diameter, vessel distribution, morphometry (shape), tortuosity, arteriole:venule (A:V) ratio, and

hemosiderin deposits) and dynamic (vessel sludging/sludged flow, boxcar blood (trickled) flow, and abnormal flow velocity) abnormalities. A severity index (SI) was computed to quantify the degree of vasculopathy for comparison between groups. The severity of vasculopathy differed significantly between the pediatric and adult patients (SI: 4.2 ± 1.8 vs. 6.6 ± 2.4; P = 0.028), indicative of a lesser degree of overall severity in the pediatric patients. Specific abnormalities that were less prominent in the pediatric patients included abnormal vessel morphometry and tortuosity. Sludged flow, abnormal vessel distribution, abnormal A:V ratio, and boxcar flow appeared in high prevalence in both age groups. The results indicate that SCA microvascular abnormalities develop in childhood and the severity of vasculopathy likely progresses with age. Intervention and effective treatment/management modalities should target pediatric patients to ameliorate, slow down, or prevent progressive microvascular deterioration.

B. Introduction

Sickle cell anemia (SCA) is a genetic disorder that affects millions of people worldwide, for which there is no cure despite substantial understanding of its underlying pathogenesis (1, 2). Anemia caused by ineffective erythropoiesis and hemolysis is a contributing factor, but vascular complications and abnormal blood flow dynamics account for much of SCA morbidity and mortality. However, there are few real-time in vivo studies on the microcirculation in SCA patients, except for the work by Lipowsky et al (3) on intravital microscopy of nail-fold capillary hemodynamics in SCA.

We have previously reported three real-time in vivo studies on the microcirculation of the bulbar conjunctiva in SCA patients using computer-assisted intravital microscopy (CAIM) (4–6). The microvascular bed of the bulbar conjunctiva

offers a readily accessible site for noninvasive measurements from which it is possible to extrapolate the in vivo condition of the microvasculature within soft tissues, and to quantify changes in microvascular condition of critical end organs over time. Using our imaging studies of the bulbar conjunctiva in SCA patients, we have characterized and quantified the morphometric and dynamic microvascular abnormalities (vasculopathy) of the disease (4), demonstrated that abnormal microvascular blood flow dynamics correlate with intracranial blood flow velocity in the Circle of Willis measured by transcranial Doppler ultrasonography (5), and evaluated the efficacy of the drug Poloxamer 188 (RheothRx® and Flocor™) on vaso-occlusion (6). Thus, microvascular characteristics from image analysis of the bulbar conjunctiva can serve as a reliable surrogate biomarker of the severity of microvascular pathology and the efficacy of interventions designed to treat and ameliorate complications resulting from SCA-associated vasculopathy.

These real-time in vivo studies using CAIM have included both adult (4, 6) and pediatric SCA patients (5, 6). However, in pediatric patients, these studies have focused primarily on the measurements of vessel diameter and blood flow velocity, and assessments of vasculopathy have not been reported. Moreover, there have been no direct comparisons of microvascular abnormalities and severity of vasculopathy between pediatric and adult SCA patients. Accordingly, the goal of this study was to characterize and compare real-time measurements on the degree of in vivo vasculopathy in pediatric and adult SCA patients, and to test the hypothesis that the severity of vasculopathy increases with age as a natural course of the disease.

C. Methods

1. Patient Groups Studied

The University of California Davis Institutional Review Board approved the study, and written informed consent was obtained from all patients or from their parents or guardians. Pediatric SCA patients (HbSS; ages 6–18 years) were recruited from the Pediatric Sickle Cell Clinic at the University of California Davis Medical Center (UCDMC). Adult SCA patients (HbSS; ages 27–58 years) were recruited from the Adult Sickle Cell Clinic at UCDMC. Before initiation of the study, all patient records were evaluated to ensure that each patient was not having any sickling complications (i.e., in steady-state condition) and had not suffered a vaso-occlusive (painful) crisis for at least a month before the study. SCA patients on chronic transfusion were allowed to participate in this study.

2. Computer-Assisted Intravital Microscopy

A CAIM system substantially modified and adapted from the earlier prototype originally designed to study the conjunctival microcirculation in adult subjects (4, 10) has been utilized successfully thereafter to study pediatric patients (5, 6, 11). The CAIM system uses macro-optics in which image acquisition is based on real-time video documentation of selected regions in the in vivo conjunctival microcirculation. The procedural details of this technique have been described in detail in previous publications (7–9).

3. Quantification of Severity of Vasculopathy and Prevalence of Microvascular Abnormalities

Videotape sequences made of the conjunctival microcirculation in each patient were coded for subsequent viewing and analysis to ensure objectivity, with the medical history and identity of each pediatric and adult patient blinded to the investigators prior to and during data analysis. Data analysis, which was described in detail in previous reports (4, 7–9), was conducted in two phases:

1. Visualization phase—Identification of morphometric characteristics. Videotape sequences of each patient were viewed in their entirety. Key landmark features (characteristics), including comma signs, vessel sludging (sludged flow), boxcar (trickled) blood flow pattern, microaneurysms (micro-pools), ischemia, vessel morphometry (pattern or shape), vessel distribution, distended vessels, tortuous vessels, sacculated (beaded) vessels, damaged vessels, and hemosiderin deposits were identified and tabulated for their presence in each experimental subject (**Tables 5-1 and 5-2**). The same coded videotape sequences were analyzed by at least two observers. Differences in the identification of the morphometric features, though infrequent, were discussed and reconciled through a third adjudicator.
2. Quantification phase—computer-assisted image analysis. Four to five short coded videotape sequences of ~30 sec each from each experimental subject were selected and frame captured for data quantification, including vessel diameter, total lengths of arterioles and venules per area for A:V ratio computation, and measurement of red cell flow velocity (4, 7–9).

Based on previous studies on microvascular abnormalities in various vascular diseases, 15 possible aberrations can be found in the conjunctival microvasculature (7–11). A SI is computed to quantify the degree (severity) of vasculopathy in each patient, based on the arithmetic summation of the presence of any of the 15 microvascular abnormalities listed above on a binary (yes = 1; no = 0) basis. The SI ranges from a score of 0 (no abnormalities present) to 15 (all 15 abnormalities present). This SI computation methodology has been validated in previous studies (7–11) and has an inter-investigator variation coefficient of <5%.

4. Statistical analysis

Results were reported as means ± standard deviation and medians with ranges. The two-sided Wilcoxon rank-sum test was used to compare SI, which is a numerical variable, between the two groups. The two-sided Fisher's exact test was used to compare the prevalence of each of the 15 microvascular abnormalities between the two groups, which can be constructed as a 2 × 2 contingency table. An OR for each abnormality in the adult patients with 95% CI was reported with the pediatric patients serving as the reference group. This OR is the ratio of the odds of an abnormality appearing in the adult patient group to the odds of it appearing in the pediatric patient group. An OR with 95% CI represents a statistically significant difference in the appearance of a specific microvascular abnormality between the two patient groups. All statistical analyses in this study were performed using the SAS v9.2 software (SAS Institute, Cary, NC). A P-value ≤0.05 was considered statistically significant.

D. Results

Fourteen pediatric and eight adult SCA patients participated in the study. Mean ages of the two groups were significantly different (13.6 ± 4.4 years vs. 36.8 ± 11.9 years, $P < 0.001$). Conjunctival microvasculature was compared between the pediatric and adults patients, and contrasted with that of healthy, non-SCA control subjects analyzed in previous studies (4, 7, 8). **Figure 5-1A** shows a typical image of the conjunctival microvasculature in a non-SCA subject frame captured from a videotape sequence from an unrelated study (7, 9). There is an orderly presence of anastomosing networks of capillaries, arterioles, and venules without the presence of ischemic (avascular) zones (**Figure 5-1A**). The normal A:V ratio is typically ~1:2, and the arterioles and venules exhibit an even distribution without the presence of dilations, narrowing, distension, micro-aneurysm, sacculated (beaded) vessels, broken/damaged vessels, or hemosiderin deposits. Normal conjunctival blood flow, though variable in red cell velocity, is smooth and non-intermittent. Blood sludging, tortuous vessels and boxcar blood flow (trickled flow) patterns are typically not observed.

Figure 5-1: Conjunctival Microvasculature

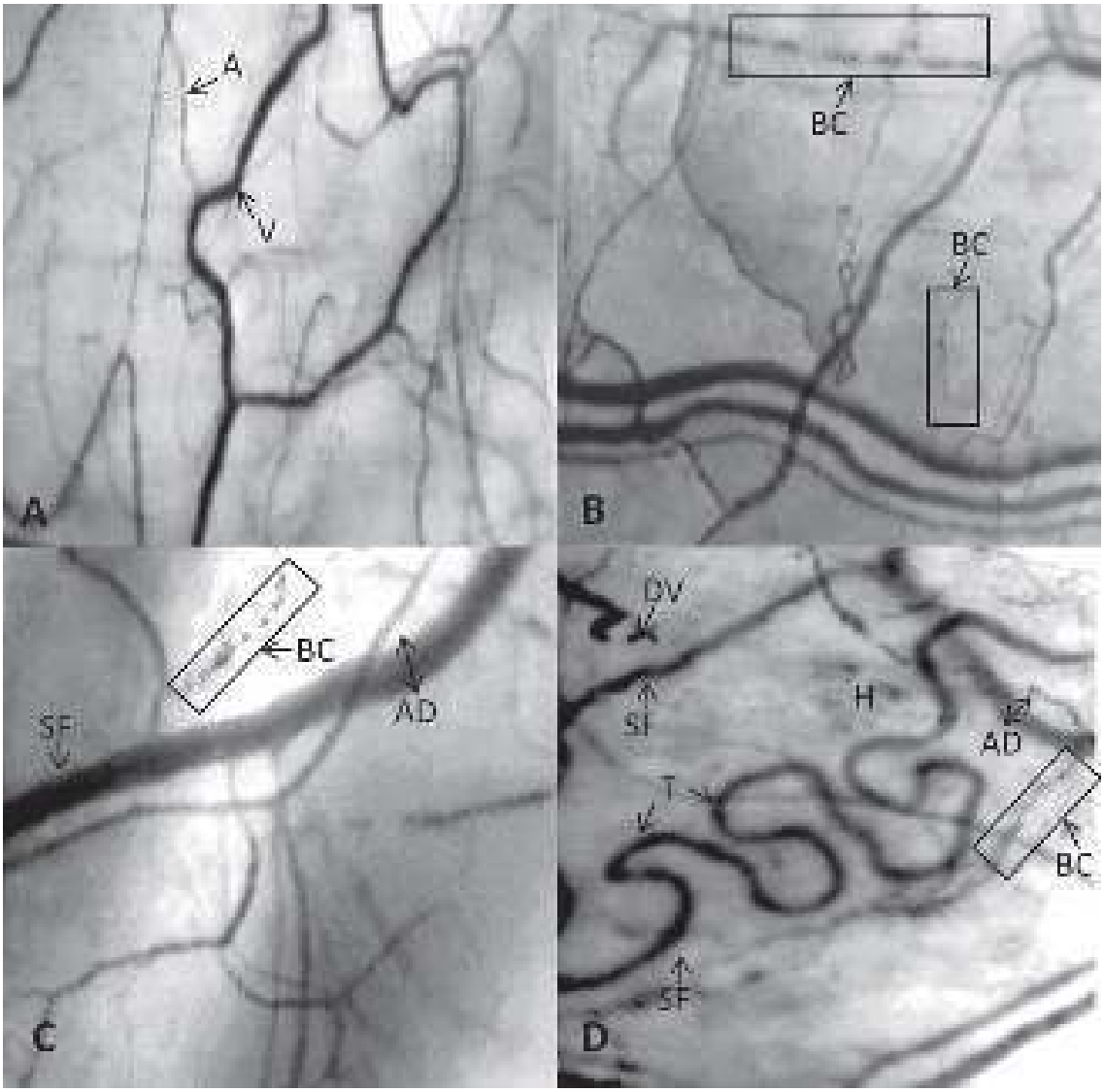


Figure 5-1A: A frame-captured image of the conjunctival microcirculation in a healthy non-SCA control subject (7, 9). Optical magnification 4.5; onscreen magnification 3125. This image illustrates a typical view of the conjunctival microcirculation in a healthy (non-SCA) control subject who has no history of any

vascular disease. Note the even and orderly distribution of normal-sized arterioles, venules, and capillaries in a richly vascularized network.

Figure 5-1B: A frame-captured image of the conjunctival microcirculation in a pediatric SCA patient (Patient #P-3; age 8 years). Optical magnification 34.5; onscreen magnification 3125. The SI of this patient is 3 and the microvascular abnormalities include only sludged blood flow (vessel sludging), boxcar (trickled) blood flow, and abnormal A:V ratio. Overall, the vasculopathy observed is mild.

Figure 5-1C: A frame-captured image of the conjunctival microcirculation in another pediatric SCA patient (Patient #P-8; age 15 years). Optical magnification 34.5; onscreen magnification 3125. Patient P-8 is 7 years older than the patient described in Figure 5-1B. The microcirculation shows a greater level of vasculopathy, which includes abnormal vessel diameter, sludged blood flow, boxcar (trickled) blood flow, abnormal vessel distribution, hemosiderin deposits, and abnormal A:V ratio in this captured frame. The overall vasculopathy in this pediatric patient is severe, with an SI of 7 (compared with the SI of 3 in the pediatric patient described in **Figure 5-1B**).

Figure 5-1D: A frame-captured image of the conjunctival microcirculation in an adult SCA patient (Patient #A-7; age 58 years). Optical magnification 34.5; onscreen magnification 3125. The microvascular abnormalities in this adult patient include abnormal vessel diameter, pronounced vessel tortuosity, abnormal vessel distribution, abnormal A:V ratio, sludged (trickled) blood flow, boxcar flow pattern, damaged vessel, and hemosiderin deposits. A, arteriole; V, venule; BC, boxcar (trickled) blood flow; SF, sludged blood flow (stop-and-go pattern of blood flow as evidenced by area(s) of darker or uneven coloration within the vessel); AD, abnormal diameter (wide); DV, damaged vessel; H, hemosiderin deposits; T, tortuosity.

The conjunctival microcirculation in the pediatric and adult SCA patients uniquely differs from those found in non-SCA control subjects (**Table 5-1**). There is a lower amount of vascularity (diminished presence of conjunctival vessels) and abnormal vascular distribution in most patients in both age groups, giving the bulbar conjunctiva a "blanched" avascular appearance. The prevalence of specific microvascular abnormalities in both patient groups is summarized in **Table 5-1 and 5-2**, and some of the abnormalities are shown in **Figure 5-1B–D**. SCA patients from both age groups exhibit, to varying degrees, the same morphometric and dynamic abnormalities, including abnormal vessel diameter, abnormal vessel distribution, abnormal vessel morphometry (shape), sludged flow, vessel tortuosity, abnormal A:V ratio, boxcar blood flow, hemosiderin deposits, and abnormal flow (red cell) velocity. These microvascular abnormalities are rarely found in the bulbar conjunctiva of healthy non-SCA subjects (4, 7–8). The severity of vasculopathy, as indicated by the severity index (SI), was significantly lower in the pediatric patients than in the adult patients (4.2 ± 1.8 vs. 6.6 ± 2.4, P = 0.028). For comparison, the mean SI values for both the pediatric and adult SCA patients were significantly higher than the mean SI value determined for a previous cohort of healthy non-SCA subjects (n = 10; SI = 0.31 ± 0.72; P < 0.05) (8). In comparing the prevalence of microvascular abnormalities between pediatric and adult SCA groups, the following significant differences were observed:

- Abnormal vessel morphometry was observed in three out of eight adult patients (38%) but was not observed in any of the pediatric patients. The odds ratio (OR) (95% confidence interval (CI)) for the difference in prevalence was ∞ (1.2, ∞) (P = 0.036).

- Vessel tortuosity was observed in seven out of eight adult patients (88%) compared with only three out of 14 pediatric patients (21%). The OR (95% CI) for the difference in prevalence was 25.7 (1.7, 1258) (P = 0.006).

In addition, several microvascular abnormalities were highly prevalent in both the pediatric and adult patients. Ten out of 14 pediatric patients (71%) and seven out of eight adult patients (88%) had vessel sludging. Ten out of 14 pediatric patients (71%) and eight out of eight adult patients (100%) had an abnormal A:V ratio. Eight out of 14 pediatric patients (57%) and seven out of eight adult patients (88%) had an abnormal vessel distribution. Eleven out of 14 pediatric patients (79%) and six out of eight adult patients (75%) exhibited boxcar flow patterns.

Table 5-1: Severity Index (SI) of Pediatric (≤18 years of age) Sickle Cell Anemia (SCA) Patients

Patient code	Age	SI	Abnormal diameter	Abnormal distribution	Uneven thickness	Abnormal morphometry	Distended vessel	Damaged vessel	Sludged flow	Tortuosity	Ischemia	Abnormal A:V ratio	Microaneurysm	Boxcar flow	Hemosiderin deposits	Comma sign	Abnormal velocity
P-1	6	6		+					+	+		+		+			+
P-2	8	2												+	+		
P-3	8	3							+			+		+			
P-4	9	4	+	+								+		+			
P-5	12	3	+	+								+					
P-6	13	4							+			+		+	+		
P-7	13	6	+	+					+			+		+	+		
P-8	15	7	+	+					+			+		+	+		+
P-9	17	4	+	+					+	+							
P-10	17	7	+	+					+	+		+		+			+
P-11	18	3							+			+		+			
P-12	18	6	+	+					+		+	+				+	
P-13	18	2							+					+			
P-14	18	2												+	+		

AGE (n = 14): 13.6 ± 4.4 SI (n = 14): 4.2 ± 1.8

Table 5-2: Severity Index (SI) of Adult (>18 years of age) Sickle Cell Anemia (SCA) Patients

Patient code	Age	SI	Abnormal diameter	Abnormal distribution	Uneven thickness	Abnormal morphometry	Distended vessel	Damaged vessel	Sludged flow	Tortuosity	Ischemia	Abnormal A:V ratio	Microaneurysm	Boxcar flow	Hemosiderin deposits	Comma sign	Abnormal velocity
A-1	29	9	+	+		+			+	+		+		+	+		+
A-2	30	6	+	+					+			+		+	+		
A-3	30	2								+		+					
A-4	31	8	+	+		+			+	+		+		+	+		
A-5	36	5	+	+					+	+		+					
A-6	53	8	+	+	+				+	+		+		+	+		
A-7	58	9	+	+		+		+	+	+		+		+	+		
A-8	27	6	+	+					+	+		+		+			

AGE (n = 8): 36.8 ± 11.9 SI (n = 8): 6.6 ± 2.4

E. Discussion

CAIM is a real-time technology that can be used to noninvasively videotape, analyze and quantify real-time microvascular abnormalities in vascular diseases. The technique has been used successfully in our laboratory to assess microvascular abnormalities in type-1 and type-2 diabetes, Alzheimer's disease, and SCA (4–12). The in vivo microvascular bed of the bulbar conjunctiva (conjunctival microcirculation) is particularly amenable to the use of CAIM because it is noninvasively and easily accessible, and yields images of excellent quality and clarity. Results from some of the studies on the identification and quantification of microvascular abnormalities in the conjunctival microcirculation (4, 11) have been used as a basis for subsequent translational research and interventional efficacy studies (6, 10).

This study was designed to extend our knowledge base on real-time vasculopathy in pediatric and adult SCA patients. Our overall goal is to understand the ontogeny of vasculopathy based on the hypothesis that, as a genetic disorder, SCA microvascular complications and vasculopathy begin to develop after birth and continue to progress into adulthood as part of the natural course of the disease. Results from this study support this hypothesis: the severity of microvascular abnormalities in the pediatric patients was significantly lower than that observed in the adult patients. Secondary analyses of specific microvascular abnormalities revealed that the observed difference in severity was primarily due to a lower prevalence of abnormal vessel morphometry and vessel tortuosity in the pediatric patients compared with the adults. These findings suggest that these two specific abnormalities develop at a slower rate than other microvascular abnormalities.

The primary limitation of this study is that it is cross-sectional. The observed difference in the severity of vasculopathy between the pediatric and adult patients could be attributable to advances in management of the disease that were not available to the adult patients during their childhood. A longitudinal study in which the microvasculature of SCA patients is evaluated at regular intervals from childhood to adulthood would be required to definitively test the hypothesis that the severity of vasculopathy progresses with age. If confirmed, the results of this study suggest that the pediatric years represent a window of opportunity during which effective treatment and management modalities may slow or ameliorate complications of SCA caused by vasculopathy that arises as a natural progression of the disease from childhood to adulthood. Specific abnormalities, e.g., abnormal vessel morphometry and vessel tortuosity, may serve as landmark biomarkers to evaluate the efficacy of treatment and disease management modalities over time. Moreover, the high prevalence of other abnormalities in both pediatric and adult patients, including vessel sludging, abnormal A:V ratio, abnormal vessel distribution, and boxcar flow patterns—indicative of rapid development of vasculopathy in childhood—suggests an urgency to identify better interventions and treatments that ameliorate or slow the progression of microvascular abnormalities and can be used to treat pediatric SCA patients more aggressively.

Conjunctival vessels have unique shapes and forms (**Figure 5-1A–D**) and can be easily reidentified for follow-up studies using CAIM—each individual vessel can serve as its own baseline (reference) control and then relocalized and reassessed in longitudinal studies (6, 10). This makes the conjunctival microcirculation an ideal arena and CAIM an excellent noninvasive real-time technology for longitudinal studies of SCA disease progression and evaluations of the efficacy of medications and other treatment or management modalities. At this time, CAIM is not yet widely used as a research tool.

However, two identical CAIM systems have been built recently and are functional in other laboratories. A blinded interventional collaborative study to compare independently obtained real-time in vivo vasculopathy data is in progress. These studies will eventually allow for independent confirmation of our results at other institutions and will validate the utility of CAIM as a clinical tool to objectively and noninvasively study vasculopathy in SCA and other vascular diseases.

F. References

1. U.S. Department of Health & Human Services, National Institutes of Health. *National Heart, Lung and Blood Institute—Diseases and Conditions Index on Sickle Cell Anemia* (all sections). 2009. pp 1–17. Available at: http://www.nhlbi.nih.gov/health/dci/Diseases/Sca/SCA_All.html.
2. Embury SH, Hebbel RP, Mohandas N, Steinberg MH, editors. *Sickle Cell Disease: Basic Principles and Clinical Practice*. New York, NY: Raven Press; 1994.
3. Lipowsky HH, Sheikh NU, Katz DM. *Intravital microscopy of capillary hemodynamics in sickle cell anemia*. J Clin Invest 1987; 80:117–127.
4. Cheung AT, Chen PC, Larkin EC, et al. *Microvascular abnormalities in sickle cell disease: A computer-assisted intravital microscopy study*. Blood 2002; 99: 3999–4005.
5. Cheung AT, Harmatz P, Wun T, et al. *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 2002; 97:3401–3404.
6. Cheung AT, Chan MS, Ramanujam S, et al. *Effects of poloxamer 188 treatment on sickle cell vaso-occlusive crisis: Computer-assisted intravital microscopy stud*y. J Investig Med 2004; 52:402–406.
7. Devaraj S, Cheung AT, Jialal I, et al. *Evidence of increased inflammation and microcirculatory abnormalities in patients with type 1 diabetes and their role in microvascular complications*. Diabetes 2007; 56:2790–2796.
8. Cheung AT, Tomic MM, Chen PC, et al. *Correlation of microvascular abnormalities and endothelial dysfunction in type-1 diabetes mellitus (T1DM): A real-time intravital microscopy study*. Clin Hemorheol Microcirc 2009; 42:285–295.
9. Smith MM, Chen PC, Li CS, et al. *Whole blood viscosity and microvascular abnormalities in Alzheimer's disease*. Clin Hemorheol Microcirc 2008;41: 229–239.
10. Cheung AT, Ramanujam S, Greer DA, et al. *Microvascular abnormalities in the bulbar conjunctiva of patients with type 2 diabetes mellitus*. Endocr Pract 2001; 7:358–363.
11. Cheung AT, Perez RV, Chen PC. *Improvements in diabetic microangiopathy after successful simultaneous pancreas-kidney transplantation: A computer-assisted intravital microscopy study on the conjunctival microcirculation*. Transplantation 1999; 68: 927–932.
12. Cheung AT, Price AR, Duong PL, et al. *Microvascular abnormalities in pediatric diabetic patients*. Microvasc Res 2002; 63:252–258.

Chapter 6: Exchange Transfusion Therapy and Its Effects on the Real-Time Microcirculation in Pediatric Sickle Cell Anemia Patients: An Intravital Microscopy Study

Cheung ATW[1], Miller JW[1], Miguelino MG[1,2], To WJ[1], Li J[1], Lin X[1], Chen PC[4],

Samarron SL[1], Wun T[2], Zwerdling T[3], Green R[1]

1 Department of Medical Pathology and Laboratory Medicine, University of California Davis School of Medicine, Sacramento, CA, USA

2 Department of Internal Medicine (Hematology/Oncology), University of California Davis School of Medicine, Sacramento, CA, and Section of Hematology and Oncology, VA Northern California Health Systems, Sacramento, CA, USA

3 Department of Pediatrics (Hematology/Oncology), University of California Davis School of Medicine, Sacramento, CA, USA

4 Shiley Center for Orthopedic Research and Education, Scripps Institute, La Jolla, CA, USA

Format modified from published manuscript:

> Cheung, A.T., et al., Miller JW, Miguelino MG, To WJ, Li J, Lin X, Chen PC, Samarron SL, Wun T, Zwerdling T, Green R. *Exchange transfusion therapy and its effects on real-time microcirculation in pediatric sickle cell anemia patients: an intravital microscopy study.* J Pediatr Hematol Oncol, 2012. 34(3): p. 169-74

A. Abstract

Periodic blood exchange transfusion is a treatment modality commonly used to manage pediatric sickle cell anemia (SCA) at the University of California Davis Medical Center. The goal of exchange transfusion therapy is to ameliorate vaso-occlusion and improve tissue perfusion by removing sickled red blood cells (RBCs) and introducing normal RBCs. Using computer-assisted intravital microscopy, pre- and post-transfusion microvascular characteristics were analyzed. In this study, the bulbar conjunctiva exhibited a “blanched” avascular appearance in all six pediatric SCA patients prior to

transfusion, indicative of tissue hypoperfusion and ischemia. Immediately following transfusion, substantial improvement in vascularization and tissue perfusion resulted, reflected by the enhanced appearance of capillaries and arterioles. In addition, a decrease in red cell velocity was observed. These observations provide evidence that exchange transfusion therapy is beneficial in ameliorating vaso-occlusion and improving tissue perfusion. However, with the paradoxical post-transfusion decrease in red cell velocity presumably due to induced hyperviscosity from the large transfusion volume, blood flow is still impaired. This decreased velocity may thwart efforts to improve oxygen delivery via transfusion and may, to some extent, promote vaso-occlusion instead. This paradoxical result warrants further investigation on the effects of transfusion volume and viscosity in the exchange transfusion process.

B. Introduction

Sickle Cell Anemia (SCA) is an autosomal recessive disease that afflicts ~1 in 500 African American births as well as an emerging population of ~1 in 20,000 Hispanic American births each year. Moreover, SCA is the most common inherited blood disorder in the United States affecting 70,000 to 100,000 Americans (1). SCA is the prototype of hereditary hemoglobinopathies arising from a single nucleotide mutation in the β-globin chain, which leads to a concomitant polymerization of sickle hemoglobin (HbS) when it is deoxygenated at low oxygen tension. Polymerization of hemoglobin (Hb) causes conformational changes that subsequently transforms the molecules into a network of insoluble fibrous polymers, ultimately resulting in sickling of the red blood cells (RBCs) in SCA patients (2, 3).

Sickled RBCs are characteristically rigid and are adherent to the intraluminal endothelium of blood vessels (4), causing changes in the mechanical shearing of the endothelial surface which, in turn, leads to endothelial dysfunction and related microvascular complications commonly seen in SCA patients (5, 6). In addition, vaso-occlusion, hyperviscosity and blood sludging can cause drastic changes in flow dynamics and tissue perfusion (hypoperfusion), which can result in end-organ complications, tissue ischemia and morbidity in SCA.

Periodic exchange or simple blood transfusion is an accepted treatment modality commonly used to manage SCA with the intended goal of ameliorating vaso-occlusion and improving tissue perfusion and blood flow. Several investigators have presented results that confirmed efficacy of transfusion therapy in the primary and secondary prevention of stroke, acute chest syndrome and symptomatic relief of severe anemia (7-10). Though also used for other indications (e.g., priapism, splenic sequestration), the practice is less evidence-based. Presumably, removing sickled RBCs and replacing them with normal RBCs improves oxygen carrying capacity and tissue perfusion. Complications of transfusion therapy include alloimmunization, hypersensitivity reactions, iron overload, and hyperviscosity (11-14). Moreover, there may be changes in blood flow dynamics which have not been previously studied.

In the pediatric Sickle Cell Clinic at the University of California Davis Medical Center (UCDMC), periodic exchange transfusion (not simple transfusion) has been adopted as a management modality of choice for children with SCA. Numerous studies have been performed to demonstrate post-transfusion improvement in oxygenation, which can be routinely measured by pulse oximetry (15-18). However, a search in the literature revealed that real-time changes in blood flow dynamics and microvascular

characteristics as well as improvement in tissue perfusion have not been directly investigated (19-21).

The paucity of information related to real-time microvascular changes in SCA arises from a lack of non-invasive, *in vivo* technology which can be used to document and quantify microvascular characteristics and longitudinal changes in SCA patients. 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 vascular diseases, including SCA and diabetes (22-24). A unique operational feature of this technology lies in its ability to identify individual conjunctival vessels and to relocate them in longitudinal studies, with each vessel serving as its own reference control. This makes CAIM an ideal platform to be used in studying pre-transfusion microvascular characteristics and post-transfusion changes.

C. Materials And Methods

1. *Patient Recruitment and Study Design*

Six pediatric SCA patients diagnosed with SCA (HbSS) between the ages of four to fifteen years from the UCDMC Sickle Cell Clinic were recruited and enrolled to participate in this study. The subjects were randomly selected from patients who were receiving transfusion therapy for either primary or secondary prevention of disease complications.

The pre- and post-transfusion microcirculation in the bulbar conjunctiva of the eye (conjunctival microcirculation) was imaged via video documentation in the clinic. Videotape sequences on each patient were analyzed for pre-transfusion microvascular

characteristics and post-transfusion changes using image analysis procedures developed in this laboratory. The experimental protocol and the study on human subjects were approved by the UCDMC Institutional Review Board. Signed informed consent was obtained from the patients or parents/guardians, whichever appropriate. This study was conducted in accordance with the Declaration of Helsinki human-use guidelines.

2. ***Exchange Transfusion***

In the UCDMC clinic, SCA patients were scheduled for exchange transfusion once a month. Upon arrival at the clinic, pre-transfusion hematocrit and blood pressure measurements were made for each patient to ensure that an exchange transfusion was warranted. Post-transfusion blood pressure measurements were made for future reference. The exchange transfusion procedure was based on a 1:1.5 (v:v) ratio. A specific volume of blood (10 ml/kg body weight) was phlebotomized from a patient and a 15 ml/kg body weight of donor blood was immediately infused. Venous blood sample was obtained from each patient prior to the transfusion process for blood typing and determination of Hb level, percentage HbS, hematocrit (Hct), and other related blood chemistry parameters. Pre- and post-transfusion measurement of oxygen saturation was performed via pulse oximetry.

3. ***Computer-Assisted Intravital Microscopy (CAIM)***

The CAIM technology was used to non-invasively study the conjunctival microcirculation and to quantify pre-transfusion microvascular characteristics and post-transfusion changes in this study. The procedural details have been presented in previous reports (5, 6) and summarized briefly as follows. Patients were asked to relax

for at least five minutes and cautioned against touching or rubbing the eye. Non-medicated sterile saline drops were administered in case of eye irritation and excessive drops were blotted. Each patient was asked to place his/her head securely against a forehead restraint and a chin rest. The lens of the CAIM system was carefully positioned to provide a clear, focused image of the perilimbal region in the bulbar conjunctiva of the left eye to give an 8.53 mm² area view of the site of interest. Various areas of the bulbar conjunctiva were videotaped in each patient using a charge-coupled device (CCD) video camera (COHU Model CCD-6415-3000) connected to a digital video recorder (Sony GV-HD700). A fiber-optics light source (Fiber-Lite Model 3100) with a Kodak #58 Wratten green (anti-red) filter was used to enhance contrast of the vessels in image display. The basic design of CAIM utilizes macro-optics as an operational platform so that focusing for image sharpness could be performed only by adjusting the physical distance between the lens and the bulbar conjunctiva. This allows video images to be obtained at the same magnification for all pre- and post-transfusion video recordings. Approximately five minutes of high-resolution video recordings of various areas in the bulbar conjunctiva were captured for subsequent viewing and computational measurements. All recordings were viewed in their entirety and sequences were selected based on image quality by investigators for subsequent analysis and measurements via image analysis. At least three video sequences with excellent image display from each patient were analyzed and all measurements were averaged.

4. ***Data Analysis***

Specific microvascular abnormalities (e.g., avascularity, tissue ischemia, abnormal vessel distribution) were identified from viewing of selected video sequences (5, 6). The same video sequences were analyzed using imaging analysis software

(AVID; co-developed at UC Davis and UC San Diego) to generate measurements of vessel diameter and red cell velocity for longitudinal comparison. Pre- and post-transfusion video sequences on the same vessel allowed for direct comparison of real-time microvascular changes to determine any increase or decrease in blood vessel diameter and red cell velocity arising as a result of the exchange transfusion process. This image analysis technique has been previously validated and described in previous studies (5, 6, 22, 23). Of specific interest to this study, emphasis was placed on post-transfusion changes in vessel diameter, red cell velocity and appearance of capillaries and arterioles in previously poorly or non-perfused areas. Other parameters of microangiopathy were also studied but are not reported in this manuscript.

5. ***Statistical Analysis***

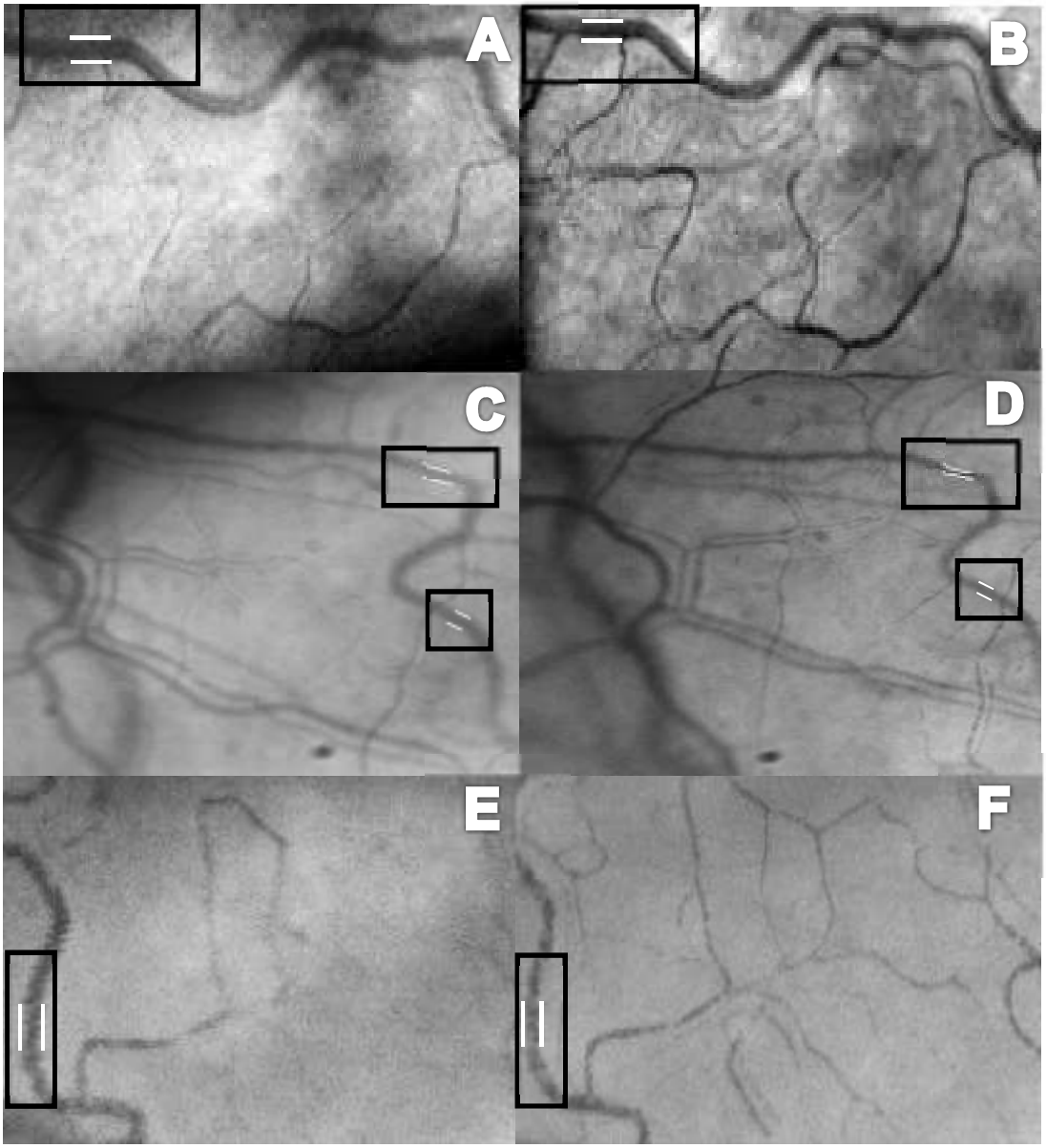
All measurements were averaged and results reported as mean ± SD. Differences between pre- and post-transfusion groups were compared using a non-parametric rank F-test. A p-value of ≤ 0.05 was statistically significant.

D. Results

The pre-transfusion hematocrit measurements of the SCA patients were low (25.68 ± 2.48; n = 6) and comparable with previous patients who needed an exchange transfusion as a disease management modality. The pre-transfusion blood pressure measurements of the patients were also low (100.40 ± 6.50 mmHg / 62.80 ± 10.03 mmHg; n = 5). Pre-transfusion images of all six SCA pediatric patients exhibited a "blanched" avascular appearance in the bulbar conjunctiva, indicative of diminished presence of capillaries and arterioles (**Figure 6-4-1A, 6-1C, 6-1E**). This "blanched"

appearance is similar to observations made in previous studies and is a unique characteristic of SCA (5, 6). The averaged diameters of the venules and red cell velocity from the six pediatric SCA patients were 48.51 ± 7.67μm and 0.95 ± 0.21mm/s respectively— these results were comparable with historical measurements (6). Oxygen saturation was measured via pulse oximetry in three out of six patients and showed below normal values in all three patients (95%).

Figure 6-1: Pre- and Post-Transfusion Frame-Captured Images



The pre- and post-transfusion frame-captured images showing microvascular changes in the bulbar conjunctiva of pediatric SCA patients. Optical magnification, 4.5x; on-screen magnification, 125x

Figure 6-1A. An image of the bulbar conjunctiva in a pediatric SCA patient (Patient #TF-2; age 8 years old). Note the characteristic "blanched" avascular appearance in the bulbar conjunctiva and a significantly diminished presence of arterioles and capillaries. The boxed area shows the presence of a typical venule with sludged blood flow in the patient. The parallel marker indicates the location where vessel diameter and red cell velocity were measured for comparison with **Figure 6-1B**.

Figure 6-1B. An image of the same location of the bulbar conjunctiva in the patient immediately following transfusion, showing a significant post-transfusion decrease in venular diameter. In addition, a substantial increase in tissue perfusion is shown as indicated by the enhanced presence of capillaries and arterioles.

Figure 6-1C. An image of the bulbar conjunctiva in a second SCA patient (Patient #TF-3; age 5 years old). Again, note the "blanched" avascular appearance similar to the appearance in Patient #TF-2 in **Figure 6-1A**. The two boxed areas and parallel markers indicate the locations where vessel diameters and red cell velocities were measured for comparison with **Figure 6-1D**.

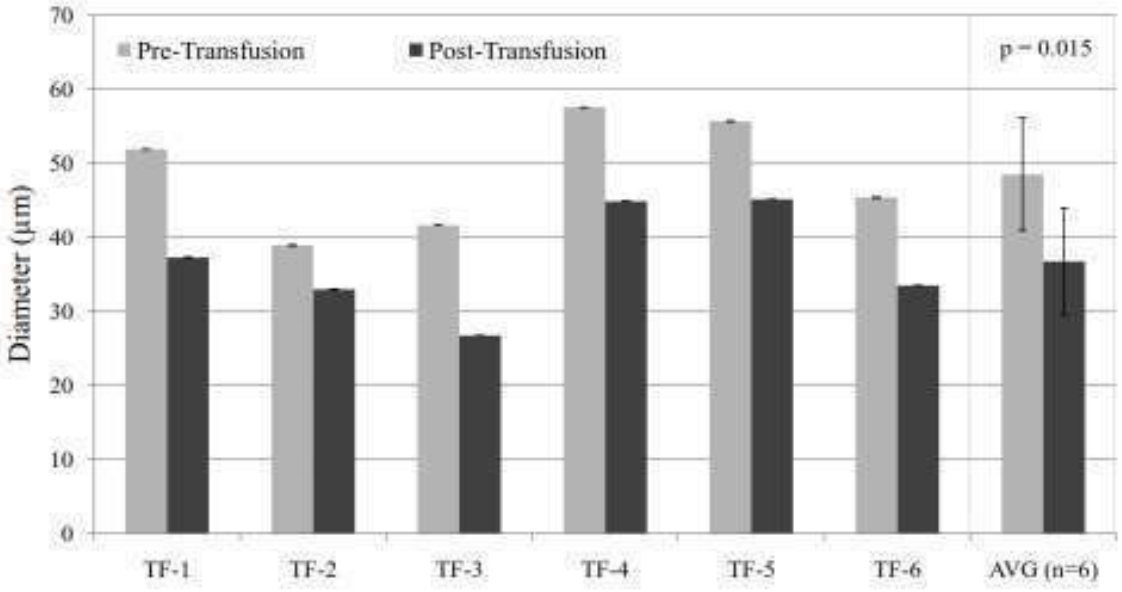
Figure 6-1D. An image of the same location in the bulbar conjunctiva shown in **Figure 1C** immediately following transfusion. Note the significant decreases in vessel diameter in the two locations (indicated by the parallel markers) and a substantial improvement in tissue perfusion.

Figure 6-1E. An image of the bulbar conjunctiva in a third SCA patient (Patient #TF-5; age 15 years old). Again, note the "blanched" avascular appearance similar to the appearance in Patients #TF-2 and #TF-3. The boxed area and parallel marker indicate the location where measurements were made for comparison with **Figure 6-1F.**

Figure 6-1F. An image on the same location in the bulbar conjunctiva shown in **Figure 6-1E** immediately following transfusion. Again, note the significant decrease in vessel diameter and substantial improvement in tissue perfusion.

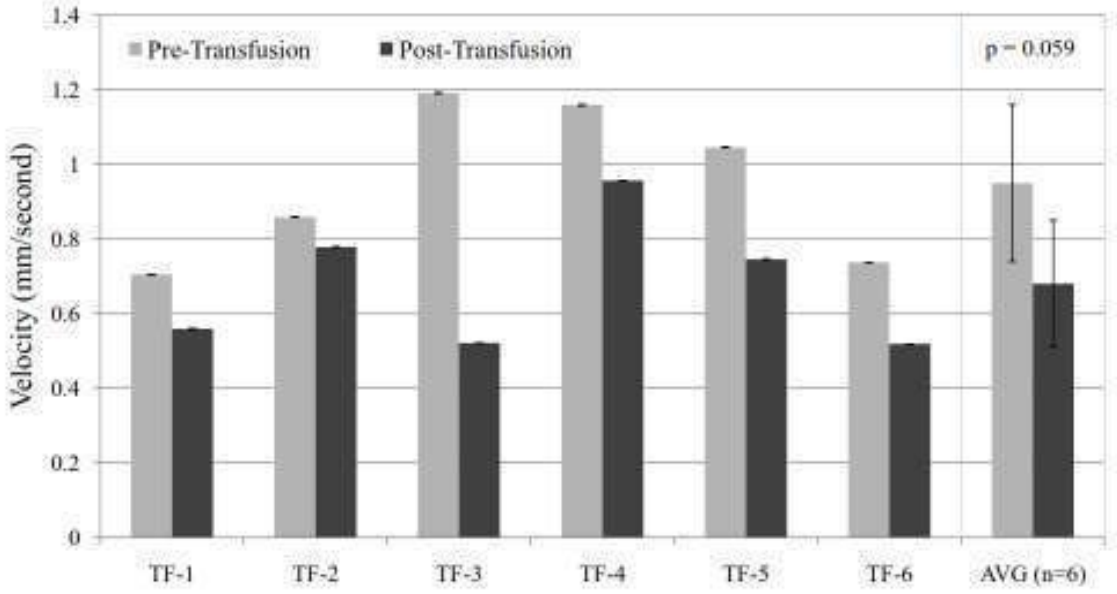
The post-transfusion blood pressure measurements of the patients showed a noticeable, albeit not significant, improvement from pre-transfusion values (104.75 ± 10.31 / 61.00 ± 7.26 mmHg; n = 4). The appearance of the post-transfusion bulbar conjunctiva and its microcirculation in the SCA patients differed from the pre-transfusion ischemic and hypoperfused state. There was substantial improvement in vascularization of the microvasculature (**Figure 6-4-1B, 6-1D, 6-1F**). In areas that were hypoperfused and devoid of capillaries and arterioles before transfusion, a significant increased presence of capillaries (and to lesser extent arterioles) was observed, indicative of improved vascular perfusion in the post-transfusion tissues. A significant decrease in averaged vessel diameter occurred immediately following transfusion (n = 6; 36.76 ± 7.23 μm; P = 0.015; Figure **6-2**). An observable and measurable, but statistically non-significant decrease in red cell velocity occurred after transfusion (n = 6; 0.68 ± 0.18 mm/s; P = 0.059; Figure **6-3**). In addition, oxygen saturation showed significant improvement in all three patients (99%) in whom this parameter was measured.

Figure 6-2: Pre- and Post-Transfusion Measurements of the Conjunctival Vessel Diameter in Six Pediatric SCA Patients



Vessel diameter measurement for each patient and averaged (mean ± SD) vessel diameter measurement for all six patients combined. Note the significant change in post-transfusion vessel diameter measurements (P = 0.015).

Figure 6-3: Pre- and Post-Transfusion Red Cell Velocity Measurements of the Conjunctival Vessels in Six Pediatric SCA Patients



Red cell velocity measurements for each patient and averaged (mean ± SD) red cell velocity measurement for all six patients combined. Note the substantial change in post-transfusion velocity measurements, albeit not statistically significant (P = 0.059).

E. Discussion

Stem cell transplantation is the only known cure for SCA (25). However, limited availability due to donor matching and risks of severe adverse events may outweigh its benefits (26). Consequently, palliative treatment modalities such as transfusion therapy are more commonly utilized in the management of SCA to ameliorate or prevent complications, including vaso-occlusion, blood sludging, tissue hypoperfusion and

ischemia, and diminished blood flow arising from SCA (2, 4). In addition, transfusion therapy has clear benefits in the primary and secondary prevention of stroke as well as for a variety of other indications with less compelling evidence (27-29).

Vaso-occlusion is commonly identified as the underlying factor causing end-organ clinical manifestations in SCA (e.g. priapism, splenic sequestration, acute chest syndrome, cerebrovascular accident, etc). Therefore, it is expected that after exchange transfusion therapy where sickled RBCs are replaced by normal RBCs in occluded and sludged vessels, there would be improvement in tissue perfusion. With the introduction of non-sickled RBCs (characteristically non-rigid, non-adherent and more deformable), vaso-occlusion and vessel sludging are ameliorated as reflected in the enhanced presence of previously inconspicuous capillaries and arterioles, thus providing direct evidence of improved perfusion (**Figure 6-1B, 6-1D, 6-1F**). Certainly, the pathophysiologic mechanism of vaso-occlusion cannot be attributed solely to RBC sickling as it is known that vaso-occlusion is multifactorial (30). There are a number of possible contributing factors in sickle cell vaso-occlusion (e.g. sickle cell non-deformability, sickle blood viscosity, endothelial cell activation, adhesion molecule upregulation, central nervous system responses) which transfusion therapy may not be able to completely remedy. Post-transfusion improvement in tissue perfusion may mitigate several of these complications.

Simple transfusion is not a management of choice for pediatric SCA patients because of the small blood volume in pediatric patients. Thus, the exchange transfusion protocol is normally preferred in pediatric clinics. In most sickle cell clinics, the amount of blood to be exchanged (phlebotomized and transfused) is computed and based on the following equation:

$$\text{Exchange Volume (ml)} = \frac{(Hct_d - Hct_i) \times TBV}{Hct_{rp} - (Hct_i + Hct_d)/2}$$

Hct_d, desired hematocrit; Hct_i, initial hematocrit;
Hct_{rp}, hematocrit of replacement cells (usually 0.7-0.8);
TBV, estimated total blood volume in ml.

The calculated amount of blood to be exchanged as based on this equation is approximately 50-60 ml/kg of body weight for an average adult. This amount of blood, which can be tolerated by an adult, is an unacceptably large blood volume in the case of a pediatric patient. In the exchange transfusion protocol used for pediatric patients at UCDMC, a volume of 10 ml/kg of blood is phlebotomized and 15 ml/kg of blood infused immediately. This 1:1.5 (v:v) ratio was used in the present study. From the results generated in this study, we can conclude that vaso-occlusion and tissue ischemia (specifically the lack of perfusing capillaries) were ameliorated with demonstrable improvements as shown in **Figure 6-1A-F**.

Despite observable improvements in perfusion, no improvement in blood flow was noted, as the averaged red cell velocity actually decreased (albeit not significantly) immediately after blood infusion in the exchange transfusion process. With the removal of sickled RBCs and introduction of normal RBCs, we expected that red cell velocity would immediately increase after an exchange transfusion. The decrease in red cell velocity was not expected though it could perhaps be attributed to a hyperviscosity condition, induced by a substantially increased presence of normal RBCs in a non-physiological infusion volume. The velocity change we observed was in agreement with the concept of induced hyperviscosity and hematocrit balance in an optimal transfusion

suggested by Vichinsky in his discussion on the balance between transfusion volume, viscosity, hemotocrit, and blood flow dynamics (15).

The *in vivo* images we generated (**Figure 6-1A-F**) represent the first documentation of its kind on real-time microvascular changes arising from the exchange transfusion process and may shed light on future transfusion studies. The concomitant decrease in vessel diameter which we observed could be attributed to a homeostatic feedback mechanism in an attempt to alter flow dynamics so as to maintain adequate venous return, cardiac output and mean arterial pressure via the sympathetic (T1-L2) adrenergic pathway. The exchange transfusion process is designed to improve blood flow and oxygen-carrying capacity, thereby improving tissue perfusion and mitigating vaso-occlusion. However, with the paradoxical post-transfusion decrease in red cell velocity, the intended benefits of exchange transfusion may be offset by the adverse effect of increased viscosity, thus thwarting improved optimal oxygen delivery and blood flow and paradoxically promoting vaso-occlusion (15, 28-30). It is apparent that the exchange volume may need adjustment for an optimal outcome to counteract the reduction in red cell velocity, which is counterproductive in the transfusion process.

Direct implications from this study suggest that future considerations for exchange transfusion guidelines should include the measurement of whole blood viscosity and optimum Hct to prevent unintended outcomes. As discussed in previous studies in the literature, there is a delicate balance between red cell transfusion and Hct target levels which merits emphasis (31). It may be useful to utilize these added parameters to develop a mathematical model to predict the redistribution of and improvement in post-transfusion blood flow dynamics. It would be valuable to conduct follow-up studies using a larger cohort of subjects, inclusion of additional time points for observations (e.g., immediately post-transfusion, 1-, 2-, 6-, and 12-hours post-

transfusion), and designing the study to measure whole blood viscosity and Hct to characterize the temporal changes in blood flow dynamics. This would provide a comprehensive evaluation of the physiologic effects of exchange transfusion therapy on the microcirculation.

F. References

1. Hassell KL. *Population estimates of sickle cell disease in the U.S*. Am J Prev Med, 2010. 38(4 Suppl): p. S512-21.
2. Kasper DL and Harrison TR. *Harrison's Principles of Internal Medicine*. 2005, New York: McGraw-Hill, Medical Pub. Division.
3. Champe PC, Harvey RA, and Ferrier DR. *Lippincott's Illustrated Reviews: Biochemistry*. 2005, Philadelphia: Lippincott Williams & Wilkins.
4. Cotran RS, Kumar V, and Collins T. *Robbins Pathologic Basis of Disease*. 1999, Philadelphia: Saunders.
5. Cheung AT, Chen PC, Larkin EC, Duong PL, Ramanujam S, Tablin F, Wun T. *Microvascular abnormalities in sickle cell disease: a computer-assisted intravital microscopy study*. Blood, 2002. 99(11): p. 3999-4005.
6. Cheung AT, Miller JW, Craig SM, To PL, Lin X, Samarron SL, Chen PC, Zwerdling T, Wun T, Li CS, Green R. *Comparison of real-time microvascular abnormalities in pediatric and adult sickle cell anemia patients*. Am J Hematol, 2010. 85(11): p. 899-901.
7. Nichols FT, Jones AM, and Adams RJ. *Stroke prevention in sickle cell disease (STOP) study guidelines for transcranial Doppler testing*. J Neuroimaging, 2001. 11(4): p. 354-62.
8. Cohen AR, Martin MB, Silber JH, Kim HC, Ohene-Frempong K, Schwartz E. *A modified transfusion program for prevention of stroke in sickle cell disease*. Blood, 1992. 79(7): p. 1657-61.
9. Emre U, Miller ST, Gutierez M, Steiner P, Rao SP, Rao M. *Effect of transfusion in acute chest syndrome of sickle cell disease*. J Pediatr, 1995. 127(6): p. 901-4.
10. Adams RJ, McKie VC, Hsu L, Files B, Vichinsky E, Pegelow C, Abboud M, Gallagher D, Kutlar A, Nichols FT, Bonds DR, Brambilla D. *Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography*. N Engl J Med, 1998. 339(1): p. 5-11.
11. Vichinsky EP, Earles A, Johnson RA, Hoag MS, Williams A, Lubin B. *Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood*. N Engl J Med, 1990. 322(23): p. 1617-21.
12. Vichinsky EP, and Ohene-Frempong K. *Approaches to Transfusion Therapy and Iron Overload in Patients with Sickle Cell Disease: Results of an International Survey*. Pediatr Hematol Oncol, 2010.
13. Orlina AR, Unger PJ, and Koshy M. *Post-transfusion alloimmunization in patients with sickle cell disease*. Am J Hematol, 1978. 5(2): p. 101-6.
14. Fung EB, Harmatz PR, Milet M, Balasa V, Ballas SK, Casella JF, Hilliard L, Kutlar A, McClain KL, Olivieri NF, Porter JB, Vichinsky EP. *Disparity in the management of iron overload between patients with sickle cell disease and thalassemia who received transfusions*. Transfusion, 2008. 48(9): p. 1971-80.

15. Vichinsky EP. *Transfusion Therapy*. In: Sickle cell disease: Basic principles and clinical practice. 1994, New York: Raven Press: p. 781-798

16. Raj A, Bertolone SJ, Mangold S, Edmonds HL Jr. *Assessment of cerebral tissue oxygenation in patients with sickle cell disease: effect of transfusion therapy*. J Pediatr Hematol Oncol, 2004. 26(5): p. 279-83.

17. Smith MJ, Stiefel MF, Magge S, Frangos S, Bloom S, Gracias V, Le Roux PD. *Packed red blood cell transfusion increases local cerebral oxygenation*. Crit Care Med, 2005. 33(5): p. 1104-8.

18. Thorling EB, and Erslev AJ. *The "tissue" tension of oxygen and its relation to hematocrit and erythropoiesis*. Blood, 1968. 31(3): p. 332-43.

19. Miller DM, Winslow RM, Klein HG, Wilson KC, Brown FL, Statham NJ. *Improved exercise performance after exchange transfusion in subjects with sickle cell anemia*. Blood, 1980. 56(6): p. 1127-31.

20. Kleinman SH, Hurvitz CG, and Goldfinger D. *Use of erythrocytapheresis in the treatment of patients with sickle cell anemia*. J Clin Apher, 1984. 2(2): p. 170-6.

21. Warrier PR, Ducos R, and Yu LC. *Acute chest syndrome in sickle cell disease*. J Pediatr, 1986. 109(4): p. 731.

22. Cheung AT, Price AR, Duong PL, et al., *Microvascular abnormalities in pediatric diabetic patients*. Microvasc Res, 2002. 63(3): p. 252-8.

23. Cheung AT, Price AR, Duong PL, Ramanujam S, Gut J, Larkin EC, Chen PC, Wilson DM. *Microvascular abnormalities in the bulbar conjunctiva of patients with type 2 diabetes mellitus*. Endocr Pract, 2001. 7(5): p. 358-63.

24. Cheung AT, Tomic MM, Chen PC, Miguelino E, Li CS, Devaraj S. *Correlation of microvascular abnormalities and endothelial dysfunction in Type-1 Diabetes Mellitus (T1DM): a real-time intravital microscopy study*. Clin Hemorheol Microcirc, 2009. 42(4): p. 285-95.

25. Walters MC, Patience M, Leisenring W, Eckman JR, Scott JP, Mentzer WC, Davies SC, Ohene-Frempong K, Bernaudin F, Matthews DC, Storb R, Sullivan KM. *Bone marrow transplantation for sickle cell disease*. N Engl J Med, 1996. 335(6): p. 369-76.

26. Platt OS, Guinan EC. *Bone marrow transplantation in sickle cell anemia--the dilemma of choice*. N Engl J Med, 1996. 335(6): p. 426-8.

27. Hulbert ML, Scothorn DJ, Panepinto JA, Scott JP, Buchanan GR, Sarnaik S, Fallon R, Chu JY, Wang W, Casella JF, Resar L, Berman B, Adamkiewicz T, Hsu LL, Smith-Whitley K, Mahoney D, Woods G, Watanabe M, DeBaun MR. *Exchange blood transfusion compared with simple transfusion for first overt stroke is associated with a lower risk of subsequent stroke: a retrospective cohort study of 137 children with sickle cell anemia*. J Pediatr, 2006. 149(5): p. 710-2.

28. Hurlet-Jensen AM, Prohovnik I, Pavlakis SG, Piomelli S. *Effects of total hemoglobin and hemoglobin S concentration on cerebral blood flow during transfusion therapy to prevent stroke in sickle cell disease*. Stroke, 1994. 25(8): p. 1688-92.

29. Charache SC, Scott JC, Charache P. *"Acute chest syndrome" in adults with sickle cell anemia*. Microbiology, treatment, and prevention. Arch Intern Med, 1979. 139(1): p. 67-9.

30. Embury S, Hebbel R, Steinberg M, et al. *Pathogenesis of vasoooccclusion. In: Sickle cell disease: Basic principles and clinical practice*. 1994, New York: Raven Press: p. 311-326

31. Schmalzer EA, Lee JO, Brown AK, Usami S, Chien S. *Viscosity of mixtures of sickle and normal red cells at varying hematocrit levels*. Implications for transfusion. Transfusion, 1987. 27(3): p. 228-33.

Chapter 7: Summary and Future Directions

Microvasculopathy underlies much of the morbidity associated with SCD. At present, there is little that can be done clinically to lessen or ameliorate the severity or progression of the microvascular damage in SCD. There is also great variability in severity of symptoms among SCD patients. However, there are no identified biomarkers to elucidate which SCD patients will be more symptomatic or have more rapid disease progression.

The objective of this study was to identify predictive biomarkers of microvascular abnormalities in SCA patients with the long-term goal of designing novel interventions to reduce morbidity and mortality caused by vascular damage in this patient population. The specific aims were to assess and quantify microvascular abnormalities in SCA patients, and to determine the cross-sectional relationships between B vitamin status, inflammatory cytokines, vascular adhesion molecules, and microvascular characteristics in SCA patients under steady-state conditions.

A. Summary of Results

1. Cross-Sectional Relationships of Biomarkers

The results focused on the independent associations of each variable with severity index after controlling for age and sex, as well as the change in the severity index explained by the independent variable over the model that includes age and sex alone.

It was found that age is a strong determinant of microvasculopathy severity, as determined by CAIM, with the CAIM severity index (SI) increasing about 0.1 units per 1 year increase in age ($P<0.001$). Homocysteine was also significantly associated with SI after controlling for age and sex ($P=0.047$), as was MCP-1 ($P=0.026$). No other measured parameters were found to be associated with microvasculopathy.

2. Comparison of Microvascular Abnormalities

The conjunctival microcirculation in the pediatric and adult SCA patients uniquely differs from those found in non-SCD control subjects. There was diminished conjunctival vessels and abnormal vascular density in most SCA patients in both age groups, giving the bulbar conjunctiva a “blanched” avascular appearance. SCA patients from both age groups exhibit, to varying degrees, the same morphometric and dynamic abnormalities, including abnormal vessel diameter, abnormal vessel distribution, abnormal vessel morphometry (shape), sludged flow, vessel tortuosity, abnormal A:V ratio, boxcar blood flow, hemosiderin deposits, and abnormal flow (red cell) velocity.

These microvascular abnormalities are rarely found in the bulbar conjunctiva of healthy non-SCD subjects. The severity of microvasculopathy, as indicated by SI, was significantly lower in the pediatric patients than in the adult patients (4.2 ± 1.8 vs. 6.6 ± 2.4, $P = 0.028$). For comparison, the mean SI values for both the pediatric and adult SCA patients were significantly higher than the mean SI value determined for a previous cohort of healthy non-SCD subjects ($n = 10$; age 15-60 years; $SI = 0.31 \pm 0.72$; $P < 0.05$) (1).

3. Exchange Transfusion Therapy

Pre-transfusion images of six pediatric SCA patients exhibited a “blanched” avascular appearance in the bulbar conjunctiva, indicative of diminished capillaries and arterioles. The averaged diameters of the venules and red cell velocity from the six pediatric SCA patients were 48.51 ± 7.67 μm and 0.95 ± 0.21 mm/s respectively.

The appearance of the post-transfusion bulbar conjunctiva and its microcirculation in the SCA patients differed from the pre-transfusion ischemic and hypoperfused state. There was substantial increase in vessel density of the microvasculature. In areas that were hypoperfused and devoid of capillaries and arterioles before transfusion, increased presence of capillaries (and to lesser extent arterioles) was observed, indicative of improved vascular perfusion following transfusion.

A significant decrease in average vessel diameter occurred immediately following transfusion ($n = 6$; 36.76 ± 7.23 μm; $p = 0.015$). An observable and measurable, but statistically non-significant decrease in red cell velocity occurred after transfusion ($n = 6$; 0.68 ± 0.18 mm/s; $P = 0.059$). In addition, oxygen saturation showed significant improvement in all three patients (99%) in whom this parameter was measured.

B. Conclusion and Future Directions

With current improvements in treatment including general supportive measures, the population of patients with SCD is progressively living long than previous generations. Consequently, chronic vascular damage (pulmonary hypertension, renal disease, silent cerebral infarctions) will account for proportion of the morbidity of SCD.

Interventions aimed at improving vascular function and decreasing damage have the potential to mitigate such morbidity.

The microvascular abnormalities are fewer in children than in adults, suggesting SCD is a progressive microvascular disease and intervention therapies should probably start very early in life. Currently, there is little in the way of safe and effective interventions or therapies. Hydroxyurea is a frequently prescribed therapy that increases the concentration of fetal hemoglobin and reduces hemoglobin S polymerization (2); however, it is not without potential risk. Side effects of hydroxyurea treatment run the gamut from nausea to a possible increased risk of acute myeloid leukemia (3). Another example of a common therapy for SCD is blood transfusion, which also comes with potential risks. High frequency of transfusions has been shown to increase damage to the vascular, liver, heart and pancreas through iron overload (4). Moreover, elevated iron status correlates with MCP-1 release, which may contribute to vascular damage (5).

The goal of this project was to find biomarkers that might potentially provide clues for target interventions to reduce microvasculopathies in SCA. Of the many biomarkers measured, one that stood out was homocysteine; another was MCP-1. Our study demonstrated that age was a strong determinant of microvascular severity, and that homocysteine and MCP-1 were also significantly associated with severity index after controlling for age and sex in SCA patients.

Although the mechanistic role of homocysteine in the development of vascular disease has yet to be definitively determined, in the general population or SCD, there is clear evidence that elevated homocysteine has a strong associated with all forms of

vascular disease (6-10). Additionally, studies have demonstrated that B-vitamin supplementation, specifically folate, B12 and B6, lowers homocysteine levels (11-14). Therefore, we conclude, from our studies, that high dose B vitamin therapy started early in life will maintain low blood levels of homocysteine, and possibly could prevent or slow the progression of microvasculopathy in SCD patients.

Additionally, B vitamin intervention might affect MCP-1 and other markers of inflammation and microvasculopathy. MCP-1 is a cytokine that is elevated when there is endothelial activation and inflammation (15, 16), and in our study, SCD patients showed MCP-1 correlated with the extent of their microvasculopathy. It may be the case with B vitamin supplementation that we would see a lowering of MCP-1, and therefore MCP-1 could be an intermediate biomarker of improved microvascular health used to determine how well an intervention is working. It should be noted, however, that we did not see a direct correlation between homocysteine and MCP-1.

B-vitamin supplementation could effectively ameliorate or reduce microvascular damage, and subsequently extend life expectancy in SCD patients. If effective, B vitamin supplementation for patients with SCD would represent a low-cost, minimal risk nutritional intervention with clinical outcomes that would lessen the morbidity and mortality of the disease and substantially improve the quality of life for these patients. Hence, our findings suggest that there should be aggressive B vitamin, placebo controlled trials in pediatric SCD patients to determine whether this would slow the progression of microvasculopathy by maintaining healthy levels of homocysteine. At the same time, MCP-1 levels could be monitored as a potential marker of vascular inflammation. The noninvasive CAIM procedure could be used to monitor microvasculopathy to determine efficacy of the intervention.

C. References

1. Cheung A, Tomic M, Chen P, Miguelino E, Li C, Devaraj S. *Correlation of microvascular abnormalities and endothelial dysfunction in Type-1 Diabetes Mellitus (T1DM): a real-time intravital microscopy study*. Clin Hemorheol Microcirc 2009;42(4):285-95.
2. Cokic VP, Smith RD, Beleslin-Cokic BB, Njoroge JM, Miller JL, Gladwin MT, Schechter AN. *Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase*. J Clin Invest 2003;111(2):231-9.
3. Platt OS. *Hydroxyurea for the treatment of sickle cell anemia*. N Engl J Med 2008;358(13):1362-9.
4. Lee MT, Piomelli S, Granger S, Miller ST, Harkness S, Brambilla DJ, Adams RJ. *Stroke Prevention Trial in Sickle Cell Anemia (STOP): extended follow-up and final results*. Blood 2006;108(3):847-52.
5. Valenti L, Dongiovanni P, Motta BM, Swinkels DW, Bonara P, Rametta R, Burdick L, Frugoni C, Fracanzani AL, Fargion S. *Serum hepcidin and macrophage iron correlate with MCP-1 release and vascular damage in patients with metabolic syndrome alterations*. Arterioscler Thromb Vasc Biol 2011;31(3):683-90.
6. Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PWF, Belanger AJ, O'Leary DH, Wolf PA, Schaefer EJ, Rosenberg IH. *Association between Plasma Homocysteine Concentrations and Extracranial Carotid-Artery Stenosis*. New England Journal of Medicine 1995;332(5):286-91.
7. Choy PC, Mymin D, Zhu Q, Dakshinamurti K, O K. *Atherosclerosis risk factors: the possible role of homocysteine*. Mol Cell Biochem 2000;207(1-2):143-8.
8. Stubbs PJ, Al-Obaidi MK, Conroy RM, Collinson PO, Graham IM, Noble IM. *Effect of plasma homocysteine concentration on early and late events in patients with acute coronary syndromes*. Circulation 2000;102(6):605-10.
9. Collaboration HS. *Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis*. JAMA 2002;288(16):2015-22.
10. *Genomic resource centre: Genes and human disease*. Internet: http://www.who.int/entity/genomics/public/Maphaemoglobin.pdf. World Health Organization. (accessed April 2012).
11. Segal JB, Miller ER, 3rd, Brereton NH, Resar LM. *Concentrations of B vitamins and homocysteine in children with sickle cell anemia*. South Med J 2004;97(2):149-55.
12. Balasa BV, Kalinyak KA, Bean JA SD, Gruppo RA. *Hyperhomocysteinemia is associated with low plasma pyridoxine levels in children with sickle cell disease*. J Pediatr Hematol Oncol 2002;24:374-9.
13. van der Dijs FP, Fokkema M, Dijck-Brouwer D, Niessink B, Wal Tvd, Schnog J, Duits A, Muskiet F, Muskiet F. *Optimization of folic acid, vitamin B(12), and vitamin B(6) supplements in pediatric patients with sickle cell disease*. Am J Hematol 2002;69(4):239-46.

14. Kennedy TS, Fung EB, Kawchak DA, Zemel BS, Ohene-Frempong K, Stallings VA. *Red blood cell folate and serum vitamin B12 status in children with sickle cell disease*. J Pediatr Hematol Oncol 2001;23(3):165-9.

15. Papayianni A, Alexopoulos E, Giamalis P, Gionanlis L, Belechri A-M, Koukoudis P, Memmos D. *Circulating levels of ICAM-1, VCAM-1, and MCP-1 are increased in haemodialysis patients: association with inflammation, dyslipidaemia, and vascular events*. Nephrol Dial Transplant 2002;17:432-41.

16. Schwartz CJ, Valente AJ, Sprague EA, Kelley JL, Nerem RM. *The pathogenesis of atherosclerosis: an overview*. Clin Cardiol 1991;14 (2 Suppl 1):I1-16.

Appendix A: Supplementary Tables

1. Patient Demographics, Detailed

Study ID	Gender	Age	Transfused	Hydroxyurea	Folic Acid Supplement[1]	Multivitamin Supplement
SCD-B6-28	F	4	√			√
SCD-B6-11	F	6				
SCD-B6-12	F	8	√			
SCD-B6-04	F	9	√		√	
SCD-B6-03	F	13	√	√		
SCD-B6-15	F	13	√			
SCD-B6-29	F	15	√			
SCD-B6-10	F	17	√			
SCD-B6-16	F	17	√		√	
SCD-B6-24	F	17	√			
SCD-B6-07	F	18	√			
SCD-B6-19	F	18				
SCD-B6-27	M	5	√			√
SCD-B6-08	M	8	√			√
SCD-B6-30	M	8	√			
SCD-B6-02	M	12	√	√	√	
SCD-B6-18	M	15				
SCD-B6-17	M	17	√			
SCD-B6-01	M	18	√			
SCD-B6-13	F	22	√			√
SCD-B6-26	F	23	√			
SCD-B6-25	F	26	√	√	√	√
SCD-B6-23	F	27			√	√
SCD-B6-35	F	27	√		√	
SCD-B6-09	F	29	√	√	√	
SCD-B6-21	F	30			√	
SCD-B6-05	F	31	√	√	√	√
SCD-B6-22	F	31		√	√	√
SCD-B6-32	F	31			√	√
SCD-B6-33	F	33	√	√	√	
SCD-B6-06	F	36		√		
SCD-B6-38	F	39				
SCD-B6-20	F	53			√ **IC**	
SCD-B6-31	F	61		√	√ **IC**	
SCD-B6-36	M	26	√	√	√	
SCD-B6-37	M	31			√ **IC**	
SCD-B6-34	M	48			√	
SCD-B6-14	M	58		√	√	√

[1] The folic acid data represent those who have been prescribed folic acid supplementation; this is not reflective of compliance. Folic acid values labeled √ **IC** are those subjects who were in compliance with their hematologist's recommendations.

2. B-Vitamins and Related Analytes

Study ID	Gender	Age	PLP, plasma (ng/mL)	PLP, RBC (nM/L)	PL, plasma (nM/L)	PL, RBC (nM/L)	4-PA, plasma (nM/L)	Kyn, plasma (µM/L)	Try plasma (µM/L)	Trp/Kyn Ratio
SCD-B6-28	F	4	162.0	691.5	121.7	115.95	174.93	2.6	85.4	32.58
SCD-B6-11	F	6	47.1	121.7	10.2	0.00	27.40	2.49	42.59	17.10
SCD-B6-12	F	8	69.9	227.1	26.8	4.01	20.31	3.97	72.53	18.27
SCD-B6-04	F	9	38.2		13.7		29.74			
SCD-B6-03	F	13	41.3		9.1		10.71			
SCD-B6-15	F	13	79.5		189.9					
SCD-B6-29	F	15	39.3	223.4	8.2	0.00	14.41	1.6	46.8	29.08
SCD-B6-10	F	17	36.2	218.4	6.5	0.04	30.22	1.62	60.87	37.57
SCD-B6-16	F	17	44.9	233.3	12.5	6.86	38.86	1.41	37.79	26.80
SCD-B6-07	F	18	71.8	490.3	23.2	46.33	81.94	3.16	64.41	20.38
SCD-B6-19	F	18	24.7	145.1	4.8	485.17	7.63	1.73	65.39	37.80
SCD-B6-27	M	5	139.4	671.6	87.9	88.87	90.73	2.5	98.2	38.68
SCD-B6-08	M	8	23.5	232.0	5.2	0.00	4.57	2.55	45.37	17.79
SCD-B6-30	M	8	28.1	256.4	13.7	8.13	36.56	1.7	52.6	31.30
SCD-B6-02	M	12	61.1		20.2		12.65			
SCD-B6-18	M	15	22.9	242.2	5.3	6.12	13.23			
SCD-B6-17	M	17	22.6		5.4		29.27			
SCD-B6-01	M	18	27.4							
SCD-B6-13	F	22	28.7		5.8		15.33	2.2	78.26	35.57
SCD-B6-26	F	23	19.8		0.0					
SCD-B6-23	F	27	42.8	398.1	11.2	15.66	16.06	2.5	60.1	23.94
SCD-B6-35	F	27			0.0					
SCD-B6-09	F	29	42.2	265.2	8.9	4.11	29920	1.03	42.66	41.42
SCD-B6-21	F	30	102.6					2.21	59.11	26.75
SCD-B6-05	F	31	21.7	375.6	5.4	0.00	13.12	0.87	21.21	24.38
SCD-B6-22	F	31	30.8	246.78	9.2	11.22	21.51	3.56	57.78	16.23
SCD-B6-32	F	31	36.6		6.4	0.00	21.99	2.5	57.9	22.96
SCD-B6-33	F	33	11.6		3.8	0.00	6.83	1.7	75.6	45.26
SCD-B6-06	F	36	56.9	316.7	10	9.91	31.95	1.67	45.11	27.01
SCD-B6-38	F	39	39.5		6.9					
SCD-B6-20	F	53	16.7				236.1	2.81	43.7	15.55
SCD-B6-31	F	61	49.3		29.4	8.01	1418.2	1.0	43.7	45.02
SCD-B6-36	M	26	13.1		0.0					
SCD-B6-37	M	31	29.7		8.1					
SCD-B6-34	M	48	68.0		15.4	15.22	236.1	3.0	53.0	17.60
SCD-B6-14	M	58	52.3	661.5	22.4	41.21	138.42	4.87	35.03	7.19

3. B-Vitamins and Related Analytes – continued

Study ID	Gender	Age	Folate, plasma (ng/mL)	Vitamin B12, plasma (pg/mL)	Homocysteine, plasma (umol/L)	Cysteine, plasma (ng/mL)
SCD-B6-28	F	4	> 20	508.0	4.6	275.8
SCD-B6-11	F	6	18.7	629	4.6	237.0
SCD-B6-12	F	8	> 20	793	2.6	166.9
SCD-B6-04	F	9	> 20	431	7.0	294.3
SCD-B6-03	F	13	11.4	365	7.6	241.1
SCD-B6-15	F	13	> 20	375	3.9	201.6
SCD-B6-29	F	15	12.5	757.0	3.6	206.7
SCD-B6-10	F	17	16.0	450	6.4	322.9
SCD-B6-16	F	17	19.8	678	4.2	250.6
SCD-B6-07	F	18	7.7	338	6.4	271.1
SCD-B6-19	F	18	12.1	313	5.3	217.7
SCD-B6-27	M	5	> 20	722.0	4.3	263.1
SCD-B6-08	M	8	> 20	758	5.8	286.0
SCD-B6-30	M	8	> 20	590.0	4.9	255.4
SCD-B6-02	M	12	> 20	345	6.6	286.4
SCD-B6-18	M	15	13.8	659	7.8	226.0
SCD-B6-17	M	17	4.1	333	7.4	233.3
SCD-B6-01	M	18	9.5	607	9.6	346.8
SCD-B6-13	F	22	18.8	652	4.4	248.3
SCD-B6-26	F	23	12.0	1744	6.9	274.6
SCD-B6-23	F	27	5.0	504.0	7.4	305.5
SCD-B6-35	F	27	>20	460.0	7.4	261.7
SCD-B6-09	F	29	> 20	493	4.9	268.7
SCD-B6-21	F	30	8.4	663	6.8	254.5
SCD-B6-05	F	31	15.2	385	5.1	297.2
SCD-B6-22	F	31	> 20	485	3.6	239.0
SCD-B6-32	F	31			7.8	287.3
SCD-B6-33	F	33	4.9	459.0	6.5	295.7
SCD-B6-06	F	36	10.3	384	7.5	316.1
SCD-B6-38	F	39	6.1	428.0	18.9	207.3
SCD-B6-20	F	53	> 20	331	9.6	300.0
SCD-B6-31	F	61	> 20	376.0	7.0	274.2
SCD-B6-36	M	26	9.3	321.0	10.3	323.5
SCD-B6-37	M	31	12.8	358.0	10.1	341.6
SCD-B6-34	M	48	11.0	741.0	8.0	256.6
SCD-B6-14	M	58	> 20	478	17.0	393.0

4. Vascular Adhesion Molecules and Inflammatory Markers

Study ID	Gender	Age	BNP, plasma (pg/mL)	VCAM-1, serum (ng/mL)	ICAM-1, serum (mg/mL)	E-Selectin, serum (mg/L)	P-selectin, serum (mg/L)	VEGF, serum (ng/L)
SCD-B6-28	F	4	26	1220.00	330.50	181.24	95.54	0
SCD-B6-11	F	6	119	1688.60	392.18	130.51	66.47	0
SCD-B6-12	F	8		1361.18	372.11	120.30	119.21	0
SCD-B6-04	F	9	93	518.82	332.52	414.73	229.28	18.12
SCD-B6-03	F	13	42	617.20	296.81	125.21	160.29	7.74
SCD-B6-15	F	13	19	557.24	198.52	56.53	55.05	0
SCD-B6-29	F	15		869.00	310.41	49.53	128.75	47
SCD-B6-10	F	17	39	498.84	254.88	97.71	80.91	0
SCD-B6-16	F	17	45	744.78	323.33	154.94	105.71	0
SCD-B6-07	F	18	126	976.88	237.87	127.11	70.42	28.12
SCD-B6-19	F	18	35	898.50	218.47	30.42	88.02	0
SCD-B6-27	M	5	47	1151.00	416.65	112.84	100.94	0
SCD-B6-08	M	8	78	1450.34	341.59	171.13	83.90	0
SCD-B6-30	M	8		895.00	301.04	83.02	89.09	0
SCD-B6-02	M	12	66	1281.24	311.71	186.91	84.18	0
SCD-B6-18	M	15	121	1379.62	341.12	216.17	95.99	0
SCD-B6-17	M	17	55	734.02	314.69	102.65	142.90	0
SCD-B6-01	M	18	16	944.6	301.84	46.29	80.91	0
SCD-B6-13	F	22		555.00	287.17	82.31	63.86	0
SCD-B6-26	F	23	57					
SCD-B6-23	F	27	69	1042.00	254.51	67.07	75.99	0
SCD-B6-35	F	27	20					
SCD-B6-09	F	29	41	873.90	322.23	72.47	76.98	0
SCD-B6-21	F	30	68	844.69	343.58	122.23	81.69	0
SCD-B6-05	F	31	203	1528.72	302.39	58.56	92.38	27.64
SCD-B6-22	F	31	25	697.12	416.00	98.98	53.98	0
SCD-B6-32	F	31	12	506.00	171.11	18.67	44.97	0
SCD-B6-33	F	33	81		265.98			11.13
SCD-B6-06	F	36	116	909.26	151.87	52.33	62.74	0
SCD-B6-38	F	39	4					
SCD-B6-20	F	53	157	2209.68	270.90	91.79	65.15	17.65
SCD-B6-31	F	61	13	421.00	219.47	54.64	71.44	17.81
SCD-B6-36	M	26	167					
SCD-B6-37	M	31	175					
SCD-B6-34	M	48						0
SCD-B6-14	M	58	901	3533.18	252.50	35.22	62.22	41

The values listed as “0” were below the sensitivity (out of range) of the multiplex assay.

5. Inflammatory Markers and Cytokines

Study ID	Gender	Age	CRP, plasma (mg/dL)	TNF-α, serum (pg/mL)	IL-1β, serum (ng/mL)	IL-2, serum (ng/mL)	IL-6, serum (ng/ML)	IL-8, serum (ng/mL)	MCP-1, serum (ng/mL)
SCD-B6-28	F	4	0.5	0.25	0.34	0	1.77	7.08	236
SCD-B6-11	F	6	0.3	0	0	0	0	17	404
SCD-B6-12	F	8	1.0	2.65	3.25	0	0	6.52	251
SCD-B6-04	F	9		0.25	6.92	0	0	9.79	695
SCD-B6-03	F	13	< 0.1	0.01	0.15	0	0	57	349
SCD-B6-15	F	13	< 0.1	0.05	0	0	0	33	466
SCD-B6-29	F	15	0.2	0.82	2.66	0	0.19	33	603
SCD-B6-10	F	17	< 0.1	0.05	0.15	0	0	12.1	361
SCD-B6-16	F	17	0.4	1.77	0.63	0	0	26	469
SCD-B6-07	F	18	2.5	0	0	0	18	11.99	280
SCD-B6-19	F	18	< 0.1	0	0	0	0	27	198
SCD-B6-27	M	5	< 0.1	0	1.49	0	0	7.08	185
SCD-B6-08	M	8	0.1	0	0	0	0	7.97	217
SCD-B6-30	M	8	0.2	0.14	2.08	0	0	40	449
SCD-B6-02	M	12	0.2	0.05	0	0	0	8.2	122
SCD-B6-18	M	15	0.4	1.70	1.11	0	0	17	296
SCD-B6-17	M	17	0.2	2.30	1.88	0	0	14	283
SCD-B6-01	M	18	0.1	0.88	0.91	0	5.29	86	855
SCD-B6-13	F	22	0.2	0	0.15	0	0	22	204
SCD-B6-26	F	23	0.8						
SCD-B6-23	F	27	< 0.1	8.11	8.82	0	88	12.54	304
SCD-B6-35	F	27	0.5						
SCD-B6-09	F	29	0.3	0	0.91	0	6.17	25	600
SCD-B6-21	F	30	0.2	0.25	0.91	0	0	17	688
SCD-B6-05	F	31	0.6	97	58	9.78	119	43	776
SCD-B6-22	F	31	0.7	0	0.25	0	0	13.04	247
SCD-B6-32	F	31	< 0.1	92	414	243	0.89	6.19	1180
SCD-B6-33	F	33	0.5	0	0.15	0	0	124	884
SCD-B6-06	F	36	0.3	1.32	0.72	0	0	34	290
SCD-B6-38	F	39	0.1						
SCD-B6-20	F	53	< 0.1	0.35	2.66	0	0	20	198
SCD-B6-31	F	61	0.6	0	0	0	0	18	531
SCD-B6-36	M	26	<0.1						
SCD-B6-37	M	31	0.5						
SCD-B6-34	M	48	1.4	0	0	0	4.76	12.34	720
SCD-B6-14	M	58	0.6	0	0	0	4.06	74	446

The values listed as “0” were below the sensitivity (out of range) of the multiplex assay.

6. Iron Serum Analytes

Study ID	Gender	Age	Total Iron (mcg/dL)	Total Iron Binding Capacity (mcg/dL)	Iron Saturation (%)	Ferritin (ng/mL)	Transferrin (mg/dL)
SCD-B6-28	F	4	89.0	222.0	40.1	1290.0	160.0
SCD-B6-11	F	6	173.0	192.0		2428.0	138.0
SCD-B6-12	F	8	145.0	231.0		1932.0	166.0
SCD-B6-04	F	9	107.0	335.0		87.0	241.0
SCD-B6-03	F	13	76.0	367.0		296.0	264.0
SCD-B6-15	F	13	169.0	227.0		1011.0	163.0
SCD-B6-29	F	15	199.0	206.0	96.6	4186.0	148.0
SCD-B6-10	F	17	117.0	309.0		1184.0	222.0
SCD-B6-16	F	17	110.0	211.0		3206.0	152.0
SCD-B6-07	F	18	95.0	222.0		557.0	160.0
SCD-B6-19	F	18	182.0	322.0		116.0	232.0
SCD-B6-27	M	5	96.0			1015.0	
SCD-B6-08	M	8	78.0	306.0		80.0	220.0
SCD-B6-30	M	8		224.0		4784.0	161.0
SCD-B6-02	M	12	86.0	303.0		712.0	218.0
SCD-B6-18	M	15	47.0	274.0		54.0	197.0
SCD-B6-17	M	17	177.0	197.0		1185.0	142.0
SCD-B6-01	M	18	249.0	252.0		1189.0	181.0
SCD-B6-13	F	22	68.0	348.0	19.5	49.0	250.0
SCD-B6-26	F	23	51.0	367.0	13.9	143.0	264.0
SCD-B6-23	F	27		416.0		18.0	299.0
SCD-B6-35	F	27	79.0	211.0	37.4	2744.0	152.0
SCD-B6-09	F	29	198.0	274.0		3791.0	197.0
SCD-B6-21	F	30	78.0	391.0		81.0	281.0
SCD-B6-05	F	31	53.0	< 97		5819.0	< 70
SCD-B6-22	F	31	88.0	236.0		655.0	170.0
SCD-B6-32	F	31	57.0	235.0	24.3		169.0
SCD-B6-33	F	33	192.0	197.0	97.5	5825.0	142.0
SCD-B6-06	F	36	216.0	246.0		3072.0	177.0
SCD-B6-38	F	39	88.0	279.0	31.5	85.0	201.0
SCD-B6-20	F	53	132.0	278.0		47.0	200.0
SCD-B6-31	F	61	70.0	307.0	22.8	338.0	221.0
SCD-B6-36	M	26	106.0	242.0	43.8	1281.0	174.0
SCD-B6-37	M	31	72.0	328.0	22.0		236.0
SCD-B6-34	M	48	71.0	259.0	27.4	625.0	186.0
SCD-B6-14	M	58	73.0	322.0		77.0	232.0

7. Other Analytes

Study ID	Gender	Age	Albumin (g/dL)	Creatinine (mg/dL)	Alkaline-Phosphatase (U/L)
SCD-B6-28	F	4	4.2	0.23	131.0
SCD-B6-11	F	6	4.5	0.29	178.0
SCD-B6-12	F	8	4.3	0.43	222.0
SCD-B6-04	F	9	4.3	0.34	264.0
SCD-B6-03	F	13	4.5	0.49	85.0
SCD-B6-15	F	13	3.9	0.50	198.0
SCD-B6-29	F	15	4.1	0.40	76.0
SCD-B6-10	F	17	4.5	0.44	83.0
SCD-B6-16	F	17	4.3	0.43	72.0
SCD-B6-07	F	18	4.0	0.58	58.0
SCD-B6-19	F	18	3.8	0.42	45.0
SCD-B6-27	M	5		0.18	112.0
SCD-B6-08	M	8	4.5	0.34	168.0
SCD-B6-30	M	8	4.0	0.31	
SCD-B6-02	M	12	4.6	0.33	278.0
SCD-B6-18	M	15	3.8	0.41	231.0
SCD-B6-17	M	17	4.3	0.54	73.0
SCD-B6-01	M	18	4.2	0.92	137.0
SCD-B6-13	F	22	4.1		60.0
SCD-B6-26	F	23	3.9	0.58	135.0
SCD-B6-23	F	27	4.2	0.52	
SCD-B6-35	F	27	4.5	0.63	60.0
SCD-B6-09	F	29	4.4	0.41	69.0
SCD-B6-21	F	30	4.0	0.57	67.0
SCD-B6-05	F	31	4.0	0.33	79.0
SCD-B6-22	F	31	4.4	0.35	126.0
SCD-B6-32	F	31	4.2	0.73	37.0
SCD-B6-33	F	33	4.3	0.47	80.0
SCD-B6-06	F	36	4.6	0.53	50.0
SCD-B6-38	F	39	3.9	0.87	61.0
SCD-B6-20	F	53	3.7	0.74	73.0
SCD-B6-31	F	61	3.7	0.74	77.0
SCD-B6-36	M	26	4.0	0.65	79.0
SCD-B6-37	M	31	4.3	0.89	78.0
SCD-B6-34	M	48	4.1	0.88	76.0
SCD-B6-14	M	58	3.7	1.38	70.0

8. CAIM Vascular Markers

Study ID	Gender	Age	Severity Index	Abnormal Diameter	Abnormal Distribution	Uneven Thickness	Adnormal Morpho.	Distend	Damage	Sludging	Tortuosity	Ischemia	Abnormal AV Ratio	Micro-aneurysims	Boxcar Phenom.	Hemosiderin	Comma Sign	Abnormal Velocity
SCD-B6-28	F	4	2	1	1	0	0	0	0	?	0	0	?	0	0	0	0	0
SCD-B6-11	F	6	6	0	1	0	0	0	0	1	1	0	1	0	1	0	0	1
SCD-B6-12	F	8	2	0	0	0	0	0	0	0	1	?	0	0	0	1	0	0
SCD-B6-04	F	9	4	1	1	0	0	0	0	0	0	?	1	0	1	0	0	0
SCD-B6-03	F	13	4	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0
SCD-B6-15	F	13	6	1	1	0	0	0	0	1	0	0	1	0	1	1	0	0
SCD-B6-29	F	15	4	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0
SCD-B6-10	F	17	7	1	1	0	0	0	0	1	1	0	1	0	1	0	0	1
SCD-B6-16	F	17	2	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
SCD-B6-24	F	17		Not recorded														
SCD-B6-07	F	18	6	1	1	0	0	0	0	1	0	1	1	0	0	0	1	0
SCD-B6-19	F	18	2	?	0	0	0	0	0	?	0	0	0	0	1	1	0	0
SCD-B6-27	M	5	0	0	?	0	0	0	0	0	0	0	?	0	0	0	0	0
SCD-B6-08	M	8	3	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0
SCD-B6-30	M	8	4	1	1	0	1	0	0	0	?	0	1	0	0	0	0	0
SCD-B6-02	M	12	3	1	1	0	0	0	0	0	0	?	1	0	0	0	0	0
SCD-B6-18	M	15	7	1	1	0	0	0	0	1	0	?	1	0	1	1	0	1
SCD-B6-17	M	17	4	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0
SCD-B6-01	M	18	3	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0
SCD-B6-13	F	22	4	1	?	0	0	0	0	1	0	0	0	0	1	1	0	0
SCD-B6-26	F	23	5	0	1	0	0	0	?	1	0	0	1	0	1	1	0	0
SCD-B6-25	F	26		Not recorded														
SCD-B6-23	F	27	6	1	1	?	0	0	0	1	1	0	1	0	1	0	0	0
SCD-B6-35	F	27	7	1	1	0	1	0	0	1	1	0	1	0	1	0	0	0
SCD-B6-09	F	29	9	1	1	?	1	0	0	1	1	0	1	0	1	1	0	1
SCD-B6-21	F	30	6	1	1	0	0	0	0	1	0	0	1	0	1	1	0	0
SCD-B6-05	F	31	8	1	1	0	1	0	0	1	1	0	1	0	1	1	0	0
SCD-B6-22	F	31	1	0	0	0	0	0	0	?	0	0	1	0	?	?	0	0
SCD-B6-32	F	31	7	1	1	0	0	?	0	1	1	0	1	1	1	0	0	0
SCD-B6-33	F	33	8	0	1	0	1	0	0	1	1	1	1	0	1	1	0	0
SCD-B6-06	F	36	5	1	1	0	0	0	0	1	1	0	1	0	?	0	0	0
SCD-B6-38	F	39		Not recorded														
SCD-B6-20	F	53	8	1	1	1	0	0	0	1	1	0	1	0	1	1	0	0
SCD-B6-31	F	61	9	1	1	0	0	0	1	1	1	0	1	1	1	1	0	0
SCD-B6-36	M	26	6	1	1	0	1	0	0	0	0	1	1	0	0	1	0	0
SCD-B6-37	M	31	6	1	1	0	1	0	0	0	1	0	1	0	0	1	0	0
SCD-B6-34	M	48	7	0	1	0	0	0	0	1	1	1	1	0	1	1	0	0
SCD-B6-14	M	58	8	1	1	?	1	0	0	1	1	0	1	0	1	1	0	0

9. References:

1. Ballas SK. *Sickle Cell Disease: Clinical Management*. Bailliere's clinical haematology 1998;11(1):18214.

Appendix B: Research and Funding Acknowledgements

I acknowledge the University of California Davis Medical Center Sickle Cell Center nurses, social workers, SCA patients, caregivers, and parents for their support and generous participation. I would like to thank the UCDMC Clinical and Chemistry Laboratories for their blood processing and training of biological sample processing.

I also acknowledge my mentors for their contributions:

Dr. Joshua W. Miller for concept development and study design, biochemical analysis, statistical analysis, interpretation of the data, and review of my dissertation.

Dr. Ralph Green, the principal investigator of the NIH grant R01 HL83276 which funded this research, for concept development and study design, interpretation of the data, and review of my dissertation.

Dr. Theodore Wun for concept development and study design, providing access to and assisting with patient recruitment, interpretation of the data, and review of my dissertation.

Dr. Theodore Zwerdling for concept development and study design, and providing access to and assisting with patient recruitment.

Dr. Anthony Cheung for developing the intravital microscope and CAIM methodology, for concept development and study design, and verification of CAIM results.

Dr. Imran Khan and his staff for validating the multiplex assays and sample processing.

Dr. Lin Xin for assay development and validation, blood processing, interpretation of the data, and CAIM analysis.

Ms. Soraya Foutouhi, a fellow graduate student and laboratory assistant, for instruction and assistance in laboratory practices.

This study was funded by a grant from the National Institutes of Health (NIH R01 HL83276), and a UC Davis Medical Center Medical Pathology and Laboratory Medicine Research Grant (2005).

This publication was made possible by Grant Number UL1 RR024146 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research.

There were no conflicts of interest with respect to this study.