The Effect of Atorvastatin on Endothelial Dysfunction and Albuminuria in Sickle Cell Disease (in the grant entitled: Endothelial Dysfunction in the Pathogenesis of Sickle Cell Nephropathy)

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SYNOPSIS

Title of the Protocol: The effect of atorvastatin on endothelial dysfunction and albuminuria in sickle cell disease

Overview: It is well recognized that sickle cell disease (SCD) is characterized by a vasculopathy, with involvement of multiple organs including the brain, lung, spleen, penis, and kidney. This results in multiple clinical complications, including ischemic stroke, pulmonary hypertension, autosplenectomy, priapism, as well as albuminuria and chronic renal disease. Several recent studies have confirmed the association of both albuminuria and renal dysfunction with echocardiographically-defined pulmonary hypertension and other vasculopathic complications in SCD, suggesting that they may share a similar pathophysiology. Despite the high prevalence of albuminuria in patients with SCD and the known association of renal failure with increased mortality, the pathophysiology and treatment of albuminuria in this setting remain poorly defined.

The treatment options for nephropathy in SCD remain limited. Although ACE inhibitors are the "standard of care" in the treatment of patients with proteinuria, there are, to date, no controlled, long-term studies confirming their efficacy and safety in this setting.

In this study, we will evaluate the efficacy and safety of atorvastatin in SCD patients. At the completion of this trial, we will have an improved understanding of the contribution of endothelial dysfunction and soluble fms-like tyrosine kinase (sFLT-1) to the pathophysiology of albuminuria in SCD. If the data support the hypothesis that atorvastatin is safe and effective in this population, we plan on carrying out adequately powered studies to more definitively evaluate its safety and efficacy in the treatment and/or prevention of albuminuria in SCD.

Intervention: We will conduct a randomized, double-blind, placebo-controlled, crossover trial of atorvastatin (n = 19). Subjects will receive atorvastatin 40 mg/day or placebo for 6 weeks, separated by a 4 week washout phase. At the end of the washout period, a repeat history and examination and laboratory studies will be performed which will serve as a baseline for the second treatment phase, and patients will receive either atorvastatin or placebo, depending on their first treatment administration.

IND Holder: EXEMPT

Specific Aims:

A) To evaluate the effect of atorvastatin on endothelial dysfunction, assessed using ultrasound imaging of the brachial artery, in sickle cell disease patients.

B) To evaluate the effect of atorvastatin on plasma markers of endothelial activation, plasma levels of sFLT-1 and VEGF, heme oxygenase activity, rho/rho kinase activity, and TF-mediated sFLT-1 release from monocytes in sickle cell disease patients.

C) To evaluate the effect of atorvastatin on albuminuria and estimated glomerular filtration rate in sickle cell disease patients

D) To evaluate the association of endothelial dysfunction with pulmonary arterial pressure.

Hypotheses/Estimates: Our primary hypothesis is that endothelial dysfunction is an important contributor to the pathophysiology of albuminuria in SCD. We propose that atorvastatin will

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improve endothelial dysfunction, decrease levels of sFLT-1, and decrease albuminuria in SCD patients.

Criteria for Evaluation:

Efficacy: Primary Endpoint: E*ndothelial Dysfunction:* This will be assessed non-invasively using a brachial artery shear stress method. Assessments will be obtained at Baseline, and then at week 6 during the Treatment Phase. The primary efficacy measure will be a comparison of the % change in FMD from baseline to week 6.

Secondary Endpoints: E*ndothelial Dysfunction:* The % change in NTMD from baseline to week 6 will be a secondary efficacy measure.

Endothelial Activation, sFLT-1, VEGF, heme oxygenase activity, rho/rho kinase activity and TF-mediated sFLT-1 release: We will measure plasma levels of soluble VCAM and soluble ICAM, plasma levels of sFLT-1 and VEGF; and assess heme oxygenase and rho/rho kinase activities at Baseline and then at week 6 during the Treatment Phase. Flow cytometry will be performed to assess monocyte activation state, absolute cell counts, TF expression, as well as TF-mediated sFLT-1 release from monocytes at baseline and at week 6.

Renal Function: We will assess the effect of atorvastatin on albuminuria (assessed by spot urine microalbumin/creatinine ratio) and estimated GFR at baseline and at week 6 during the Treatment Phase.

To examine the relationship of endothelial dysfunction, assessed using ultrasound imaging of the brachial artery, with tricuspid regurgitation velocity (TRV), Doppler echocardiography will be performed at baseline and at Week 6 in both treatment periods.

We will also evaluate patients for treatment-related complications by employing a history, physical examination, and routine laboratory tests (CBC, liver function tests, creatine kinase, lipid profile, and renal function). In addition to abnormal liver function tests and myopathy, we will monitor for episodes of SCD-related events.

Study Design: This is a randomized, double-blind, placebo-controlled, crossover trial of atorvastatin to evaluate the efficacy of atorvastatin in improving endothelial dysfunction and decreasing albuminuria in SCD. It will be divided into a screening/baseline phase, treatment phase, and follow-up phase.

Study Population: Nineteen patients with SCD (HbSS or HbSβ^0 thalassemia) between the ages of 16 and 60 who meet the eligibility criteria and provide consent to participate in the study, will be randomized in this crossover trial.

Clinical and Laboratory Evaluations: The Screening/Baseline Phase will occur within 28 days of study drug administration and will include: informed consent, a history and physical examination, and clinical laboratory tests including: a complete blood count, routine coagulation studies, routine chemistries to assess liver and renal function, lipid profile, creatine kinase, urine microalbumin-creatinine ratio, chest x-ray, and serum pregnancy test (if female, and of child-bearing capacity). During the first visit of each Treatment Phase, prior to treatment initiation, we will also obtain a complete blood count, routine chemistries to assess liver and renal function, lipid profile, creatine kinase, urine microalbumin-creatinine ratio, serum pregnancy test (if female). Finally, measurements of endothelial dysfunction (assessed non-invasively using a brachial artery shear stress method), endothelial activation (soluble

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VCAM, soluble ICAM), sFLT-1, VEGF, heme oxygenase activity, rho/rho kinase activity, tissue factor (TF) expression, TF-mediated sFLT-1 release from monocytes, estimated GFR and microalbumin-creatinine ratio will be obtained at specified time points during the Baseline, Treatment and Washout/Follow-up Phases. The Follow-up Phase will consist of safety assessments performed four weeks after the final dose of study treatment.
Sample Size: In a 2x2 crossover design, the minimum required sample size is 9 per sequence to detect a clinically meaningful difference of 0.65 in mean changes in FMD% between atorvastatin and placebo groups at significance level $\alpha = 0.05$ with 80% power. Assuming a 5% attrition rate, we will enroll 19 patients in the study.
Randomization: 1:1 randomization to receive atorvastatin or placebo.

BACKGROUND, SIGNIFICANCE AND RATIONALE

The increased survival into adulthood of SCD patients is associated with an increased incidence of organ dysfunction. Renal abnormalities are common in patients with SCD (1). Proteinuria (used interchangeably with albuminuria), the most common clinical manifestation of glomerular injury, is especially prevalent in SCD (2-4). Increasing proteinuria and glomerular injury lead to a progressive decline in glomerular filtration rate (GFR) and potentially end-stage renal disease (ESRD) (5-7). Multiple studies have shown an association between renal function and pulmonary hypertension (PHT) diagnosed using Doppler echocardiography (8-10), suggesting that both nephropathy and PHT may share a similar pathophysiology in SCD. Indeed, both renal failure and PHT are risk factors for death in SCD (8-11). We have recently reported that the level of soluble fms-like tyrosine kinase-1 (sFLT-1), a vascular endothelial growth factor (VEGF) receptor family member, is significantly higher in SCD patients with macroalbuminuria (urine albumin excretion [UAE] > 300 mg/g creatinine) when compared to patients with microalbuminuria (UAE: 30 - 299 mg/g creatinine) and normoalbuminura (UAE < 30 mg/g creatinine) (12). sFLT-1 is also associated with proteinuria in multiple other clinical conditions, including preeclampsia, hypertension and diabetes mellitus (13-17). VEGF plays a critical role in glomerular development and function, and is important in maintaining the glomerular filtration barrier. VEGF has also been implicated in glomerular healing by facilitating glomerular capillary repair (18). Finally, sFLT-1, which lacks transmembrane and cytoplasmic domains, binds and reduces circulating VEGF, thereby abrogating its beneficial effects and inducing endothelial dysfunction.

Despite the high prevalence of nephropathy in SCD, the available treatments for this complication are limited. Although angiotensin-converting enzyme (ACE) inhibitors (as well as angiotensin receptor antagonists) are commonly used in patients with proteinuria (6), renal failure remains a significant cause of morbidity and mortality in this patient population. Understanding the role of endothelial dysfunction in the pathophysiology of SCD-associated nephropathy will have substantial implications for the treatment of this complication. An improvement in endothelial function has the potential to decrease the progression of nephropathy in SCD and improve the survival of these patients.

Endothelial Dysfunction in Sickle Cell Disease

Similar to patients with coronary artery disease, atherosclerosis and its risk factors, SCD patients exhibit endothelial dysfunction characterized by a blunted response to NO synthase inhibition and a resistance to exogenous NO (19-26). Endothelium-dependent, NO-dependent blood flow is also impaired in both children and adults with SCD, when measured by flow-mediated vasodilation (27,28). In response to hyperemia, brachial artery flow increases to a greater value in SCD patients than in controls, but the % flow increase is similar in both groups. Flow-mediated dilation (FMD) is lower in SCD patients than in controls despite the fact that the calculated wall shear stress reaches a higher value in patients following hyperemia, thus indicating blunted reactivity of the endothelium to an increase in blood flow (27). All of these provide evidence of marked alteration in wall shear stress-induced, endothelium-dependent vasodilation in SCD. Despite the evidence for endothelial dysfunction in SCD, its role in the pathophysiology of disease complications remains poorly defined.

The plasma level of sFLT-1 is reported to be increased in SCD (12,29). Increased plasma levels of sFLT-1 have also been reported in non-SCD patients with chronic kidney disease (30). In addition, plasma markers of endothelial dysfunction (soluble VCAM-1 and vWF), the incidence of myocardial infarction and stroke, as well as the Framingham risk score were shown to be correlated with sFLT-1 levels. The administration of sFLT-1 or a VEGF neutralizing antibody to rats has been shown to produce glomerular endothelial cell damage and proteinuria (31). Recombinant sFLT-1 antagonizes VEGF action, and decreases the bioavailability of nitric oxide (NO) by inhibiting the phosphorylation of the protein kinase, Akt (30). Studies in newly diagnosed and untreated patients with hypertension show a relationship between microalbuminuria and markers of endothelial activation, including soluble E-selectin, soluble ICAM-1 and soluble VCAM-1 (32,33). Finally, endothelial dysfunction has

been reported to be related to albumin excretion rate in patients with type 1 diabetes mellitus (34). All of these findings suggest an association between endothelial dysfunction and both albuminuria and kidney disease.

We propose that in SCD patients, endothelial dysfunction mediated, at least in part by increased levels of sFLT-1, contributes to the pathophysiology of albuminuria.

Albuminuria in Sickle Cell Disease

Renal abnormalities, including abnormalities of distal nephron function, supratubular proximal tubule function, hemodynamic changes and glomerular abnormalities are common in SCD (1). Proteinuria, the most common clinical manifestation of glomerular injury (1) is also common in SCD (2-4). Progressive decline in glomerular filtration rate (GFR) is often associated with increasing proteinuria and evidence of glomerular injury (5,6,35). Less often, SCD patients develop the nephrotic syndrome due to membranoproliferative glomerulonephritis (MPGN) (36). This may be related to a direct effect of sickled RBC, but patients may also have MPGN associated with hepatitis C infection acquired from multiple blood transfusions. In addition to the nephropathy associated with SCD, it is also possible that the medullary ischemia induced by sickling can exacerbate the course of other underlying renal diseases. There is, for example, some evidence that black patients with sickle cell trait and autosomal dominant polycystic kidney disease develop end-stage renal disease earlier than those without sickle cell trait (37).

The pathophysiology of albuminuria in SCD remains uncertain. However, a positive correlation has been found between the presence of increased GFR (hyperfiltration) and the likelihood of increased albumin excretion in young SCD patients (38). Hyperfiltration is particularly common in children with SCD (39). Progressive decline in GFR is often associated with increasing proteinuria and evidence of glomerular injury (5-7). Focal segmental glomerulosclerosis (FSGS) is the most common glomerular finding in sickle cell nephropathy and is intimately associated with glomerular hypertension and glomerular hypertrophy (6,40). Multiple groups have observed an association of echocardiography-defined pulmonary hypertension with measures of renal function (8-10), suggesting that both pulmonary hypertension and SCD-related glomerulopathy may share a similar pathophysiology. Macroalbuminuria is associated with increased tricuspid regurgitant jet velocity in SCD (10,12). The plasma level of sFLT-1 is higher in SCD patients with macroalbuminuria compared to patients with microalbuminuria and normoalbuminuria (12). We have also observed an association between macroalbuminuria and plasma levels of soluble VCAM (12). . Finally, we observed an association of sFLT-1 with soluble VCAM (12). This finding, combined with the observations in other disease states such as preeclampsia (13-17), suggests that endothelial dysfunction due, at least in part to increased sFLT-1 levels, contributes to the pathophysiology of albuminuria in SCD.

Despite the high prevalence of albuminuria and its association with progressive renal failure in SCD, the treatment options remain limited. ACE inhibitors, and more recently angiotensin receptor blockers are the “standard of care” in the treatment of patients with proteinuria (6). The amount of proteinuria was observed to fall by over 60% following a brief course of ACE inhibitor therapy (6). Other studies suggest that ACE inhibitors may also be beneficial in patients with microalbuminuria (41,42). Small studies suggest that hydroxyurea may decrease proteinuria in SCD patients (2,42). There are, however, no controlled, long-term studies confirming the efficacy of ACE inhibitors or hydroxyurea in this setting. Despite treatment with these agents, many SCD patients experience progressive glomerulopathy and renal failure. As a result, renal transplantation is increasingly employed as treatment for ESRD. Although comparable short-term allograft results have been reported when compared with age- and race-matched transplant recipients with other causes of ESRD, a somewhat shorter cadaveric graft survival beyond 1 year and a greater adjusted 3-year risk of graft loss in the SCD group has been reported (43). Whether survival is improved compared to dialysis remains uncertain (44-47). Furthermore, as the underlying pathogenetic processes are still present, recurrent disease is common following renal transplantation in SCD (47).

Atorvastatin

Statins inhibit 3-hydroxyl-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, which is an early rate-limiting step in the biosynthesis of cholesterol (48). By inhibiting HMG-CoA reductase, these drugs reduce hepatocyte levels of cholesterol, resulting in upregulation of LDL receptors and consequently increasing clearance of LDL cholesterol from plasma (49,50). They also cause smaller reductions in triglyceride levels and minor increases in HDL-cholesterol (49). The cardiovascular benefits of statins may also be mediated by their potent anti-proliferative and pro-apoptotic effects on vascular smooth-muscle cells through inhibition of ras and rho GTPase activities important for cell proliferation (51); enhanced endothelial production of nitric oxide through synergistic mechanisms of stabilizing endothelial NO synthase messenger RNA (52) and augmenting endothelial NO synthase phosphorylation and catalytic activity (53); and increased release of circulating endothelial progenitor cells that stabilize endothelial barrier function in response to injury (54-56). Statins also clearly possess anti-inflammatory and antithrombotic (57-59) effects that contribute to their potency in preventing atherogenesis.

Among the statins, atorvastatin exhibits a number of pharmacokinetic characteristics that are different from other members of the class. Atorvastatin is extensively metabolized to ortho- and para-hydroxylated derivatives and various beta-oxidation products (60) and around 70% of the HMG-CoA reductase inhibition achieved with atorvastatin is ascribed to these ortho- and para-hydroxylated metabolites (50). Both atorvastatin and its metabolites are principally eliminated through biliary excretion, with only small amounts excreted in the urine. The half-life of HMG-CoA reductase inhibition is approximately 20 - 30 hours as a result of the action of its active metabolites (48).

Atorvastatin is not associated with neurological, cognitive or renal adverse effects and does not require dosage adjustment in patients with renal dysfunction due to its favorable pharmacokinetic profile. In patients aged ≥ 65 years, atorvastatin is well tolerated with no dose-dependent increase in adverse events up to the maximum daily dosage of 80 mg/day. Various risk factors increase the likelihood of developing myopathy or rhabdomyolysis, including advanced age (61), small body frame, alcohol abuse and the presence of other systemic diseases (62), as well as the use of combination statin-fibrate therapy (63) and concomitant use of agents that inhibit the cytochrome (CYP) 3A4 enzyme, thereby blocking the oxidative metabolism of atorvastatin and increasing drug plasma concentrations (64). A retrospective analysis of pooled data in 14,236 patients treated for up to 52 months comparing the safety of atorvastatin 10 mg, atorvastatin 80 mg, and placebo (65) showed that withdrawals due to treatment-related adverse events were 2.4%, 1.8%, and 1.2%; persistent elevations in hepatic transaminases >3 times upper limits of normal were observed in 0.1%, 0.6%, and 0.2%; and treatment-associated myalgia was observed in 1.4%, 1.5%, and 0.7% of patients in the atorvastatin 10 mg, atorvastatin 80 mg, and placebo groups respectively. No cases of rhabdomyolysis were reported in any group.

The benefit of statin therapy on endothelial function remains somewhat controversial, with several studies showing no benefit (66-68). However, atorvastatin treatment has been reported to produce significant improvement in endothelium-dependent vasodilation (60,69-72) in patients with diabetes (types I and II). The improvement in FMD does not appear to be related to basal cholesterol levels or cholesterol reduction (72).

Statins have been reported to provide protection against chronic renal damage independent of their cholesterol lowering effects (73–75). The protective effects are thought to derive from anti-inflammatory and antioxidant actions, fibrogenesis attenuation and endothelial cell protection (76). Atorvastatin was found to improve effective renal plasma flow (ERPF) and GFR in aging rats subjected to ischemic-reperfusion injury, accompanied by an increase in the expression of endothelial NO synthase and NO production (77). Atorvastatin also dramatically reduced albuminuria and histological changes in the kidneys of autoimmune mouse model of anti-glomerular basement membrane glomerulonephritis compared to vehicle-treated control animals (78). Although transient and mild proteinuria has been reported in patients treated with high doses of statins (79,80), a randomized study comparing rosuvastatin 10 mg (titrated to 40 mg) with atorvastatin 10 mg (titrated to 80 mg) in 469 patients with type 2 diabetes with dyslipidemia showed no significant change from baseline in urinary albumin excretion (UAE) for either treatment group or between-treatment groups at 16 weeks, and

median UAE for both treatment groups remained within normal limits, providing evidence that patients can be treated with higher efficacy statins without clinically meaningful effects on UAE (81). Atorvastatin has been reported to produce a significantly greater GFR and effective renal plasma flow after the release of unilateral ureteral obstruction in rats at 1 hour, 6 hours and 12 hours, as well as significant reduction of urinary microalbumin/creatinine ratios at 12 hours, but not earlier (82). Atorvastatin also produces significantly higher GFR as well as decreased urinary microalbumin in rats following 12 days of unilateral ureteral obstruction but not following 3 days of unilateral ureteral obstruction (83). In a large study of patients with type 2 diabetes and prior cardiovascular disease, atorvastatin treatment for a median of 3.9 years was associated with a modest improvement in annual change in estimated GFR (net, 0.18 mL/min/1.73 m(2)/y; 95% confidence interval [CI], 0.04 to 0.32; P = 0.01) that was most apparent in those with albuminuria (net improvement, 0.38 mL/min/1.73 m(2)/y; P = 0.03), although it did not influence the incidence of albuminuria (hazard ratio, 1.49; 95% CI, 0.73 to 3.04; P = 0.3) or regression to normoalbuminuria (hazard ratio, 1.19; 95% CI, 0.57 to 2.49; P = 0.6). Although atorvastatin did not influence albuminuria incidence, there was a low incidence rate of albuminuria and transition to more severe kidney status resulting in limited power to detect treatment effects (84).

There are no published reports on the effect of atorvastastin in patients with SCD. Pretreatment with lovastatin eliminated excessive tissue factor (TF) expression in the pulmonary veins (~ 16% positive) following a period of hypoxia for 3 hours followed by reoxygenation in a mild sickle cell transgenic mouse model (NY1DD) and decreased VCAM expression modestly in the posthypoxic NY1DD mouse (85). Simvastatin has also been reported to prolong survival following pneumococcal challenge (86). In a study involving 26 SCD patients at steady state, treatment with simvastatin for 21 days was well tolerated and resulted in increased plasma levels of nitric oxide metabolite, and decreased levels of both high sensitivity CRP and IL-6 in a dose-dependent manner (87).

With the evidence of chronic inflammation and endothelial dysfunction in SCD, and the ability of statins to decrease inflammation following hypoxia-reoxygenation injury and improve endothelial dysfunction, we propose that atorvastatin will have a beneficial role in patients with SCD.

PRELIMINARY DATA

Nephropathy is Associated with Vasculopathic Complications in Sickle Cell Disease: We have previously shown that PHT, diagnosed using Doppler echocardiography is associated with nephropathy (p = 0.01) in SCD patients (9). Serum creatinine (1.1 ± 0.5 mg/dL vs. 0.8 ± 0.3 mg/dL, p = 0.02) and blood urea nitrogen (16.1 ± 11.7 mg/dL vs. 10.1 ± 6.6 mg/dL, p = 0.01) were higher in patients with PHT compared to those without PHT. We have recently confirmed the high prevalence of albuminuria (assessed by spot urine measurements of microalbumin/creatinine ratio) in SCD (Figure 1). In a cross-sectional study of 73 SCD patients, normoalbuminuria (urine albumin excretion [UAE] < 30 mg/g creatinine) was observed in 34 patients (46.6%), microalbuminuria (UAE: 30 – 299 mg/g creatinine) in 24 patients (32.9%) and macroalbuminuria (UAE: ≥ 300 mg/g creatinine) in 15 patients (20.5%) (12). Albuminuria was correlated with age (r = 0.32, 95% CI: 0.09–0.51; p = 0.006). As expected, albuminuria was inversely correlated with estimated GFR (MDRD method) (r = -0.24; p = 0.04) and positively correlated with cystatin C (r = 0.25; p = 0.03). We also observed significant associations between albuminuria and both echocardiography-defined

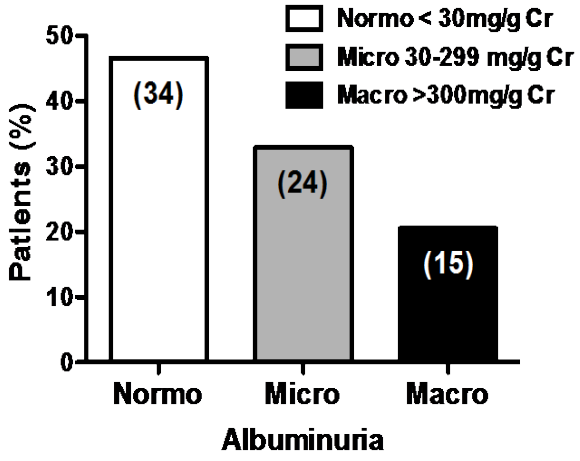

Figure 1: The Prevalence of Albuminuria in SCD

PHT (p = 0.04) and a history of stroke (p = 0.002). In patients with measurable tricuspid regurgitant jet velocities (TRV), the TRV was significantly higher in those with macroalbuminuria compared to patients with microalbuminuria and normoalbuminuria (3.15 m/s vs. 2.45 m/s vs. 2.45 m/s; p = 0.00068). We were unable to detect significant associations between albuminuria and the number of acute pain episodes, history of acute chest syndrome, history of leg ulcers, history of priapism or retinopathy.

Role of Endothelial dysfunction in Pathophysiology of Albuminuria and Other Vasculopathic Complications: We observed that sickle cell anemia and sickle β^0 thalassemia patients with macroalbuminuria had significantly lower hemoglobin values compared to those patients with microalbuminuria and normoalbuminuria (7.1 g/dL vs. 9.1 g /dL vs. 8.7 g /dL, p = 0.033), although no significant differences in the levels of lactate dehydrogenase (1175 U/L vs. 901 U/L vs. 914.5 U/L; p = 0.25) or other hemolytic measures were observed (12). However, a significantly higher level of soluble VCAM-1 (1347 n/mL vs. 765.4 ng/mL vs. 753.4 ng/mL; p = 0.033) was observed in patients with macroalbuminuria compared to those with microalbuminuria and normoalbuminuria. The level of sFLT-1 was elevated in patients with SCD compared with healthy control subjects (98.2 pg/mL vs. 61.6 pg/mL; p = 0.0005) (88). sFLT-1 is a member of the vascular endothelial growth factor receptor (VEGFR) family (89) and is a splice variant of the VEGFR1. In addition to binding free VEGF and placenta growth factor (PlGF), sFLT-1 forms dominant-negative complexes with membrane-bound VEGFRs; hence, although VEGF can continue to bind to the cell surface, no signal is initiated, thereby inducing endothelial dysfunction. The level of sFLT-1 was significantly higher in patients with macroalbuminuria, compared to patients with microalbuminuria and normoalbuminura (120.1 pg/mL vs. 99.7 pg/mL vs. 85.4 pg/mL; p = 0.016). We also observed significant correlations between sFLT-1 and soluble VCAM (r = 0.42, p = 0.0003), suggesting that sFLT-1 may contribute to endothelial dysfunction in SCD.

There was a negative correlation between sFLT-1 and hemoglobin (r = -0.52, p < 0.0001) (Figure 2A), and significant positive correlations with various measures of hemolysis (lactate dehydrogenase [r = 0.64, p < 0.0001] [Figure 2B], total bilirubin [r = 0.3, p = 0.012], indirect bilirubin [r = 0.3, p = 0.012] and reticulocyte count [r = 0.33, p = 0.005]). Interestingly, sFLT-1 was correlated with absolute monocyte count (r = 0.25, p = 0.039), suggesting that monocytes may be a source of sFLT-1 in SCD. Furthermore, sFLT-1 was correlated with D-dimer (r = 0.42, p = 0.0017) suggesting that sFLT-1 may contribute to coagulation activation in SCD. Finally, sFLT-1 was correlated with TRV (r = 0.33, 95% CI: 0.04 – 0.58; p = 0.021) and was significantly associated with the presence of echocardiography-defined PHT (120.0 pg/mL vs. 86.5 pg/mL, p = 0.017). The level of sFLT-1 also appeared to be higher in patients with histories of priapism (114.1 pg/mL vs. 91.0 pg/mL, p = 0.052) and stroke (132.9 pg/mL vs. 93.3 pg/mL, p = 0.087) compared to patients without these complications, although the differences were not statistically significant.

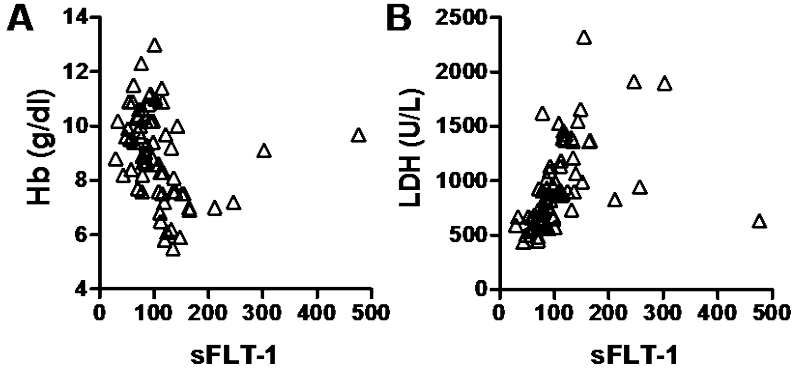

Figure 2: Association of sFLT-1 with hemoglobin and LDH in subjects with sickle cell disease.

RESEARCH DESIGN AND METHODS

We will conduct a randomized, double-blind, placebo-controlled, crossover trial of atorvastatin to evaluate the efficacy of atorvastatin in improving endothelial dysfunction and decreasing albuminuria in SCD. The study will consist of three phases: 1) *Screening/Baseline*; 2) *Treatment*; and 3) *Follow-up*. The Screening/Baseline Phase will occur within 28 days of study drug administration and will include: informed consent, a history and

physical examination, and clinical laboratory tests including: a complete blood count, routine coagulation studies, routine chemistries to assess liver and renal function, lipid profile, creatine kinase, urine microalbumin-creatinine ratio, chest x-ray, and serum pregnancy test (if female, and of child-bearing capacity). We will also obtain baseline levels of biomarkers and ultrasound imaging of the brachial artery to assess endothelial dysfunction. The Treatment Phase is a 16-week phase with cross-over design and will consist of two, six-week treatment periods separated by a four week wash-out. The sequence of treatment assignment will be determined randomly via block permuted randomization procedure. At the start of the first treatment period, half the patients will be assigned to receive atorvastatin while the other half will be assigned to receive placebo. In the second treatment period, following the washout period, patients will be crossed-over to receive either atorvastatin or placebo, depending on their first treatment assignment. Each treatment period will consist of drug administration, safety assessments and other study assessments. Appendix 2 of the protocol provides a detailed schedule of assessments for the study period.

An Investigational New Drug (IND) application to support the administration of atorvastatin to SCD patients is pending. ***(FDA determined study to be EXEMPT from IND. Letter on file.)***

Inclusion Criteria*:*
a) HbSS or HbSβ^0 thalassemia between ages of 18 and 70; ***(Maximum age revised 06/03/2013and 04/07/2015.)***
b) albuminuria (micro- or macroalbuminuria, defined as ≥ 30mg/g creatinine);
c) serum ALT ≤ 2 times upper limits of normal and/or GGT ***(added 05/12/2015, revised 06/13/2017)*** ≤ 3 times upper limits of normal;
d) platelet count ≥ 150,000 cu/mm;
e) normal baseline coagulation profile (PT/INR, PTT);
f) non-crisis, steady state with no severe pain episodes during the preceding 4 weeks, and no documented infection in the 2 weeks prior to enrollment; ***(infection added 8/26/11)***
g) ability to understand the requirements of the study;
h) if a woman of childbearing potential, must use an adequate method of contraception; and
i) if receiving hydroxyurea, ACE inhibitors or ARBs, should be on a stable dose for at least 3 months.

Exclusion Criteria:
a) hypersensitivity to any component of atorvastatin, ***or history of adverse reaction to statins***; (***ADDED 8/26/2011)***
b) pregnant or breastfeeding;
c) on statin therapy;
d) history of metastatic cancer;
e) current history of alcohol abuse;
f) history of diabetes mellitus or poorly controlled systemic hypertension;
g) end-stage renal disease;
h) total cholesterol level < 80 mg/dL and LDL cholesterol > 130 mg/dL;
i) on a chronic transfusion program;
j) ingested any investigational drugs within the past 4 weeks;
k) prior history of any myopathy;
l) allergy to nitroglycerin;
m) *taking any of the following drugs: phosphodiesterase-5 inhibitors (e.g., sildenifil), CYP3A4 inhibitors (e.g., cyclosporine, protease inhibitors), macrolide antibiotics (e.g., clarithromycin, erythromycin), fibric acid derivatives (e.g. gemfibrizol), niacin, colchicines, antifungal agents (azole derivatives), amiodarone, danazol, daptomycin, diltiazem, verapamil, eltrombopag, everolimus, fosphenytoin, or lanthanum.*

Patients will also be encouraged to avoid grape fruit juice and red yeast rice for the duration of the study.

*Atovastatin is contraindicated during pregnancy and breast-feeding.****(Added 08/26/2011.)***

Termination Criteria: Treatment with study drug will be discontinued at any time if the patient: a) withdraws consent; b) develops ALT > 3 times upper limits of normal or GGT ***(added 05/12/2015, modified 6/13/2017)*** > 5 times upper limits of normal; c) develops symptoms and signs consistent with myopathy (muscle weakness and increased levels of creatine kinase); or if, d) in the judgment of the investigator, continuation of the study drug will be hazardous to the patient. The study will be terminated at the occurrence of treatment-related serious adverse events, including transaminitis or myopathy as above, in up to 3 study participants.

Treatment: Subjects will receive atorvastatin 40 mg/day or placebo for 6 weeks and after a 4-week wash-out period will be "crossed-over" to receive 6 weeks of whichever treatment they did not receive initially. Pill counting will be used to assess compliance. If toxicity occurs the study drug will be discontinued and the patient followed until resolution of the problem. Subjects discontinued prematurely will continue to follow-up for efficacy assessments.

Assessments:

E*ndothelial Dysfunction* – Vascular endothelial dysfunction will be assessed by measuring endothelium-dependent (flow-mediated) and endothelium-independent (nitroglycerin-mediated) dilation of the brachial artery as described previously (90,91). This technique has been safely performed in SCD patients (92,93). Subjects will be asked to hold all morning medications on days they have study visits until after the brachial ultrasound test. The reason for this is to avoid any effects of vasoactive medications. They are also required to refrain from smoking, vigorous exercise, caffeine, and eating and drinking (except water) for at least 6 hours before the brachial ultrasound procedure is scheduled. ***(Revised 06/03/2013.)*** Ultrasound images of the right brachial artery proximal to the antecubital fossa will be acquired using a 12 MHz linear-array transducer. The first set of baseline images will be obtained after 10 minutes of supine rest. Reactive hyperemia will be induced by inflating a pneumatic occlusion cuff placed around the upper forearm to a suprasystolic pressure (~ 200 mmHg) for 5 minutes, and then deflating the cuff. Images of the artery will be recorded for two minutes after cuff deflation. After 10 minutes of rest, a second set of baseline images will be acquired. Sublingual nitroglycerin (0.4 mg) will then be administered with acquisition of ultrasound images for the subsequent 5 minutes.

Gated end-diastolic images will be stored in a digital format on a personal computer for subsequent offline quantification. Each set of recordings will be coded and analyzed in random order by a blinded observer. Measurements will be performed using customized software (Brachial Tools, Medical Imaging Applications, LLC). Arterial diameter will be measured from the lumen-intimal interfaces of the proximal and distal arterial walls. Data from at least ten end-diastolic frames will be averaged for each baseline measurement and from at least three frames at maximum dilation during reactive hyperemia and following administration of nitroglycerin. FMD will be calculated as the % change in arterial diameter in response to reactive hyperemia, and NTMD will be quantified as the % diameter change following administration of nitroglycerin.

Assessments will be obtained at Baseline, Week 4 and Week 6 during each Treatment Phase. The primary efficacy measure will be a comparison of the % change in FMD from baseline to week 6. NTMD is a secondary efficacy measure.

Endothelial Activation, sFLT-1, VEGF, heme oxygenase activity, rho/rho kinase activity and TF-mediated sFLT-1 release: We will measure plasma levels of soluble VCAM and soluble ICAM, plasma levels of sFLT-1 and VEGF (ELISA kits from R&D systems) and assess heme oxygenase and rho/rho kinase activities (see below) at baseline and then at week 6 of both treatment periods. Flow cytometry will be performed to assess monocyte activation state, absolute cell counts, TF expression, as well as TF-mediated sFLT-1 release from monocytes at baseline and at week 6.

Heme oxygenase activity: Heme oxygenase-1 (HO-1) protein in plasma will be determined (ELISA kit from Cusabio Biotech, Newark, DE) according to the manufacturer's directions. Tandem experiments will be performed using human microvascular endothelial cells (Lonza, Allendale, NY) to determine the ability of blood from SCD patients to induce HO-1 expression (94) and activity (95). Rho/rho Kinase Activity: The

activity of rho kinase will be assayed by western blotting with a phospho-specific and activation specific antibody (Anaspec) as previously described (96), in lysates prepared from the endothelial cells exposed to blood.

Renal Function – We will assess the effect of atorvastatin on albuminuria by spot urine microalbumin/creatinine ratio and estimated GFR at baseline, Week 4 and Week 6 of both treatment periods.

Safety – Subjects will be evaluated for safety throughout the study by history-taking, measurement of vital signs, physical examinations, and routine laboratory tests (CBCs, and chemistries including liver function tests, creatine kinase, lipid profile, and tests of renal function). In addition to abnormal liver function tests, myopathy, and other adverse events, we will monitor subjects for SCD-related events (Appendix 1).

Doppler echocardiography will be performed at baseline and at Week 6 in each study arm to examine the relationship of endothelial dysfunction, assessed using ultrasound imaging of the brachial artery, with tricuspid regurgitation velocity (TRV). ***(Added 06/04/3013).***

A hemoglobin analysis will be performed at screening to confirm diagnosis of sickle cell anemia (or HbS-β^0 thalassemia). (***Added 08/26/2011.)***

Statistical Methods: ***(****revised 08/26/2011;* ***revised 05/31/2013.)***
Statistical analysis strategy**:** Descriptive statistics of endothelial dysfunction measures (FMD%, NTMD%), soluble VCAM, soluble ICAM, sFLT-1, VEGF, heme oxygenase activity and rho/rho kinase activity will be presented by treatment and by visit, along with corresponding charts. FMD% and NTMD% will be log-transformed. To evaluate the effect of atorvastatin on endothelial dysfunction measures, we will perform a comparison of treatment by visit, using Student t-test subsequently followed by crossover ANOVA model testing carry-over effect and treatment effect. For soluble VCAM, soluble ICAM, sFLT-1, VEGF, heme oxygenase activity and rho/rho kinase activity, since they are not normally distributed, we will compare atorvastatin with placebo using Wilcoxon sign rank test. Similar analysis will be performed for the albuminuria and estimated GFR values. In addition, the relationship between outcome measures and treatment will be studied through a multiple regression analysis.

To evaluate the effect of atorvastatin on albuminuria, we will examine albuminuria both as a continuous variable using a linear mixed model to analyze the effect and as a categorical variable using a generalized estimating equation approach. Point estimates and confidence interval for the effect of albuminuria will be presented. Distribution of any complication among patients by treatment will be presented. The association of side effects by treatment will be tested using McNemar's test.

Differences in pre- and post- treatment echocardiographically-defined TRV will be described in tabular and graphical terms. ***(Added 06/04/2013.)***

Sample Size Justification: Based on previous literature (70), we assume a standard deviation of 0.894 for changes in mean FMD%. In a 2x2 crossover design, the minimum required sample size is 9 per sequence to detect a clinically meaningful difference of 0.65 in mean changes in FMD% between atorvastatin and placebo groups at significance level α =0.05 with 80% power. Assuming a 5% attrition rate, we will enroll 19 patients for this study. ***(Revised 06/03/2013.)***

Anticipated Outcomes: We anticipate that atorvastatin, by improving endothelial dysfunction, will decrease albuminuria in SCD. Furthermore, atorvastin will increase HO activity, but decrease rho kinase activity, sFLT-1 levels and TF-mediated sFLT-1 release from monocytes.

Potential Pitfalls: The main limitation in this exploratory study is that 6 weeks of treatment with atorvastatin may not be adequate to achieve an improvement in endothelial dysfunction assessed by flow mediated

vasodilation or improvement in the estimated GFR. If the results suggest a promising risk-benefit ratio for the effects of atorvastatin on endothelial function, parameter estimates from the clinical intervention will be used to determine the most appropriate design and sample size requirements for a more definitive study.

As hydroxyurea may affect endothelial function and is associated with decreased plasma levels of endothelial markers in SCD patients, the benefits of atorvastatin may be less for patients on hydroxyurea. To deal with these issues, patients on hydroxyurea will be on a stable dose for at least 3 months prior to starting the study and the dose will only be changed for safety reasons. In addition, analyses of the results will be adjusted for treatment with hydroxyurea as a post stratification variable.

Feasibility: The UNC Comprehensive Sickle Cell Program is well suited to conduct the studies proposed here. Our program consists of a dedicated group of clinical researchers, nurses and scientists who have a track record of high quality clinical and laboratory-based studies. We have extensive experience in the conduct of clinical studies as proposed here. We will use the services of the NC TraCs Institute and the CTRC at UNC, Chapel Hill, with such resources as a state of the art database system, an investigational drug pharmacy, research ultrasound laboratory, and outpatient facilities where study patients will be evaluated. The UNC Comprehensive Sickle Cell Program provides care to ~ 350 adults and 350 children with SCD. In addition to a weekly sickle cell clinic, we have a weekly clinical and research meeting where patient cases and research studies are discussed. This practice model fosters efficient accrual of subjects onto clinical research studies.

Data Management: For all study aims, a standardized set of data collection forms will be designed using the RED Cap system and a secure database will be created to support the compilation of study-specific information. All data will be stored under password protection on a secured server. A data manager will handle all data entry and the security of the database. This individual will work closely with the statistician on database design and quality assurance procedures.

Timeline: The projected study duration is 5 years. We anticipate beginning recruitment within 3-6 months of the start date. This time period is required to confirm the presence of albuminuria. Recruitment is expected to take up to 48 months. The remaining few months will be dedicated to completing the studies, data analysis and publication.

HUMAN SUBJECTS

This human subjects' research meets the definition of a clinical trial. An Investigation New Drug (IND) application to the FDA to support the administration of atorvastatin to SCD patients is pending. ***(FDA determined the study to be EXEMPT from IND. Letter on file.)***

Protection of Human Subjects

1. Human Subjects Involvement and Characteristics: This study seeks to evaluate the contribution of endothelial dysfunction to the pathophysiology of albuminuria in SCD. It involves the conduct of a study to evaluate the effect of atorvastatin on endothelial dysfunction and albuminuria in SCD. Nineteen patients with SCD (HbSS or $S\beta^0$ thalassemia) that meet the eligibility criteria will be enrolled in a randomized, double-blind, placebo-controlled, crossover trial. At the time of enrollment, all study subjects will be in the non-crisis, steady state. Informed consent will be obtained according to IRB approved procedure. Patients will be closely monitored for adverse events following randomization to the study drug. The primary goal of this study is to evaluate the effect of atorvastatin on endothelial dysfunction (FMD and NTMD). Furthermore, we will evaluate plasma markers of endothelial activation (soluble VCAM, soluble ICAM), plasma levels of sFLT-1, VEGF, nitric oxide metabolites, heme oxygenase activity, rho/rho kinase activity, tissue factor-mediated sFLT-1 release from monocytes, albuminuria, estimated glomerular filtration rate and safety as described in the application.

2. Sources of Research Material: Enrollment and study participation will involve the collection of a full range of clinical data, including medical history. We will obtain brachial artery ultrasound data, as well as clinical and laboratory data at specified intervals during the course of the studies as described in the research plan.

3. Plans for Recruitment: Patients will be recruited from amongst the patients that routinely receive their care at the UNC Comprehensive Sickle Cell Program. Dr Ataga and/or one of his co-investigators will discuss the studies with all eligible patients. Interested patients will be provided with a copy of the consent form(s), and the study coordinator or PI (or a co-investigator) will carefully go over the consent with each candidate before he/ she signs. We will strive to ensure that all patients understand the nature of the study and all their questions will be answered. The original consent form will be placed in the study files, in a secure, locked cabinet. Potential subjects will be contacted during regularly scheduled clinic visits, but may be contacted at home by study personnel if they are known to meet the eligibility criteria. The PI and study nurse involved in the study are regular staff of the program. Samples obtained from patients specifically for these studies will be collected as approved by the IRB. This is anticipated to occur during specified study visits.

4. Potential Risks: The clinical study with ancillary studies entail minor physical risk associated with venipuncture to obtain blood samples; administration of atorvastatin; administration of nitroglycerin; and pneumatic occlusion cuff placed around the upper forearm to a suprasystolic pressure (~ 200 mmHg) during the assessment of flow-mediated vasodilation. The most significant risks of blood tests are discomfort and bruising. There is extensive clinical experience with the use of atorvastatin, although there are no published studies using this drug in SCD. Atorvastatin is well tolerated in the general population. However, as the most common complications of statins are liver function test (LFT) abnormalities and myopathy, we will exclude those patients with significant liver disease or history of a myopathy. In addition, as SCD patients have low cholesterol levels, lipid profiles will be monitored closely. Nitroglycerin commonly causes a headache, and less often hypotension, dizziness and lightheadedness. We will exclude patients with systolic blood pressure < 90 mmHg, severe aortic stenosis, and hypertrophic obstructive cardiomyopathy. Headaches associated with nitroglycerin use are typically short lasting and respond to acetaminophen. Pneumatic occlusion of the forearm to a suprasystolic pressure is uncomfortable. However, this technique has been safely performed in SCD patients. Patients will be monitored very closely for any laboratory or clinical complications during the course of the studies.

5. Procedures for Minimizing Risks: We will do everything possible to minimize the risks associated with participating in the studies. For patients receiving nitroglycerin during assessment of flow-mediated vasodilation, we will ensure there are no contraindications to receiving this drug. In addition, we will obtain baseline laboratory studies (including liver function tests and creatine kinase) prior to commencement on the study and monitor them closely during the course of the study due to the small risk of worsening LFT and myopathy with atorvastatin. This study will be monitored by a DSMB, which will review all reports to determine if there is a safety concern. Finally, we have established data safety and monitoring procedures as described below.

Risks to Privacy: All clinical information, including personal identifiers obtained on research subjects will be maintained in a locked and secured file drawer with access limited to clinical staff. All data will be coded to protect patient confidentiality. We will make all effort to maintain patient confidentiality.

6. Potential Benefits of the Proposed Research to the Subjects and others: The projects proposed in this application will help to define the contribution of endothelial dysfunction to the pathogenesis of albuminuria in SCD. The available treatments of nephropathy in SCD remain limited. Treatment with atorvastatin may provide clinical benefit.

7. Importance of the Knowledge to be Gained: Despite our improving understanding of the pathophysiology of SCD, treatment options for several SCD-related complications remain quite limited. This proposal aims to increase our understanding of the pathogenesis of albuminuria in SCD and evaluate the efficacy and safety of

atorvastatin in SCD-associated nephropathy. The risks to patients from participating in this study are modest and are reasonable in relation to the potential benefits and the importance of knowledge to be gained.

8. Data and Safety Monitoring Plan: All SAEs will be reported to the IRB and the DSMB. The DSMB will review these data quarterly and notify the Principal Investigator if safety appears to be an issue in the study. In addition, after 5 patients and then 10 patients are enrolled, all of the available safety data will be presented by treatment group to the DSMB in a confidential report. The DSMB will review and determine if there is a safety concern. The study will be terminated at the occurrence of treatment-related serious adverse events, including transaminitis or myopathy, in up to three study participants.

The UNC TraCS DSMB is a committee that has been established within the UNC School of Medicine. The membership is appointed by the Dean (or his designee), and is available to review any clinical trial that is being carried out by a UNC investigator. Most multi-center clinical trials funded by the NIH or by industry have national data and safety monitoring boards organized by the sponsoring organization. However, single- or dual-site clinical trials such as the study proposed in this application depend on a local DSMB such as the one based here at UNC. The current makeup includes a chair (Dr. Ross Simpson), an ethicist, an epidemiologist/biostatistician, and several clinical researchers. In addition, *ad hoc* members are added to the DSMB when need for scientific expertise arises.

9. Inclusion of Women and Minorities: Based on the results of our studies in this patient population to date, we anticipate that enrolled subjects will include 50-55% women. We have achieved this in the past without special efforts to recruit women, and this reflects the gender distribution of our patient population. In addition, due to the nature of the disease to be studied (sickle cell disease), over 95% of patients will identify themselves as African-American. A small number identifying themselves as Hispanic or Native American may be available; such patients typically comprise less than 2% of our clinic patients with this disease.

10. Inclusion of Children: As the prevalence of albuminuria in children is thought to be lower than in adults, we anticipate that most of our study patients will be adults.

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Endothelial function and renal disease, Specific Aim #3

	Screening/ Baseline*		Treatment Phase #1				Washout Phase		Treatment Phase #2		
	Between Day -28 to Day 0		Day 0	Week 4	Week 6		4 Weeks		Day 0	Week 4	W
Informed Consent	X										
Medical History and Assessment of Eligibility	X										
Physical Exam	X		X		X				X		
Vital signs	X		X	X	X				X	X	
Hematology (CBC w/diff and retics)	X		X		X				X		
Chemistries (including creatine kinase)	X		X	X	X				X	X	
Lipid profile	X				X						
PT(INR) and PTT	X										
Urine microalbumin/ creatinine ratio	X		X	X	X				X	X	
Urinalysis	X										
Serum pregnancy test (for women of child bearing capacity)	X		X						X		
Urine pregnancy test (for women of child bearing capacity)				X	X					X	
Randomization/ initiation of study treatment	X		X						X		
Brachial ultrasound	X		X	X	X				X	X	
Measures of endothelial function	X		X		X				X		
Measures of hemolysis	X		X		X				X		
Adverse event probe			X	X	X		X		X	X	

*Screening/Baseline Phase and Day 0 of the 1st Treatment Phase may be consolidated into one visit as long as eligibility is confirmed prior to initiation of study treatment