PHASE I/II SAFETY AND DOSE ESCALATION TRIAL OF THE OMEGA-3 FATTY ACIDS DOCOSAHEXAENOIC ACID (DHA) and EICOSAPENTAENOIC ACID (EPA) IN CHILDREN AND YOUNG ADULTS WITH SICKLE CELL DISEASE (SCD)

NCT02947100

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STUDY DRUG: SCD-Omegatex™ (Enteric Fish Oil 250 DHA/27 EPA Soft Gelatin Capsule, 450 mg)

SPONSOR: National Institute of General Medical Sciences of the National Institutes of Health (NIH/NIGMS) under Award Number P20GM109021

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Version 5, April 2018

TRIAL SUMMARY

Full Title	Phase I/II Safety and Dose Escalation Trial of the Omega-3 Fatty Acids (n-3 FAs) Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) in Children and Young Adults with Sickle Cell Disease (SCD)
Abbreviated Title	Omega-3 Fatty Acids in SCD
Primary Objectives	1) To determine the clinical safety of DHA/EPA supplementation with SCD-Omegatex™ in children with Sickle Cell SS Disease (HbSS), Sickle C Disease (HbSC) and Sickle β^0 Thalassemia (HbSβ^0thal) as evidenced by an absence of adverse events (AEs) related to the fatty acid (FA) supplementation 2) To determine whether 6 months of supplementation with SCD-Omegatex™ will reduce thermal sensitivity as measured by Quantitative Sensory Testing (QST) to below pre-treatment levels
Secondary Objectives	1) To determine whether 6 months of supplementation with SCD-Omegatex™ will increase health-associated Quality of Life (QoL) responses using the Varni™ Pediatric QoL Inventory™ SCD Module. 2) To determine the effect of treatment by assessing pain days using an iPad daily report pain diary. 3) To measure the effect of treatment on individual thermal sensitivity parameters by QST
Exploratory Objectives	1) To measure the effect of treatment on select biomarkers of inflammation including pro and anti-inflammatory cytokines, adhesion molecules, hsCRP.and plasma lipid mediators derived from DHA, EPA and Arachidonic Acid (AA). 2) To assess the effect of SCD-Omegatex™ treatment on thrombin generation as assessed by Computerized Automated Thrombogram (CAT).
Study Drug	SCD-OmegatexTM (the Drug Product, DP) consists of a fish-oil derived FA concentrate, containing mainly omega-3 fatty acid ethyl esters (EE), predominantly docosahexaenoic acid (DHA-EE). Drug Substance (DS) also contains eicosapentaenoic acid (EPA-EE) but at a concentration lower than DHA-EE. DHA and EPA ratio is approximately 9:1 Alpha-tocopherol is added during manufacturing the drug substance at a concentration of 2 mg/g as an antioxidant.
Study Design	Phase I/II Safety and Dose Escalation Trial
Research Setting	Single Site – Nemours/AI duPont Hospital for Children (NAIDHC), Wilmington, Delaware
Trial Blinding	Open Label

Treatment Groups	• **Phase I Portion**: The Dose Limiting Toxicity (DLT) portion of the study will follow a "3+3" design. Three subjects will be enrolled at the starting dose of 25 mg/kg body weight of omega-3 FAs (combined DHA and EPA). If none of the subjects has a DLT after 2 months of treatment, the dose is escalated for the next 3 subjects to 37.5 mg/kg. The original 3 enrollees will remain on the 25 mg/kg dose for the remainder of the trial. If one DLT is observed at 25 mg/kg, the next 3 subjects are enrolled at 25 mg/kg. If one out of 6 subjects has a DLT at 25 mg/kg, the dose is escalated to 37.5 mg/kg for the next group of subjects. If 2 or more of 6 subjects develops a DLT at 25 mg/kg, escalation is stopped and no Maximum Tolerated Dose (MTD) is found. If the dose is escalated to 37.5 mg/kg and 1 or fewer DLT is observed in the first 3 subjects after 2 months, 3 more subjects will be enrolled at the 37.5 mg/kg. If in 2 more months, fewer than 2 out of 6 subjects show toxicities at 37.5 mg/kg, 37.5 mg/kg is the recommended Phase II dose. If 2 or more DLTs are noted, 25 mg/kg of DHA is the MTD. The length of treatment at each dose level in Phase I will be 2 months, with carryover to Phase II of subjects receiving the MTD. The maximum sample size for this portion of the study is 12 subjects. • **Phase II Portion:** A sample size of 30 is needed to have 80% power to detect a moderate 1.5 degree (0.5 standard deviation) mean thermal sensitivity reduction at 6 months following treatment using a 2-sided paired-t test of size 0.05. Since 6 of the subjects for this phase will come from the Phase I portion, the additional sample size needed for this Phase is 24 subjects. Allowing for approximately 20% attrition we will recruit 30 additional subjects in this phase with a total maximum sample size for both Phases I and II combined being 42 subjects (Phase I=12, Phase II=30).
Number of trial subjects	42
Estimated duration of trial	3 years
Duration of Participation of each subject	9 months

ABBREVIATIONS

AA	arachidonic acid
AACC	American Association for Clinical Chemistry
ACS	acute chest syndrome
AE	Adverse Event
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BHT	butylated hydroxyl-toluene
CAP	College of American Pathologists
CAT	Computerized Automated Thrombogram
CBC	complete blood count
CiC	Child in Care
CL	calibrator
CLIA	Clinical Laboratory Improvement Ammendments
CP	cold pain
CRF	case report form
CS	cold sensitivity
CTCAE	Common Terminology Criteria for Adverse Events
DHA	Docosahexaenoic Acid
dL	deciliter
DLT	dose limiting toxicity
EDTA	ethylenediaminetetraacetic acid
EE	ethyl ester
ELISA	enzyme-linked immunosorbent assay
EMR	electronic medical record
EPA	eicosapentaenoic acid
ER	emergency room
ET-1	endothelin-1
ETP	endogenous thrombin potential
FA	fatty acid
FAMES	fatty acid methyl esters
F1.2	prothrombin fragment 1.2
FDA	Food and Drug Administration
fmol	femtomole
g	gram
GC	gas chromatography
GCP	Good Clinical Practice
GI	gastrointestinal
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GMR	Geometric Mean Ratio
Hb	hemoglobin
HbF	fetal hemoglobin
HbS	sickle hemoglobin
HbSβthal	Sickle β Thalassemia
HbSC	Sickle C Disease
HbSS	Sickle Cell SS Disease
17-HDHA	17-hydroxydocosahexaenoic acid
18-HEPE	18-hydroxyeicosapentaenoic acid
12-HETE	12-hydroxyeicosatetraenoic acid
HP	heat pain

HRQoL	health related quality of life
HS	heat sensitivity
hsCRP	high sensitivity C reactive protein
HU	hydroxyurea
IB	Investigator's Brochure
IL	interleukin
IND	Investigational New Drug
IRB	Institutional Review Board
ISO	International Organization for Standardization
iu	international units
kg	kilogram
l	liter
LC-MS	liquid chromatography-mass spectrometry
LDH	lactate dehydrogenase
LFT	liver function tests
LT	leucotriene
MaR1	Maresin 1
µg	microgram
mg	milligram
µl	microliter
ml	milliliter
min	minutes
µM	micromolar
MTD	maximum tolerated dose
n3-FAs	omega-3 fatty acids
n6-FAs	omega-6 fatty acids
NAIDHC	Nemours/Alfred I duPont Hospital for Children
NCLLS	National Committee for Clinical Laboratory Standards
NFκB	nuclear factor kappa B
ng	nanogram
NHANES	National Health and Nutrition Examination Survey
NIH/NIGMS	National Institute of General Medical Sciences of the National Institutes of Health
nM	nanomolar
NSAID	non-steroidal anti-inflammatory Drug
O3I	omega-3 index
PC	phosphatidylcholine
PD1	Protectin D1
PE	phosphatidylethanolamine
pg	picogram
PI	Principal Investigator
PLPs	phospholipids
pM	picomolar
PPT	pressure pain threshold
PRBCs	packed red blood cells
PS	phosphatidylserine
PT	prothrombin time
PTT	partial thromboplastin time
PUFA	polyunsaturated fatty acid
QoL	quality of life
QST	Quantitative Sensory Testing
RBC	red blood cell
Retic	reticulocyte count
Rv	resolvin

SAE	Serious Adverse Event
SCD	Sickle Cell Disease
SD	standard deviation
SL selectin	soluble L selectin
SPM	specialized pro-resolving mediators
SP selectin	soluble P selectin
TCD	transcranial doppler
TG	thrombin generation
TxB2	thomboxane B2
TLC	thin layer chromatography
TNFα	tumor necrosis factor α
TRPV1	transient receptor potential vanilloid 1
TRPA1	transient receptor potential ankryn 1
Tt peak	time to peak
UA	urinalysis
ULN	upper limit of normal
USA	United States
VAS	visual analog scale
VCAM-1	vascular cell adhesion molecule-1
VOC	vaso-occlusive Crisis
VS	vital signs
WBC	white blood cell
xg	x gravity

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1.0 Background and Significance:

Sickle Cell Disease (SCD) is a chronic disorder caused by a mutant hemoglobin. The substitution of the amino acid valine by glutamic acid in the sixth position of the beta subunit of hemoglobin produces sickle hemoglobin (Hb S). This mutation promotes the hydrophobic interaction and polymerization of deoxygenated Hb S molecules, producing rigid, elongated red blood cells (RBCs), or sickle cells, that obstruct the microcirculation. The gene for Hb S arose in historically malarious regions of the world because the heterozygous state offered protection against the malaria parasite, and the gene is now found in individuals of African, Arabic, Hispanic, Greek, and Italian descent. The gene frequency of Hgb S is particularly high in individuals of African ancestry in the United States (USA), among whom approximately 1 in 12 are heterozygotes. SCD occurs in individuals who are homozygous for Hb S or are compound heterozygotes for Hb S and certain other abnormal hemoglobins. Approximately 1 in 500 African-Americans has SCD and 90-100,000 individuals are affected in the US (CDC Data and Statistics 2015). Globally, the numbers of affected individuals are much larger with more than 275,000 individuals born annually (Modell and Darlison 2008).

1.1 Pain in SCD

The painful vasocclusive crisis (VOC) is the hallmark of microvessel occlusion in SCD and its most common and debilitating feature (Dampier, Setty, et al. 2004; Platt et al. 1991; Smith et al. 2008; Stuart and Nagel 2004). While the initiating event is the polymerization of Hg S, investigations into its pathophysiology have led to the delineation of a complex process, encompassing interactions between sickle RBCs, white cells (WBCs), endothelium, plasma proteins and other factors (Frenette and Atweh 2007).

There is very strong evidence suggesting that SCD is a chronic inflammatory state (Belcher et al. 2003; Platt 2000). Hebbel and Kaul (Kaul and Hebbel 2000) have linked the reperfusion injury paradigm to the VOC event. These investigators and the work of Frenette (Frenette 2002; Turhan et al. 2002) have focused on activated leucocytes and their released inflammatory mediators as playing a critical role in VOC pathobiology. Enhanced production of inflammatory cytokines including interleukin (IL)-1ß and tumor necrosis factor (TNF)α and leukotrienes (LTs) such as LTB4 in SCD promote cell-cell adhesion, endothelial activation, up regulation of endothelial adhesion molecules, activation of transcription factors including nuclear factor (NF) kB, and further leukocyte recruitment (Belcher et al. 2003; Hebbel, Osarogiagbon, and Kaul 2004; Platt 2000). These inflammatory cytokines and leukotrienes also sensitize nociceptors acting as agonists for the transient receptor potential vanilloid 1 (TRPV1) and ankyrin (TRPA1) which together with other TRP channels play a role in neural and nociceptive pain (Bautista, Pellegrino, and Tsunozaki 2013; Fernandes, Fernandes, and Keeble 2012; Geffeney and Goodman 2012) via both peripheral and spinal cord mechanisms. Of note, increased levels of the biomarker high sensitivity C-Reactive Protein (hsCRP), produced in the liver in response to a variety of these inflammatory cytokines, have been shown to correlate positively with pain hospitalizations and vasoocclusive events (Krishnan et al. 2010).

SCD is a chronic hypercoagulable state. Evidence for this includes elevated plasma levels of multiple markers of thrombin and fibrin generation, increased tissue factor expression, and decreased levels of circulating anticoagulants. Platelets are activated at baseline in SCD patients, with further activation during VOC (Heeney et al. 2016). SCD patients are at significantly increased risk for thrombotic complications such as pulmonary embolism and stroke. Inflammation, ischemia-reperfusion injury and hemolysis are all likely contributors to coagulation activation (Ataga et al. 2012; Noubouossie, Key, and Ataga 2015; Tomer et al. 2001). We have used the Calibrated Automated Thrombogram (CAT),a semiautomated high throughput method as the closest relevant representation of in vivo clotting (Baglin 2005). Using this assay in children and adults with SCD we have noted striking alterations in the initial phase of thrombin generation (a shorter lag time, and lesser time to peak) although overall endogenous thrombin generation was not significantly different (Betal et al. 2009).

For SCD patients, the experience of recurrent acute VOCs has a devastating impact on quality of life (Dampier et al. 2010; Dampier et al. 2011) and is a risk factor for early mortality (Platt et al. 1991). In addition, VOCs place a heavy burden on the USA health care system, accounting for a large number of Emergency Room (ER) visits and hospital admissions annually at a cost of approximately $500 million (Weisberg et al. 2013). Despite

an extensive literature addressing pain in SCD, the nature of vasoocclusive pain, factors underlying the transition from acute to chronic pain and the wide variability in the pain experience between patients with SCD remain poorly understood. Optimal management strategies for this complex pain syndrome remain highly problematic despite over three decades of basic and clinical research in the field (Ballas, Gupta, and Adams-Graves 2012). There are major overlaps between the areas of neuropathic pain, ie pain initiated by dysfunction of the peripheral or central nervous system and inflammatory or nociceptive pain, where pain is the result of tissue injury. Such clear differentiation between these types of pain in any given subject with SCD may not be practically feasible. SCD subjects use neuropathic pain descriptors, and may have the hypersensitivity to tactile stimuli usually observed in neuropathic pain (hyperalgensia and allodynia) and a recent study by Brandow and coworkers found that a third of the SCD patient group (median age 20 years) had evidence of neuropathic pain when screened with a validated neuropathic screening tool (Brandow, Farley, and Panepinto 2014).

To preserve survival, the human body has evolved complex protective mechanisms to detect and interpret noxious stimuli. Tissue injury from a variety of mechanical, thermal or chemical stimuli produce biochemical mediators, stimulating afferent peripheral nerve fibers (nociceptors) that synapse in the dorsal horn of the spinal cord, transmitting signals through ascending pathways to the brain **(figure 1).** Descending neural pathways arising in multiple areas of the brain also regulate transmission of pain signals in both a positive and negative fashion at the level of the spinal cord (Ossipov, Dussor, and Porreca 2010).

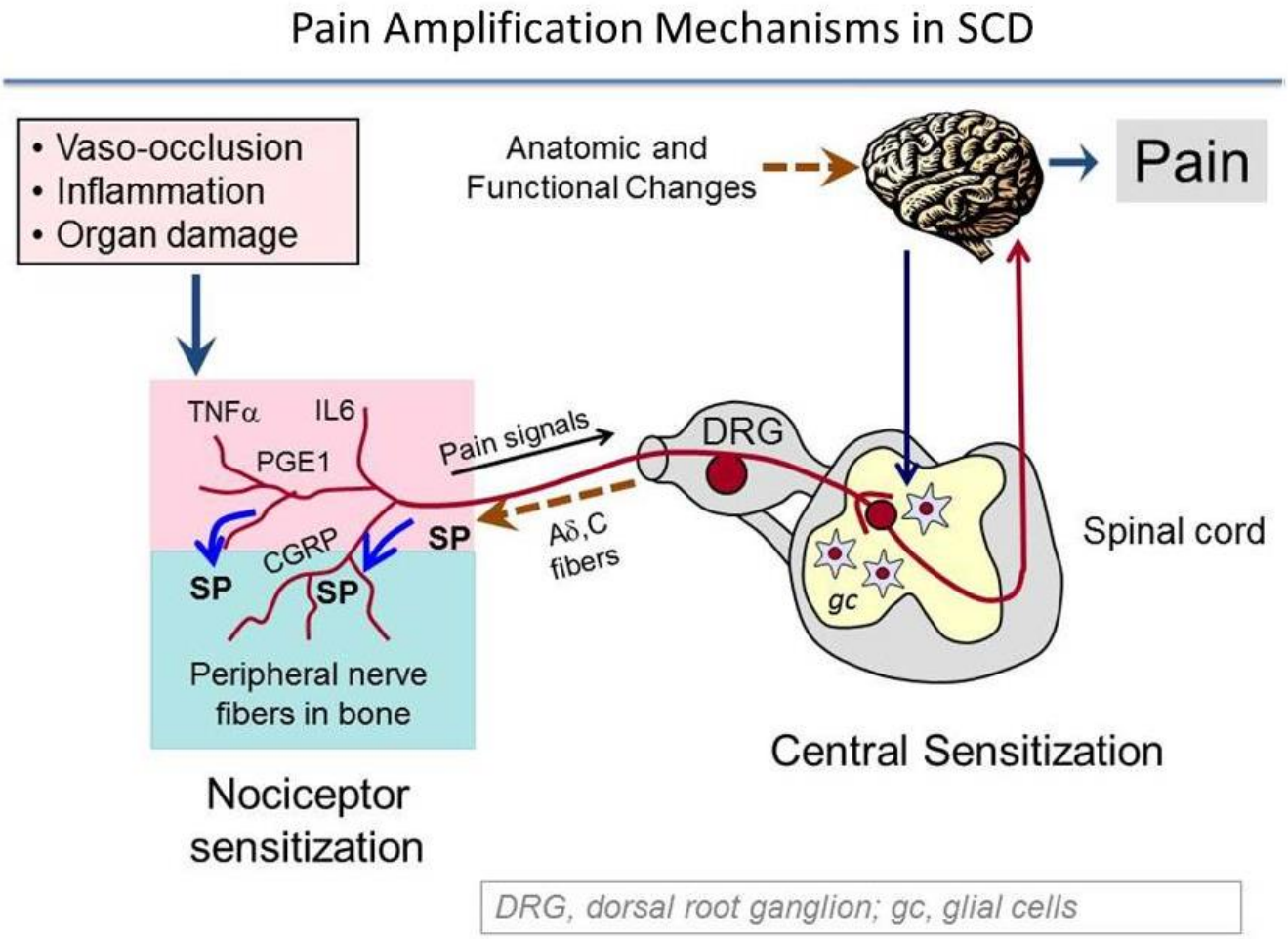


Figure 1

However, alterations in this system may occur which can result in the pathologic experience of pain. Tissue damage with associated accumulation of inflammatory factors released from activated nociceptors and other infiltrating cells can enhance the excitability of the nerve fiber resulting in an augmentation in sensitivity to painful stimuli. This process is known as **peripheral sensitization** (Basbaum et al. 2009). Repetitive exposure to

noxious stimuli can result in amplification in the pain response through changes in signaling pathways in the spinal cord and cortex, known as **central sensitization** (Kuner 2010). Mounting data supports the idea that peripheral and/or central sensitization may play a key role in the experience of sickle cell vasoocclusive pain. A recent study has implicated mast cell activation with neurogenic inflammation and hyperalgesia in SCD mice (Vincent et al. 2013).

1.2 Quantitative Sensory Testing (QST)

QST refers to a set of noninvasive psychophysical procedures performed to measure subjective somatosensory responses to various physical stimuli in order to evaluate sensory function of the peripheral nervous system. QST has been successfully performed and shown to be feasible in children as young as three years (Hilz et al. 1996) with good reproducibility over short intervals of retesting (Meier et al. 2001). In prior studies, pediatric subjects have not reported severe pain or distress and have not withdrawn from study enrollment (Meier et al. 2001; Zohsel et al. 2006). Reference values for thermal and mechanical detection and pain thresholds are available for children and adolescents aged 6 and up (Blankenburg et al. 2011; Blankenburg et al. 2010). Thermal testing for these and many other recent studies, including prior studies where QST testing was done in children (Brandow et al. 2013; O'Leary et al. 2014; Jacob et al. 2015) and adults with SCD (Campbell et al. 2016; Ezenwa et al. 2016), used the Medoc TSA II analyzer (Medoc Advanced Medical Sciences) and subjects tolerated the testing well, with no reports of significant pain or distress.

There is strong evidence that sickle mice exhibit marked hypersensitivity to thermal and mechanical stimuli (Garrison et al. 2012; Hillery et al. 2011; Kohli et al. 2010). Brandow et al have documented significantly lower median cold and heat detection and pain thresholds in SCD subjects (all genotypes; median age 14 years) evaluated by QST (Brandow et al. 2013). Studies by O'Leary et al and Jacob et al have also demonstrated altered thermal sensitivity in children with SCD, though with somewhat variable results (Jacob et al. 2015; O'Leary et al. 2014).

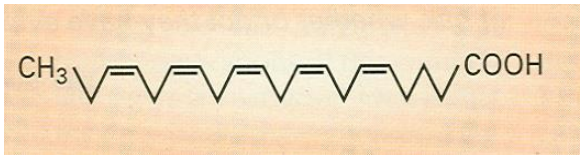
Although experimental pain does not replicate many features of clinical pain such as emotional overlay and actual tissue injury, it can provide relevant and valuable insights. Clinical relevance of QST in assessing patients with a variety of pain syndromes, including fibromyalgia, temporomandibular disorders, headache disorders and pelvic pain syndromes has been well documented in the literature. Suprathreshold experimental pain responses can predict pain intensity following surgical procedures. Of particular relevance to the current protocol, QST has been shown to be a sensitive index of treatment outcomes (Edwards et al. 2005). This technology will be a valuable tool to aid in assessing the efficacy of interventions, such as omega-3 fatty acid (n-3 FA) supplementation, to ameliorate VOC in children with SCD.

1.3 The Omega-3 Fatty Acids in SCD

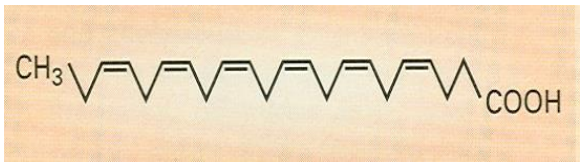
While current treatment strategies can ameliorate some of the suffering experienced by SCD patients with VOC, the development of effective and minimally toxic preventative strategies is clearly the most desirable approach. The ideal drug for the efficacious treatment of SCD should be non-toxic and orally administered so that it can be used daily to prevent VOC, rather than as an acute intervention after VOC has occurred. It should also be inexpensive to facilitate use in underdeveloped countries where SCD is so prevalent. Hydroxyurea (HU) is the only known drug with proven efficacy in preventing VOC and its efficacy rests on its pleiotropic erythrocyte and extra-erythrocytic effects (Charache et al. 1995; Stuart and Nagel 2004; Wang et al. 2011). However, there are many SCD patients who do not respond adequately, or at all, to this medication and safe administration requires burdensome and expensive laboratory monitoring.

Interest in marine oils has passed through many phases from the 19th C lighting of New England lamps to the "Eskimo Paradox" where despite their high fat intake the Inuit experience little cardiovascular disease. The essential n-3 FAs Docosahexaenoic Acid or DHA (c22:6n-3) and Eicosapentaenoic Acid or EPA (C20:5n-3), depicted in **figure 2** and characterized by a double bond (3 carbons from the methyl terminal of the molecule), have profound biologic effects (**Table 1**). A multitude of clinical trials and studies have documented their role in dyslipidemias and cardiovascular health (Bradberry and Hilleman 2013; Davidson 2013; Davidson et al. 2012), immune function and inflammatory disorders (Calder 2015; Levy et al. 2001; Serhan and Savill 2005) , brain

development and neurologic disorders (Das 2006; Vesco et al. 2015; Yassine et al. 2016) and pain (Serhan 2014).



Eicosapentaenoic Acid (C20:5n-3)

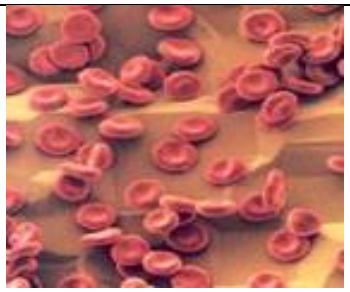
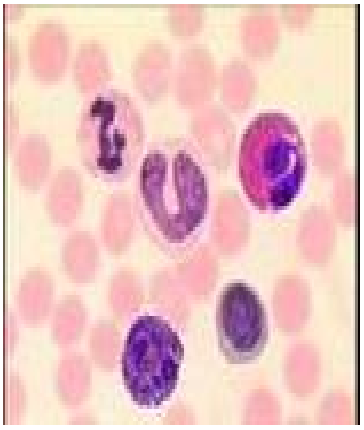
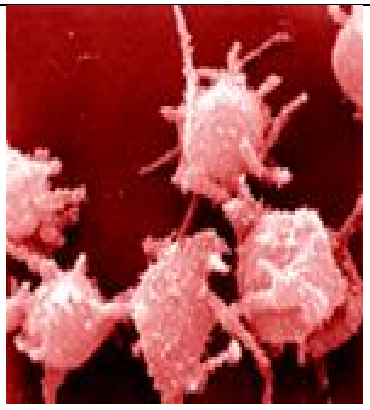
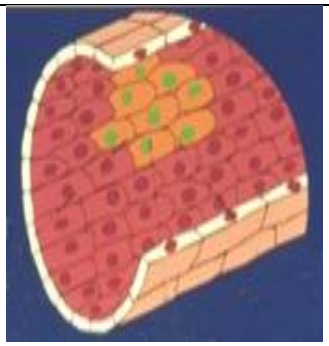


Docosahexaenoic Acid (C22:6n-3)

Figure 2

To emulate the success of HU, we need to identify drugs with effects both on the RBC and the other circulating cellular elements of blood, and the endothelium which serves as the template on which cell-cell interactions occur. The n-3FAs DHA and EPA are ideal candidates to fulfill this role since (a) Following incorporation into cell membranes DHA and EPA give rise to potent anti-inflammatory mediators the Resolvins and Protectins of the D (from DHA) and E (from EPA) series which "stop the clock" on the inflammatory process (Ji et al. 2011; Serhan 2007). (b) Resolvins modulate pain hypersensitivity by inhibiting TRPV1 and TRPA1 channels with DHA metabolites having much greater potency than those from EPA (Park et al. 2011; Xu et al. 2010; Xu and Ji 2011) (c) DHA supplementation reduces the increase in thermal pain sensitivity exhibited by the SCD mouse (Wandersee 2012; Wandersee et al. 2015). (d) Preliminary data shows that children with HbSS have a cell membrane content that favors the pro-inflammatory FA Arachidonic Acid (AA) over the anti-inflammatory FAs, DHA and EPA, and that DHA/EPA supplementation can favorably modulate this (Daak, Ghebremeskel, Hassan, et al. 2013; Setty et al. 2015). (f) In addition, DHA/EPA possess many other salutary effects on RBCs, platelets, and endothelium that can modulate SCD pathology **(Table 1).**

Table 1: Pleiotropic effects of omega-3 fatty acids on circulating blood cells and endothelium

RBCs ● Improves membrane deformability and fluidity (Lund et al. 1999; Mills, Galey, and Dixon 1993; Popp-Snijders et al. 1986; Terano et al. 1983) ●Decreases viscosity of RBC suspensions (Popp-Snijders et al. 1986; Terano et al. 1983) ●Increases enzyme levels including glutathione peroxidase (De Caterina, Liao, and Libby 2000) ● Effects on Fetal Hb via TR4-mediated CD36 transactivation (Campbell et al. 2011; Xie et al. 2009)	
WBCs ●Suppresses production of inflammatory cytokines (Calder 2013; Ji et al. 2011; Li et al. 2014; Serhan 2007; Song et al. 2016) ●Protects against ischemia-reperfusion injury in vital organs by modulating leukocyte-mediated inflammation (Chen et al. 2003; Lehr et al. 1991; Marcheselli et al. 2003; McGuinness et al. 2006; Serhan 2007) ●Induces the potent lipid mediators Resolvins, Protectins and Docosatrienes (Levy et al. 2001; Serhan and Savill 2005; Serhan 2007, 2014) ●Decreases monocyte activation and gene expression of IL-1β and TNF-α (Calder 2013; Hudert et al. 2006; Mayer et al. 2002; Saku, Kobayashi, and Kitamura 1999; Taccone-Gallucci et al. 2006) ●Inhibits the 5-lipoxygenase pathway in neutrophils and monocytes with decreased LTB4 levels(Goodnight, Harris, and Connor 1981; Saku, Kobayashi, and Kitamura 1999; Taccone-Gallucci et al. 2006)	
Platelets and Hemostasis ●Decreases platelet aggregation, adhesion and release of intracellular constituents (Goodnight, Harris, and Connor 1981; Terano et al. 1983; Weiner et al. 1986) ●Decreases monocyte procoagulant activity (Hansen et al. 1989) ●Decreases fibrinogen, vWF and plasma viscosity (Piatti et al. 1993; Saynor and Gillott 1992) ●Decreases production of proaggregatory Thromboxane A2 (Rao, Radha, and White 1983; Weiner et al. 1986) ●Prevents vascular thromboses and re-stenosis after angioplasty (Dehmer et al. 1988)	
Endothelium ●Inhibits cytokine-induced adhesion molecule expression and transcription (VCAM-1, ICAM-1 and E-selectin) (Collie-Duguid and Wahle 1996; Dehmer et al. 1988; Nohe, Johannes, and Dieterich 2003) ●Inhibits PAF generation (Hudert et al. 2006) ● NFkB inhibited by DHA (Marsella and Borgna-Pignatti 2014; Serhan 2007) ●Increases PGI3 levels (Hishinuma, Yamazaki, and Mizugaki 1999) ● DHA causes predominant endothelial-independent vasodilation (Mori et al. 2000) ● Inhibits sickle red cell-endothelial adhesion (Stuart preliminary data)	

Based on what is known about the pathophysiology of SCD, these demonstrated effects of the n-3 FAs on blood cells, the endothelium, inflammatory mediators and the coagulation system have great potential, by way of multiple mechanisms, to favorably impact the vasoocclusive complications in SCD. A wealth of scientific data as well as some small clinical trials (Okpala et al. 2011; Tomer et al. 2001; Wandersee 2012) have indicated that the n-3 FAs EPA and DHA, with their pleiotropic effects on circulating blood cells and endothelium, represent a promising approach to ameliorate the experience of pain in SCD, with essentially no toxicity **(Table 2).** A 2001 study by Tomer demonstrated a reduced frequency of VOC with reduction in some prothrombotic mediators (Tomer et al. 2001). In a small Nigerian study, a decrease in the number of VOC was seen in 16 SCD patients treated with oral DHA and EPA for 6 months (Okpala et al. 2011). In Khartoum (Sudan), Daak and coworkers, in a recent placebo controlled evaluation, treated 128 subjects with Hb SS with an n-3 FA supplement (using a capsule with a DHA to EPA ratio of 7:1) daily for 1 year (Daak, Ghebremeskel, Hassan, et al. 2013). Their study demonstrated a reduction in VOC events, and a modulation of severe anemia in the treated group with no side effects or bleeding. Dyspepsia occurred in 4% of both treated and control groups. In a follow up study, the same group demonstrated lower expression of NFκB gene expression in the patients treated with their n-3 FA supplement compared with untreated subjects (Daak et al. 2015). NFκB is a transcription factor which plays a key role in the up-regulation of inflammatory cytokines and adhesive molecules (Hoesel and Schmid 2013; Min et al. 2005)**,** providing further evidence that n-3 FA supplementation may have a beneficial effect on inflammation and adhesion in SCD. Additional support comes from recent studies in SCD transgenic mice. Wandersee et al. demonstrated that DHA supplementation improved RBC flexibility and reduced irreversibly sickled cells in SCD mice (Wandersee et al. 2015). In addition, DHA treated SCD mice demonstrated reduced cold hypersensitivity, whereas no change was seen in mice who remained on a normal diet (Wandersee 2012). Kalish treated SCD mice with a diet rich in n-3 FAs for 6 weeks. The investigators were able to clearly demonstrate a reduction in vascular activation, inflammatory response and SCD related end organ damage in SCD mice on the n-3 FA diet (Kalish et al. 2015).

Table 2: Studies related to n-3 FAs in SCD

Table 2a: Human Studies

(Tomer et al. 2001)	Prospective, double-blind trial comparing treating adults with SCD and frequent VOC with menhaden fish oil (n=5) vs olive oil control (n=5). Observed **decreased frequency of VOC** in n-3 FA treated subjects ($p<0.01$). Pretreatment, significant decreases in platelet activation markers, platelet secreted proteins, thrombin generation and fibrinolysis (approaching control values) and decrease in prothrombin fragment 1.2 (F1.2), d-dimer and plasmin-antiplasmin complexes (PAP) were also noted in n-3FA treated subjects. The dosages of DHA and EPA used in this study were approximately 30 mg/kg/day EPA and 45 mg/kg/day DHA **There were no significant adverse events.**
(Okpala et al. 2011)	16 subjects with SCD in Nigeria treated with EPA 15 mg/kg/day and DHA 10 mg/kg/day for 6 months showed a **drop in median number of VOC** ($p<0.0001$) **Here were no significant adverse events.**
(Daak, Ghebremeskel, Hassan, et al. 2013)	Double blind, placebo controlled trial in which 128 subjects with SCD in Sudan (mean age 8.1 years) were randomized to treatment with DHA/EPA (7:1 ratio, approx 25 mg/kg/day n-3 FA) vs placebo for one year. N-3 FA treated group demonstrated **significant reduction in VOC**, severe anemia, blood transfusions, WBC and missed school days as compared to controls. **There were no significant adverse events.**
(Daak, Ghebremeskel, Mariniello, et al. 2013)	80 children with SCD in Sudan treated with DHA/EPA (7:1 ratio, approx 25 mg/kg/day n-3 FA) vs placebo for 6 mos. N-3 FA treated children showed significant decrease in CU/Zn superoxide dismutase activity and cytosolic glutathione peroxidase compared to pretreatment baseline suggestive of antioxidant protection.
(Setty et al. 2015)	Ratio of pro-inflammatory AA to anti-inflammatory DHA and EPA was significantly increased in RBC membranes of children with SCD as compared with control. This correlated positively with hs-CRP levels.
(Daak et al. 2015)	Follow up to Daak 2013 study. Compared children with SCD treated with DHA/EPA 7:1 (n=24), HU treated (n=18) and placebo (n=21) for minimum 1 year with healthy controls (n=25). N-3FA treated group showed lower NFκB gene expression compared with HU and control groups ($p<0.05$)

Table 2b: Animal Studies

(Wandersee 2012; Wandersee et al. 2015)	SCD mice whose diet was supplemented with DHA for 8 weeks were compared with control SCD mice. RBCs from SCD mice on DHA diet showed deceased stiffness, decreased osmotic fragility and fewer irreversibly sickled cells compared to control mice. In addition, DHA treated SCD mice demonstrated reduced cold hypersensitivity, whereas no change was seen in mice who remained on normal diet ($p = 0.007$).
(Kalish et al. 2015)	SCD and control mice fed standard diet versus n-3 FA enhanced diet. SCD mice fed n-3 FA diet demonstrated lower neutrophil counts and diastolic BPS. N-3 FA diet resulted in reduced aortic expression of endothelin-1 (ET-1, vasoactive, proinflammatory cytokine), VCAM-1 (marker of endothelial activation) and heme-oxygenase-1 (anti-oxidant). Pulmonary vascular permeability and ET-1 expression were also reduced in mice fed n-3 FA diet. In a hypoxia-reoxygenation model of VOC, n-3 FA reduced inflammation and protected against SCD related end-organ injury.

Since AA, EPA and DHA are the precursors of lipid mediators with effects on inflammatory/anti-inflammatory tone, the content of these FAs in the RBC membranes in children with HbSS disease and age- and race-matched controls was evaluated by Setty, et al (Setty et al. 2015). While AA gives rise to proinflammatory eicosanoid mediators, EPA and DHA are the precursors of recently identified potent anti-inflammatory products including Resolvins, Protectins and Maresins. **Table 3** shows preliminary data from Drs. Setty and Stuart's research lab (presented at the 9th Annual Sickle Cell Disease Research and Educational Symposium and 38th National SCD Scientific Meeting, Hollywood, Fl., April 2015).

1.4 Research Studies from Our Group: Preliminary Data

1.4.1 Deficiency of RBC Membrane n-3 FAs EPA and DHA in Children with SCD is Associated with Increased Levels of Inflammatory Biomarker hs-CRP

In a study population included 38 children with HbSS (ages 1.7 to 18.3 years, mean age 10.8 years) and 18 age- and race- matched controls (mean age 11.9 years), Setty et al. measured the FA content of membrane phospholipids (PLPs) by capillary gas chromatography (GC) (Setty et al. 2015). PLP Recovery was monitored using diheptadecanoyl-Phosphatidylcholine (PC) and dipentadecanoyl-Phosphatidylethanolamine (PE), and the FA values presented were corrected for recovery. An ELISA assay was used to measure plasma levels of hs-CRP. Data Analysis was performed using unpaired t-test, Mann-Whitney Rank-Sum test and/or Spearman test. This data demonstrates a marked increase in the AA to EPA+DHA ratio in the HbSS patient group. This abnormal ratio is due to a decrease in n-3, and an increase in omega-6 (n-6) FAs in one or both PLP fractions as presented in Table 3. Since the n-6 FA AA is proinflammatory while the n-3 FAs EPA and DHA are membrane precursors of crucial anti-inflammatory effector metabolites, this imbalance should increase proinflammatory tone. In **figure 3** the data demonstrates a significant correlation between the AA to EPA+DHA ratio and levels of the biomarker of inflammation hsCRP in children with SCD.

Table 3: Red Cell Membrane Fatty Acid Composition in Controls and HbSS subjects (nMol/10^9 RBCs)

Fatty Acid	PhosphatidylCholine Fraction		PhosphatidylEthanolamine Fraction	
	Control	HbSS	Control	HbSS
AA	14.1 (13.3, 18.2)	¶17.3 (15.9, 20.5)	26.4 (21.3, 50.1)	26.6 (24.8, 30.7)
EPA	0.60 (0.00, 0.67)	0.45 (0.00, 0.75)	1.13 (0.69, 1.30)	‡0.58 (0.34, 0.70)
DHA	2.72 ± 0.90	†2.22 ± 0.82	4.29 (2.90, 5.84)	**3.50 (2.25, 4.08)
Ratio of AA to (EPA+DHA)	4.98 (3.99, 6.52)	§6.91 (5.19, 9.08)	6.13 ± 2.16	*7.37 ± 1.93

Data presented are the median (25th and 75th percentile) levels or mean ± SD from 18 controls (mean age 11.9 y) and 38 HbSS patients (mean age 10.8 y).

*p=0.035, ¶p=0.039, **p=0.042, †p=0.047, §p=0.003, and ‡p<0.001.

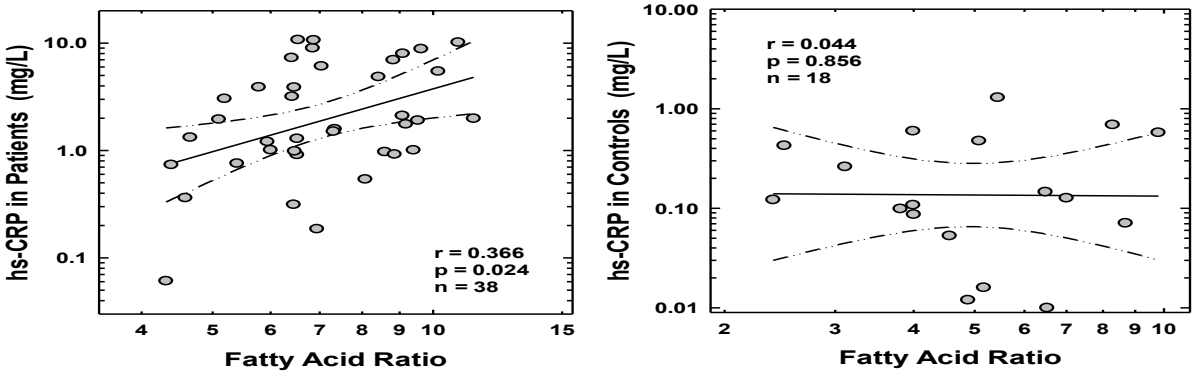


Figure 3

1.4.2 Quantitative Sensory Testing in SCD

In a recently completed trial, we tested thermal detection and pain thresholds in children with SCD compared with African American controls. In this study, SCD subjects at baseline health and healthy African American controls age 8-20 years were administered heat and cold sensitivity and pain threshold testing using a Medoc TSAII in triplicate on the forearm. To exclude outliers, the average of the two closest values was taken, discarding the third. Controls were tested at baseline and 3 months; SCD subjects were also tested at 6 months. Subjects were tested for four parameters: Cold sensitivity (CS), cold pain (CP), heat sensitivity (HS) and heat pain (HP). Sixty controls and 57 subjects with SCD completed thermal testing. Although data analysis is still underway, we have the following preliminary findings:

●Aggregating thermal sensitivity across these parameters into one thermal composite score endpoint (TCS, described in section 8.1.2) at baseline and 6-months showed a mean reduction in sensitivity of only 0.75°C ± 2.9ºC for SCD patients.
●There were no significant differences in thermal thresholds between SCD and control subjects.
●Age did not significantly associate with thermal thresholds except among SCD subjects whose CS increased on average by 0.14°C per year (p=0.01).
●In SCD subjects, there was no significant association between thermal thresholds and chronic transfusion therapy, but heat pain thresholds were significantly higher by approximately 2°C among SCD subjects on hydroxyurea therapy (p=0.04).
●The QST measurements were consistent for both SCD and control subjects for CP, HS and HP (each ICC>0.6), but not necessarily for CS (ICC=0.52 for SCD subjects and ICC=0.46 for controls). Although internally consistent, there was some variability in mean thermal thresholds with in SCD subjects over time. Results for HS varied by a mean of +0.47°C (95% CI 0.02, 0.93), CS by a mean of -0.61°C (95% CI -1.00, -0.21), and HP by a mean of +0.81°C (95% CI 0.28, 1.35) from baseline to 6 months, but the only significant difference between the 3- and 6-month means was for HP (+0.66°C; 95% 1.19, 0.14). See Table 4.

Table 4: Test-retest in SCD only.

Modality	Day 1	Month 3	Month 6	Difference (M3-D1)			Difference (M6-D1)			Difference (M6-M3)		
				Mean	95% CI	p-value	Mean	95% CI	p-value	Mean	95% CI	p-value
CS	29.01	28.43	28.40	-0.58	(-0.96,-0.20)	0.003	-0.61	(-1.00,-0.21)	0.003	-0.03	(0.36,-0.41)	0.886
CP	22.23	21.62	21.24	-0.60	(-1.74,0.54)	0.298	-0.99	(-2.18,0.20)	0.102	-0.39	(0.77,-1.54)	0.510
WS	35.73	36.24	36.21	0.51	(0.08,0.95)	0.022	0.47	(0.02,0.93)	0.040	-0.04	(0.41,-0.48)	0.870
HP	39.75	39.90	40.57	0.15	(-0.36,0.66)	0.563	0.81	(0.28,1.35)	0.003	0.66	(1.19,0.14)	0.013
TCS	6.58	7.08	7.33	0.50	(-0.19,1.19)	0.154	0.75	(0.01,1.49)	0.047	0.25	(0.91,-0.41)	0.457

There were 6 subjects who started hydroxyurea therapy either after their baseline QST testing or immediately prior, before one would expect any significant clinical effect. Thus, at the time of baseline QST, they were "hydroxyurea naïve" and their 6 month QST testing would be expected to reflect the effect of hydroxyurea treatment. This data shows a very significant change (p<0.001) in all 4 thermal thresholds (Table 5).

Table 5: Subjects who started hydroxyurea within one month of baseline QST testing or just after baseline QST testing

	Day 1	Month 3	Month 6	Change from Baseline to 3 Months			Change from Baseline to 6 Months		
Modality	**Mean**	**Mean**	**Mean**	**Mean Change**	**95% CI**	**P**	**Mean Change**	**95% CI**	**P**
CS	29.76	29.20	27.77	-0.56	(-1.34,0.22)	0.141	-1.99	(-2.77,-1.21)	**<.001**
CP	23.43	22.29	17.28	-1.13	(-3.03,0.76)	0.213	-6.14	(-8.04,-4.24)	**<.001**
WS	34.85	35.31	37.53	0.46	(-0.55,1.46)	0.333	2.68	(1.68,3.69)	**<.001**
HP	38.67	41.87	43.01	3.19	(2.11,4.27)	<.001	4.33	(3.25,5.42)	**<.001**

Summary:

• Thermal testing results tended to be consistent over a 6 month period in subjects with SCD, which is critical to inform on the use of QST in evaluating the efficacy of preventative pain therapies.

• Results between the second and third testing sessions were highly consistent, suggesting that reliability may improve once subjects have some experience with the testing procedures.

• We did not demonstrate any significant differences in thermal thresholds by QST between sickle cell and control subjects. These results differ from the findings of Brandow et al. {Brandow, 2013 #29} in which thermal hypersensitivity was seen in SCD subjects.

•The data showing that SCD patients newly on hydroxyurea exhibited significant decreases in sensitivity across all thermal parameters over the six month study period strongly supports the utility of QST as an outcome measure in a clinical trial testing a new medication to prevent vasoocclusive pain.

Based on this data which has laid the groundwork and methodologic set up, and supported by the literature, we have chosen to use thermal sensitivity by QST as the primary efficacy endpoint in this Phase I/II Safety and Dose Escalation Trial of SCD-Omegatex™, a novel formulation of DHA and EPA.

1.4.3 Thrombin Generation in SCD: Insights from Computerized Automated Thrombography (CAT) (Betal et al. 2009)

SCD is considered to be a hypercoagulable state. The Setty and Stuart Lab evaluated hemostatic perturbations in subjects with SCD by employing CAT, a novel thrombin generation assay that provides a global measure of coagulation potential and a direct assessment of the coagulation phenotype (Hemker et al. 2006). A total of 23 adults with **Sickle Cell SS Disease (Hb SS) and Sickle β°-thalassemia (Hb Sβ°thal) 18 to 53 years old were studied as well as 6 age matched controls.** Plasmas were obtained during routine visits. Platelet Poor Plasma (with and without corn trypsin inhibitor to minimize variability from activation of the contact pathway during sample collection) was analyzed by CAT using 5 picomolar (pM) Tissue Factor with 4 micromolar (μM) phospholipid. Five CAT assay parameters were evaluated including Lag Time, Endogenous Thrombin Potential (ETP), Peak thrombin (Peak), time to peak (tt peak) and start tail. Student t test was used to compare means of CAT parameters between SCD subjects and controls. Significant differences were noted in SCD compared to the controls with shortened Lag Times of 1.9 +/- 0.39 minutes (min) (mean +/- standard deviation (SD)) and tt peak 3.72 +/- 0.55 min when compared to controls whose values were 2.39 +/- 0.42 min and 4.77 +/- 0.49 min for Lag Time and tt Peak respectively ($p < 0.001$). However there was overall a lower and less sustained thrombin generation with SCD subjects demonstrating an early start tail (16.76 +/- 2.29 min) and less total ETP (1377 +/- 360 nanomolar (nM)) when compared to the controls whose values for start tail and ETP were 20 .33 +/- 1.71 and 1629 +/- 212 nM respectively ($p < 0.01$). Peak thrombin levels were similar in both groups

at 354 and 336 nM respectively. Thus the subject with SCD has a significant early upsurge in thrombin generation followed by the phenomenon of a rapid attenuation.

1.4.4 hsCRP

In a study of 70 children with SCD at steady state evaluated by a broad panel of biomarkers representing previously examined mechanisms of pathogenicity in SCD, hs-CRP, a marker of low-grade, systemic inflammation, emerged as the most significant laboratory correlate of hospitalizations for VOC. While markers of increased hemolytic status, endothelial activation and coagulation activation all correlated positively with VOC events by univariate analysis, baseline hs-CRP levels provided the most significant contribution to the association in multiple regression models (22%), and, hs-CRP, along with age, provided the best fit in negative binomial models (Krishnan et al. 2010). These data highlight the clinical relevance of the role of inflammation in pediatric VOC, providing both a rationale for future therapeutic strategies targeting inflammation in microvessel occlusive complications of SCD, and the potential clinical use of hs-CRP as a biomarker that correlates with VOC induced pain.

1.4.5 Pain Diary

Daily diaries are a well documented approach to provide qualitative and quantitative data on the pain experienced by SCD patients in the home setting. The validity and reliability of this type of data has been well described in published reports describing the use of pain diaries in both paper (Dampier et al. 2002a; Dampier et al. 2002b; Dampier, Ely, et al. 2004; Ely et al. 2002; Smith et al. 2008) and electronic form (Bakshi et al. 2015; Jacob et al. 2013; McClellan et al. 2009). The content of the diary to be used in this study is included in **Appendix 1**. We have piloted use of this pain diary in our QST trial. All families have reported access to WiFi. Overall compliance has been fair with the iPad version with some subjects using paper version of diary. Approximately 15% of participants declined the iPAD Mini, preferring a paper pain diary or choosing to use their own electronic device for diary completion. Preliminary data reveals that 5862 diary entries were completed. Approximately 30% of study participants completed the electronic pain diary greater than or equal to 75% of the time, approximately 46% of the participants completed the electronic pain daily greater than or equal to 50% of the time and approximately 70% of the participants completed the electronic pain daily greater than or equal to 25% of the time. Because paper diaries allow for backfilling of entries which provide a less accurate assessment of the pain experience (Stone and Broderick 2007; Stone et al. 2003), we would like to assess pain in real time on a daily basis. Thus, for the current trial we will only offer the diary in iPad format. The diary is designed so that an assessment can occur only between 6:30 PM and 3 AM each day, after which that day is no longer accessible to the subject.

2.0 Study Objectives and Hypotheses

We are undertaking a safety and dose escalation trial of SCD-Omegatex™(the Study Drug or Drug Product), a novel formulation of DHA ethyl ester (EE) and EPA EE)(approximately 9:1 ratio), in pediatric and young adult patients with Hb SS, Sickle C Disease (Hb SC) and Hb Sβ°thal.

Primary Objective

1) To determine, in a dose escalation trial, the clinical safety of DHA/EPA supplementation with SCD-Omegatex™ as evidenced by an absence of AEs related to the FA supplementation.
2) To determine whether 6 months of supplementation with SCD-Omegatex™ will reduce thermal sensitivity as measured by QST to below pre-treatment levels.

Pain Related Secondary Objectives

1) To determine whether 6 months of supplementation with SCD-Omegatex™ will increase health-associated Quality of Life responses using the Varni™ Pediatric Quality of Life (QoL) Inventory™ SCD Module**.**
2) To determine the effect of treatment by assessing pain days using an iPad daily report pain calendar.
3) To measure the effect of treatment on individual thermal testing parameters by QST.

Exploratory Objectives

1) To measure the effect of treatment on select biomarkers of inflammation including pro and anti-inflammatory cytokines, adhesion molecules, hsCRP and plasma lipid mediators derived from DHA, EPA and AA..
2) To assess the effect of SCD-Omegatex™ treatment on thrombin generation as assessed by CAT.

Primary Hypotheses:

SCD-Omegatex™ will be safe at both the lower and higher dosages to be administered and will significantly reduce thermal sensitivity measured by QST after 6 months of use.

Secondary Hypotheses:

Following 6 months of supplementation with SCD-Omegatex™ we hypothesize that the following will occur:

● An increase in the Varni™ Pediatric QoL Inventory™ SCD Score
● A decrease in pain days
● A decrease in the proinflammatory cytokines and chemokines as well as hsCRP and an increase in anti-inflammatory markers.
● Normalization of the altered kinetics of thrombin generation

3.0 Rationale for Dose Selection and Monitoring of Study Drug Intake

The DHA/EPA EE formulation to be used in this trial is similar to the formulation used in the studies by Daak et al (Daak, Ghebremeskel, Hassan, et al. 2013; Daak et al. 2015), with a significantly higher concentration of DHA as compared to EPA. Daak's studies used DHA/EPA EE in a 7:1 ratio while the SCD Omegatex™ formulation used in the current study contains an approximate 9:1 ratio DHA/EPA EE. The Resolvin metabolites of DHA possess more potent anti-inflammatory and analgesic effects than those from EPA (Park et al. 2011; Xu et al. 2010; Xu and Ji 2011) as well as a greater effect on membrane fluidity in endothelial cells (Hashimoto et al. 1999). Thus, the rationale for the 9:1 DHA/EPA ratio is:

● to maximize the anti-inflammatory and analgesic properties of the DHA metabolites.
● to utilize a formulation similar to the n-3 FA product shown in the Daak study to be safe and effective in children with SCD.
● to utilize a starting dosage based on the Daak trial, in which the dose was approximately 25 milligrams/kilogram/day (mg/kg/day) body weight DHA+EPA. The resulting EPA dose with this formulation was approximately 3 mg/kg/day. Using a two dose escalation protocol will enable us to evaluate safety and preliminary efficacy data at 2 dosages (25 and 37.5 mg/kg/day DHA+EPA) to inform dosing in a later Phase III trial.

DHA is the major omega-3 FA in all of the cells and tissues evaluated to date (Arterburn, Hall, and Oken 2006). **We have chosen to evaluate the packed total blood cell incorporation of the n-3 FAs DHA and EPA during therapy as a monitor of subject protocol adherence.** (Note that our preliminary results presented in Appendix 5 demonstrate that the FA indices between red cell membranes and packed total blood cells are comparable). Incorporation of DHA and EPA into RBC membranes appears to reflect the incorporation of these FAs into other tissues (Harris 2008; Neubronner et al. 2011). Studies performed to date on long-term (6 to 12 months) n-3 FA supplementation, in general, demonstrate that steady state levels of DHA and EPA in circulating cells and tissues are achieved between 2 and 6 months (depending on the nature of FA supplement) (Arterburn, Hall, and Oken 2006; Harris 2008; Katan et al. 1997; Marangoni et al. 1993). While EPA steady state levels in the RBC are achieved within 2 months, DHA incorporation into erythrocyte phospholipids during supplementation continues to increase between 4 to 6 months using either the fish oil preparation, ethyl ester or the re-esterified triglyceride form (Arterburn, Hall, and Oken 2006; Harris 2008; Katan et al. 1997; Marangoni et al. 1993).

We have attempted to standardize the mechanics of ingestion as much as possible by requiring that the capsules be ingested with the biggest meal of the day (dinner) which in general also will accommodate the highest fat content to enhance absorption. We emphasize that our monitoring of total blood cell incorporation of n-3 FAs is not because total blood cell increase in DHA and EPA is our major consideration regarding it's mode of action in SCD therapy. Rather, the effects of these FAs on pain sensation as assessed by standardized Qualitative Sensory Testing (QST) is our primary and critical goal. We are testing whether the effect of the DHA/EPA metabolites on the enhanced pain hypersensitivity that has been documented in SCD by other investigators (Brandow et al. 2013) will be rectified by the n-3 FAs, especially by DHA. Metabolites of DHA have markedly greater potency in modulating pain hypersensitivity in the SCD mouse model (Wandersee 2012; Wandersee et al. 2015) and this FA is particularly abundant in neural tissue where it is several hundredfold more abundant than EPA (Arterburn, Hall, and Oken 2006). Moreover, since the half life of RBCs is markedly decreased in SCD (to a few days) the use of red cell membrane n-3 FA content is fraught with interpretive difficulty as to when RBC membrane saturation is attained such that its main utility for us as investigators is to monitor compliance. When the RBC phospholipid accumulation of DHA and EPA reaches saturation point is not our focus. The effect of the n-3 FAs on pain hypersensitivity, i.e. on nerve tissue, as measured by our primary QST endpoint is the focus of this clinical trial, in particular whether thermal sensitivity by QST undergoes a significant change from baseline in subjects with SCD post DHA/EPA supplementation. Further supporting the effect of n-3 FAs on neural tissue is recent randomized, double blind, placebo controlled trial in the Sudan which documented a significant reduction in seizure frequency in patients with drug-resistant epilepsy treated with DHA and EPA (Ibrahim, Daak, and Ghebremeskel 2016).

We are using the 37.5 mg/kg n-3 FA dose in a cautious dose escalation phase to a maximum of 4 grams (g) DHA and EPA since this level has been shown to be without toxicity in numerous previous studies. In addition, as in the literature, if levels of DHA content of cell lipids continues to increase in cells and tissues till 4 to 6 months post treatment inception, by using the higher dose we hope to achieve maximal benefit on our primary end point during the time frame of our study.

4.0 Trial Design

This is a Phase I/II, single site, open-label safety and dose escalation trial in which children with Hb SS, Hb SC and Hb Sβ^0thal will be given a new n-3 FA formulation (SCD-Omegatex™) containing DHA and EPA at a ratio of approximately 9:1 DHA:EPA at one of two daily oral doses for a period of 6 months.

4.1 Dose Escalation (see also figure 4)

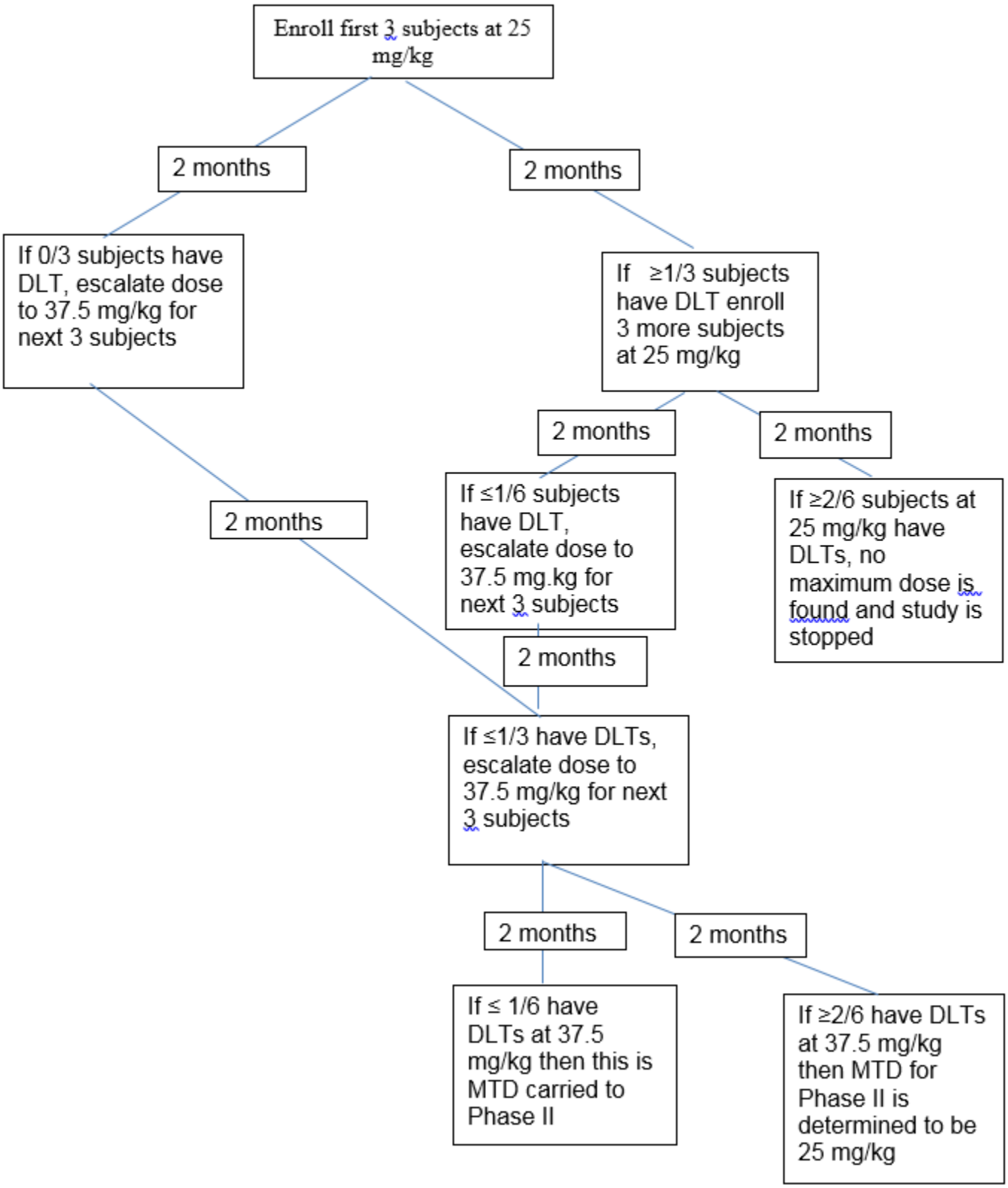
4.1.1 Phase I Portion:

The Dose Limiting Toxicity (DLT) portion of the study will follow a "3+3" design. Three subjects will be enrolled at the starting dose of 25 mg/kg body weight of SCD Omegatex™. If none of the subjects has a DLT after 2 months of treatment, the dose is escalated for the next 3 subjects to 37.5 mg/kg. The original 3 enrollees will remain on the 25 mg/kg dose for the remainder of the trial to complete 6 months of study drug. If one DLT is observed at 25 mg/kg, the next 3 subjects are enrolled at 25 mg/kg. If one out of 6 subjects has a DLT at 25 mg/kg, the dose is escalated to 37.5 mg/kg for the next group of subjects. **Note that the maximum total daily dose for this trial will be 4 g per day.** If 2 or more of 6 subjects develops a DLT at 25 mg/kg, escalation is stopped and no Maximum Tolerated Dose (MTD) is found. If the dose is escalated to 37.5 mg/kg and 1 or fewer DLT is observed in the first 3 subjects after 2 months, 3 more subjects will be enrolled at the 37.5 mg/kg. If in 2 more months, fewer than 2 out of 6 subjects show toxicities at 37.5 mg/kg, 37.5 mg/kg is the recommended Phase II dose. If 2 or more DLTs are noted, 25 mg/kg is the MTD. The length of treatment at each dose level in Phase I will be 2 months, with carryover to Phase II of subjects receiving the MTD. The maximum sample size for this portion of the study is 12 subjects. Efficacy data for all subjects will be analyzed. DLTs will be managed as described in section 7.0.

4.1.2 Phase II Portion:

From our unpublished pilot data, the standard deviation (SD) of thermal sensitivity reduction over a 6 month period is approximately 2.9 in African American children with SCD as measured by the TCS composite endpoint described in section 8.1.2. A sample size of 30 is needed to have 80% power to detect a moderate 1.5 degree (~0.5 SD) difference in TCS following treatment using a 2-sided paired-t test of size 0.05. Since 6 of the subjects for this phase will come from the Phase I portion, the additional sample size needed for this Phase is 24 subjects. Allowing for approximately 20% attrition we will recruit 30 additional subjects in this phase with a total maximum sample size for both Phases I and II combined being 42 subjects (Phase I=12, Phase II=30).

Figure 4: Flow Diagram for Dose Escalation



4.2 Primary and Secondary End Points, and Additional Markers of Interest

4.2.1 Primary Endpoints

Safety: Clinical safety will be demonstrated by the absence of AEs related to study drug.

Efficacy: The primary efficacy endpoint will be reduction of thermal sensitivity as measured by QST to below pre-treatment levels.

4.2.2 Secondary/Exploratory End Points

Change from pretreatment levels in QoL scores and number of pain days will be noted following 6 months of EPA and DHA supplementation with SCD-Omegatex™. Effect of supplementation on individual thermal sensitivity thresholds by QST will be measured. Pre and post treatment levels of proinflammatory cytokines, adhesion molecules, hsCRP, plasma lipid mediators derived from DHA, EPA and AA as well as CAT parameters will be assessed.

4.3 Inclusion/Exclusion Criteria

4.3.1 Inclusion Criteria

Subjects who meet all of the following criteria are eligible for enrollment into the study:

- Participant has signed the informed consent/assent with parent signing informed consent as age appropriate.
- Established diagnosis of SCD including Hg SS, Hb SC and Hb Sβ^o Thal genotypes
- Regular compliance with comprehensive care.
- Aged 8 years or greater and less than 26 years.
- At enrollment and at the time of study drug initiation, subject should be in his/her baseline steady state and not in the midst of any acute complication due to SCD. Must be at least 2 weeks from infection or VOC requiring inpatient admission at time of initiation of study drug.
- If subjects are on a disease modifying agent such as hydroxyurea or l-glutamine, the dose per kg must be stable for at least 3 months prior to enrollment with a plan in place to keep dose stable throughout the course of the study unless a change in clinical status requires a dose modification .

4.3.2 Exclusion Criteria

- Subjects with baseline hemoglobin (Hb) levels <5.5 grams/deciliter (g/dL).
- Inability to swallow capsules
- Poor compliance with previous treatment regimens.
- Hepatic dysfunction (alanine aminotransferase (ALT) >2X upper limit of normal (ULN) or conjugated bilirubin >2X the patients baseline within the last 6 weeks).
- Renal dysfunction (A creatinine level within the past 6 weeks of ≥ 1.0 mg/dL for children and ≥ 1.2mg/dL for a subject ≥ 18 years of age).
- Prothrombin time (PT) and/or partial thromboplastin Time (PTT) ≥ 20% higher than ULN
- Platelet count less than 100,000
- Allergy to fish, shell fish or soy
- Pregnancy.
- Chronic transfusion therapy.
- Transfusion within the last 30 days.
- Treatment with any investigational drug or regular fish oil supplementations in last 60 days.
- Currently receiving another investigational agent, or on such an agent within the last 60 days.
- Changes in per kilogram dosing of HU or l-glutamine in preceding 3 months if on these medications

- Diagnosed bleeding disorder or patient on concomitant anti-coagulation.
- Conditional or abnormal result on most recent Transcranial Doppler (TCD) or history of stroke.
- Other active chronic illness that could adversely affect subjects performance such as HIV, tuberculosis, cancer, autoimmune disease requiring immunosuppressive therapy.
- Children in Care (CiC): A child in care is a child who has been placed under the control or protection of an agency, organization, institution or entity by the courts, the government or a government body, acting in accordance with powers conferred on them by law or regulation. The definition of a CiC can include a child cared for by foster parents or living in a care home or institution, provided that the arrangement falls within the definition above. The determination of whether a child meets the definition of CiC should be made with the study center staff in consultation with the responsible Institutional Review Board (IRB).

4.4 Safety Assessments

The following labs and assessments will be performed at designated intervals (see **Table 7**) to assess for toxicity and safety:

- Vital signs (VS) and physical evaluation.
- Complete Blood Count (CBC), reticulocyte count (retic)
- PT/PTT
- Metabolic Panel (Creatinine, blood Glucose and liver function tests (LFTs) including ALT and aspartate aminotransferase (AST).
- Urinalysis including microscopic (UA)
- Fasting lipid panel
- Urine pregnancy test (in female subjects ≥ 9 years of age).
- Clinical Bleeding Assessment (**Appendix 2**)

4.5 Compliance Assessments

- DHA, EPA and AA levels (% of total FAs), omega-3 index (O3I, %DHA plus % EPA), fatty acid ratios (AA to DHA p lus EPA) in the packed total blood cell fraction. This assessment will monitor compliance with the therapeutic regimen.
- Pill count at Clinic Visit every 4 weeks.
- An email message will be sent to the email address provided by the family for study messages at 3 PM daily reminding them to take their study medication and to make entry into their pain diary. Subjects will be instructed to reply on daily pain diary that study medication has been taken and responses will be directly loaded into REDCap™ database.

4.6 Selection of Subjects and Screening

Subjects will be enrolled by study personnel in the Sickle Cell Clinic at Nemours/Alfred I duPont Hospital for Children (NAIDHC) after parental permission for participation in the study has been obtained by parent or legal guardian with appropriate subject assent (8 to 17 years) or informed consent by the study subject themselves (18 to <26 year olds). Enrollment is defined as the date and time of signing the informed consent document. Subjects will be approached to determine their interest in the study at the time of routine Hematology visits. In addition, flyers advertising the study will be distributed at clinical sites in the area where patients with Sickle Cell Disease receive medical care and potential subjects, including young adults who were previously followed in the NAIDHC Hematology Clinic, may be contacted by phone to determine potential interest in the study. Subjects who meet initial eligibility will be scheduled for a protocol discussion informational session via telephone, telemedicine or in person visit. Those subjects who meet inclusion criteria and wish to enroll will be scheduled for a first study visit at which time appropriate consenting documents will be reviewed and signed.

4.7 Monitoring for Toxicity and Safety

4.7.1 Dose Limiting Toxicities for Phase I Portion of Study

DLTs will be assessed using the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 from the U.S. Department of Health and Human Services with modifications for SCD. All CTCAE ≥ Grade III toxicities

judged as possibly, probably or definitely related to study medication will be considered as a DLT except for the modifications noted below:

a) Known SCD complications including, but not limited to, VOC, ACS, aplastic crisis, viral/bacterial infections and indirect hyperbilirubinemia associated with a hemolytic episode or gallbladder disease will not be considered as DLTs.
b) A DLT related to skin/subcutaneous bleeding will be defined as clinical evidence of hemorrhage of CTCAE Grades II and above, or a modified CTCAE of Grade I severity with areas of purpura not explainable by trauma, occurring on ≥4% of body surface (without antecedent injury).
c) Ophthalmic, gastrointestinal (GI) and respiratory hemorrhage as well as hematuria, not explained by trauma, of ≥ Grade II will be considered a DLT
d) A rise from baseline in serum creatinine of CTCAE Grade I or higher that persists for more than 2 weeks despite treatment discontinuation, or reappears at any time following medication reintroduction (for which no other reason can be found to explain the abnormality besides study drug) will be considered a DLT.
e) Phase I hepatic DLT are defined below:
   i). ALT ≥ 3x ULN **and** bilirubin ≥ 2xULN with ≥ 35% direct bilirubin.
   ii). ALT ≥ 5x ULN.
   iii). ALT ≥ 3x ULN if associated with the appearance or worsening of symptoms of hepatitis or hypersensitivity such as nausea, vomiting, right upper quadrant pain or tenderness, fever, rash or eosinophilia.
   iv). ALT ≥ 3x ULN which does not resolve to ≤ 2x ULN after 2 weeks of treatment discontinuation or recurs following resumption of study medication.

During the Phase I portion of the trial, prior to moving from the initial dose level to the higher dosage level, a formal meeting will be held in which the PI, Co-investigators, coordinator and the medical monitor will review any DLTs and adverse events and all must agree to proceed with the dose escalation. A similar meeting will take place prior to establishing the MTD to be used in the Phase II portion of the study.

4.7.2 Criteria for Subject Discontinuation from Study

Subjects may decide to discontinue participation at any time during the study. Investigators may discontinue any subject at their discretion, if in their professional opinion, the subject's health, safety, and/or well-being is threatened by continued participation in the study. In addition, the following circumstances require discontinuation of subjects:

a) Any SCD-related complication (such as ACS) requiring exchange transfusion or multiple transfusions such that Hb S% ≤ 40%.
b) Drop in hemoglobin to a level < 5 g/dL requiring multiple transfusions such that Hb S% ≤ 40%.
c) Phase II hepatic chemistry stopping criteria are defined below:

   i). ALT ≥ 3x ULN **and** bilirubin ≥ 2x ULN with ≥3.5% direct bilirubin.

   ii). ALT ≥ 5x ULN.

   iii). ALT ≥ 3x ULN if associated with the appearance or worsening of symptoms of hepatitis or hypersensitivity such as nausea, vomiting, right upper quadrant pain or tenderness, fever, rash or eosinophilia.

   iv). Interim dose discontinuation will occur if ALT exceeds 3x ULN. LFTs will be evaluated 2 weeks after interruption of study drug. If ALT returns to ≤ 2x ULN after 2 weeks of treatment discontinuation, study drug may be reinstituted at the initial dose. However, following re-challenge with study medication, if there is once again an increase in ALT ≥3x ULN, study drug will be discontinued and subject will be removed from study.

d) Renal dysfunction:

   i) A rise in serum creatinine of CTCAE Grade I or higher persisting for 4 weeks. If creatinine returns to within 20% of baseline 2 weeks after treatment discontinuation, the therapy may be reinstituted.

However, following reintroduction of study medication if the creatinine rises to CTCAE Grade I or higher the subject will be removed from the study

e) Focal neurological changes or stroke (excluding seizure in subject with known seizure disorder).

f) Allergic reaction to the study drug
g) Pregnancy
h) Hospitalized for >2 weeks while on study for any SCD-related event except for pain
i) Miss more than one study visit
j) Documented non-compliance with study drug
k) Interim dose discontinuation will occur if platelet count drops below 100,000. If platelet count fails to recover above 100,000 within 2 weeks or if platelet count again drops below 100,000 subject will be removed from the study.

Note: AEs caused by participation in the study may necessitate modifications to the level of participation of a subject or discontinuation of subjects from participation in the study. Subjects who discontinue from the study prior to completing month 2 of study drug (4th study visit) will be replaced. Subjects who discontinue prematurely from the study for any reason will be encouraged to complete at a minimum, a safety follow-up at one month.

4.7.3 Criteria for Interruption and Re-initiation of Drug

4.7.3.1 Criteria for Interruption and Re-initiation of Drug following Interruption for Toxicity in Phase II Portion of Study

AEs possibly related to the study drug that are of at least moderate grade will lead to study drug interruption. Study drug may be resumed when the AE has resolved as detailed below:

- *Liver function Tests:* Interim dose discontinuation will occur if ALT exceeds 3x ULN. LFTs will be evaluated 2 weeks after interruption of study drug. If ALT returns to ≤ 2x ULN normal after 2 weeks of treatment discontinuation, study drug may be reinstituted at the initial dose. However, following re-challenge with study medication, if there is once again an increase in ALT ≥ 3x ULN, study drug will be discontinued and subject will be removed from study.
- *Renal function:* Interim dose discontinuation of study drug will occur for a rise in serum creatinine of CTCAE Grade I or higher. Serum creatinine levels will be reassessed 2 weeks after interruption of study drug. If serum creatinine levels fall to within 20% of pre-study baseline level, study drug will be reinitiated at the same dose prior to discontinuation. However, following re-challenge with study medication, if there is once again an increase to CTCAE Grade I or higher, study drug will be stopped and subject will be removed from study.
- *Platelet count:* Interim dose discontinuation of study drug will occur if platelet count drops below 100,000. If platelet count rises to ≥ 100,000 after 2 weeks, study drug will be reinitiated. If count fails recover to >100,000 after 2 weeks or if platelet count again drops to below 100,000 after reinitiation of study drug, patient will be removed from study.

4.7.3.2 Criteria for Re-initiation of Drug following Interruption for Hospitalization, Minor Surgery, Dental Procedures or Minor Trauma

● Study drug should be held for at least the first 48 hours of any hospital admission to determine the severity of illness and address any concerns that illness may be related to study drug. After 48 hours study drug may be restarted at the discretion of study investigator.

● Subjects undergoing minor surgical or dental procedures e.g. tooth extraction, will have the study drug held for 48 hours prior to, and 48 hours after the procedure. In the event of minor trauma with bleeding for which medical attention is sought by the study subject, study drug will be held for 48 hours (e.g. lacerations requiring suture).

4.7.3.3 Special Considerations Related to Continued Study Participation

Subjects admitted for any acute, SCD-related event (e.g. fever or infection, ACS, VOC), and subjects who receive a packed red blood cell (PRBC) transfusion at any time during the study can remain on study and continue receiving study drug. However, at the study visit immediately following the admission or PRBC transfusion, all other study-related measures will be collected EXCEPT for efficacy outcome measures, including QST, QoL and research laboratory measures which may need to be delayed depending on timing. The next efficacy outcome measurement will be performed at the next scheduled study visit that is ≥ 42weeks from either the hospitalization and ≥ 4 weeks from a PRBC transfusion. If the patient has not yet started on study drug, initiation of drug must be delayed until baseline research studies can be performed and subject will continue to keep pain diary. Subjects can continue to take non-steroidal anti-inflammatories (NSAID) and other medications for pain control while on study but amount taken will be documented. Subjects should not take pain medication within 24 hours of a QST testing session, and QST testing should not take place within 2 weeks of a VOC severe enough to require inpatient hospital admission. If necessary, QST testing should be rescheduled for an appropriate time prior to or at the next scheduled study visit.

5.0 Treatment of Subjects

5.1 Description of Study Drug and Dosing Regimen

5.1.1 Description of Study Drug

The active ingredient (the Drug Substance Solutex0656SCD) of the study drug, SCD-Omegatex™ has been produced by Solutex GC, S.L. (Mallen, Spain). Solutex0656SCD (the Drug Substance, DS) is a fish-oil derived fatty acid concentrate, containing mainly omega-3 fatty acid ethyl esters (EE), predominantly docosahexaenoic acid (DHA-EE). DS also contains eicosapentaenoic acid (EPA-EE) but at a concentration lower than DHA-EE. Other constituents include naturally occurring product-related substances (ex. other polyunsaturated FAs). Alpha-tocopherol is added during manufacturing the drug substance at a concentration of 2 mg/g as an antioxidant.

The drug substance was filled in soft gelatin capsule under Good Manufacturing Practice (GMP) quality encapsulation and tested for stability by Olds Softgels, Inc (Alberta, Canada). Each 450 mg soft gel capsule contains no less than 250 mg DHA and 27 mg EPA per capsule. Capsules are enteric coated to reduce fishy after-taste. Please see Investigator's Brochure (IB) and Quality Overall Summary (QOS) for detailed information on Physical, Chemical and Pharmaceutical Properties and Formulation of study drug. The soft gelatin capsules are stored at controlled room temperature for at least 24 months in 200-counts bottles.

While SCD-Omegatex™ is a new formulation of DHA and EPA, the n-3 FAs have been widely used as over the counter supplements. There are currently 3 Food and Drug Administration (FDA) approved prescription formulations of n-3 FA available on the market, Lovaza®, Vascepa® and Epanova®. Lovaza® and Vascepa® are ethyl ester formulations while Epanova® is a free fatty acid formulation. Vascepa® is a high purity EPA agent. Lovaza®, which contains approximately 465 mg EPA and 375 mg DHA in each 1 gram capsule, is the product which most closely resembles SCD-Omegatex™ although the EPA/DHA ratio is much higher. There is a great deal of safety information on Lovaza®. This medication and the n-3 FAs in general, are very well tolerated and have shown minimal toxicity. Since its FDA approval in 2004, Lovaza® has been widely used as a triglyceride lowering agent in adults. See IB for detailed prescribing and toxicity data on Lovaza®. In a pediatric trial at our institution, 42 children with hypertriglyceridemia were given Lovaza® at a dose of 4 g/day for 8 weeks. The only reported AEs were GI symptoms, fishy taste and more frequent nosebleeds, all of which were mild to moderate in nature, did not require discontinuation of study medication and resolved on their own (Gidding et al. 2014). Given the composition of Lovaza®, the dosage of DHA that a 50 kg patient received on this study would have been approximately 30 mg/kg, which exceeds the Phase I dose in the current protocol. The EPA dose on this study far exceeded the per kg dose to be used in the current protocol. Additionally, there are a growing number of published studies treating children with a variety of diagnoses including autism spectrum, psychiatric disorders and non-alchoholic liver disease with EPA and DHA containing supplements without significant side effects (Janczyk et al. 2015; Mankad et al. 2015; Vesco et al. 2015). A study from Denmark reported on the

administration of a fish oil supplement (55% EPA and 37% DHA) at a dose of 2.4g versus placebo daily to 736 pregnant women starting at 24 weeks gestation without reported significant side effects, and demonstrated a reduction in asthma and lower respiratory tract infections in the offspring of the treated women {Bisgaard, 2016 #487}. Two recent studies in infants have used significant per kg doses of DHA than is planned for this study without significant bleeding concerns or other adverse events. Collins et al randomized 31 infants born less than 30 weeks gestation to receive either 40, 80 or 120 mg/kg/day of DHA as an emulsion via feeding tube starting within 4 days of first enteral feed. They reported that all levels of DHA in the emulsion were well tolerated without adverse effects {Collins, 2015 #494}. The same group published a study randomizing 1273 infants <29 weeks gestation to receive an enteral emulsion providing DHA at a dose of 60 mg/kg/day beginning within 3 days after their first enteral feed until 36 weeks postmenstrual age. They reported no significant difference in adverse events between the treatment and control groups, including no increased incidence of intraventricular hemorrhage {Collins, 2017 #499} Of note, as of June 2016 there were 234 trials (excluding those with unknown status) listed on ClinicalTrials.gov which aim to treat children with n-3 FAs.

Most relevant to the current protocol however, is the study in the Sudan by Daak (Daak, Ghebremeskel, Hassan, et al. 2013; Daak et al. 2015) in which children with SCD were treated with a formulation of EPA/DHA very similar in concentration and dosage to the formulation used in this trial, with a significantly higher concentration of DHA as compared to EPA. The dosage used in the Daak trial was approximately 25 mg/kg DHA+EPA, which matches the initial dose to be used in the current trial. There were no significant AEs reported in these trials and the only reported side effect was dyspepsia and increased appetite.

5.1.2 Dosing Regimen

The starting dose for the current trial is based on the dose used in the studies by Daak et al for children with SCD (Daak, Ghebremeskel, Hassan, et al. 2013; Daak et al. 2015), as previously discussed, which was well tolerated and showed evidence of efficacy. Subjects receiving the starting dose will receive 25 mg/kg n-3 FA (DHA plus EPA) given once daily, while subjects treated at dose level 2 will receive 37.5 mg/kg once daily. The maximum total daily dose for subjects on this trial will be 4g per day. Doses will be rounded up or down for capsule size. Doses that calculate to ≥ 0.5 capsule will be rounded up while doses calculating to < 0.5 will be rounded down. Dosing will be once a day, taken after the evening meal, which is the usually the most fatty meal of the day, to enhance absorption. If subjects are unable to tolerate taking the capsules as one single daily dose, after discussion with the study team, they will be permitted to divide into two doses, one taken after breakfast and a second after dinner. Capsules must not be broken, crushed or chewed. Dosing will be based on the actual DHA and EPA content of the capsules for the batch of SCD-Omegatex™ to be used in the trial. Based on testing done February, 2017 each capsule contains 336 mg EPA + DHA (34 mg EPA and 302 mg DHA). See dosing calculator in Appendix 6 for weight based dosing.

5.2 Packaging, Labeling, Storage and Return of Study Drug

The study drug will be dispensed to subjects enrolled in this trial as per the study protocol, in a light resistant container. The study drug container will be labeled to indicate that the drug is being used for investigational purposes in this trial. Per FDA regulation, the package of an investigational new drug intended for human use shall bear a label with the statement “Caution: New Drug – Limited by Federal (or United States) law to investigational use. The label or labeling of an investigational new drug shall not bear any statement that is false or misleading in any particular way and shall not represent that the investigational new drug is safe or effective for the purposes for which it is being investigated. The dispensing label will abide by Federal and State law.

5.2.1 Drug Accountability

The PI and pharmacy delegate are responsible for maintaining accurate records of study drug receipt, use and destruction. The study site will maintain center level and subject level accountability records of all received, dispensed, expired, and destroyed study medication. Each study drug accountability log will be uniquely numbered. The logs will be kept current at all times and available for inspection. Any measures to resolve a discrepancy will be documented appropriately.

5.2.2 Medication Storage
The capsules will be stored at room temperature with specific instructions that the capsules should not be stored in the freezer or refrigerated. The pharmacy storage room is locked with badge access to pharmacists only. The room is dual temperature monitored with Dickson devices. Store at 25°C (77°F); excursions are permitted between 15°C and 30°C (59°F and 86°F); do not freeze.

5.2.3 Destruction
Once therapy is completed, all returned medication will be adjudicated and destroyed on-site by the study pharmacist per standard operating procedures in compliance with Federal and State Laws. Any remaining inventory at the end of the study including expired inventory will be destroyed on site.

5.3 Prior and Concomitant Therapy
Subjects enrolled in this trial will receive all therapy and monitoring that is considered standard of care for patients with SCD. Patients on HU or l-glutamine can participate in the study but must have been on a stable per kilogram dose for at least 3 months prior to enrollment and may not have per kilogram dose escalated during the study period. If subjects have been taking a vitamin preparation, it should be determined that it does not contain EPA/DHA from any source (i.e. from plant or fish source). Subjects can continue to take non-steroidal anti-inflammatory medications and opioids for pain control while on study. Subjects may not take anti-coagulant medications while enrolled on study.

5.4 Subject Compliance
Compliance will be assessed by querying the study patient and through laboratory testing that includes FA measurement in total packed blood cells (see also section 3.0). An email message will be sent to the subject at 3PM each afternoon reminding them to take their study medication and complete their pain diary. Participants will be instructed to bring the bottles with all remaining capsules to each study visit. Remaining capsules will be counted and number recorded by the study pharmacist or research coordinator to document adherence.. Patients are required to stay on drug therapy beyond week 24 until their visit 8 appointment occurs. This is essential to accommodate critical end of study data collection requirements. A six week supply of study drug will be dispensed at study visits 2 through 6. The extra 14 day supply will ensure the subject has an adequate supply in the event that a study visit is delayed for unavoidable reasons. An eight week supply of study drug will be dispensed at visit 7 to account for any delay in the scheduled visit 8 appointment. All remaining dispensed bottles and unused capsules will be collected at visit 8 for final adjudication.

5.5 Study Procedures

5.5.1 Recruitment
Prospective study participants will be recruited at the NAIDHC Center for Cancer and Blood Disorders outpatient clinic. All prospective participants who are approached about study enrollment will be given consent documents to take home and review. A phone call, telemedicine visit or an in person meeting will be offered to discuss study enrollment and study procedures. Ample time will be given for questions to be solicited and answered. Should participants wish to enroll in the study they will meet with a study investigator and study coordinator at the time consent documents are signed and study procedures initiated.

Once on study medication, subjects will have study evaluations at one-month intervals. These visits should occur within ± 7 days of when they are due and are depicted in Table 6. However subjects will be given 6 week supply of study drug at visits 2 through 6 and an 8 week supply at visit 7 to accommodate any unavoidable delays in scheduled study visits (section 5.4). Patients are required to stay on drug therapy beyond week 24 until their visit 8 appointment and associated studies occur. This is essential to accommodate critical end of study data collection requirements which will be performed at that visit.

5.5.2 Visit One (Study Entry: Month - 2)
If subjects meet initial eligibility requirements, informed consent discussion will take place and appropriate consents and assents will be signed. Inclusion and exclusion criteria will be checked to make sure that the patient

is eligible for the study. Subject's hemoglobin electrophoresis and any available globin genotyping results must be verified and entered into database. Medical and demographic information about the subject including type of SCD, current medications including HU use and dosing, number of health care provider contacts for illness (e.g., clinic visits, ER visits, hospitalizations), and history of previous SCD complications (e.g. VOC, ACS, invasive infection, splenic sequestration) over the preceding 3 years will be gathered from the electronic medical record (EMR). For each subject, the number of pain episodes leading to a visit to the hospital or outpatient clinic over the 3 years preceding entry into the study will be extracted from the EMR. Pain episodes per year will be used to provide an assessment of disease severity (Platt et al. 1991). A clinical bleeding assessment will be taken (**see Appendix 2**). A physical examination will be performed.

Blood and urine will be collected for baseline hematology, chemistry and research labs. These labs will include: CBC, retic, PT/PTT, chemistries including creatinine and LFTs, fasting lipid panel, hsCRP, lipidomic analysis, UA and female subjects ≥ 9 years of age will receive a urine pregnancy test.

If subject meets all eligibility criteria for entry into the study, the study coordinator will instruct subject on use of the i-Pad pain diary and provide them with an iPad mini (Apple Inc). Subjects will be instructed to begin filling out the diary each day immediately. At either visit One or Visit Two, a registered dietician or research coordinator will administer a validated questionnaire to assess n-3 FA content in the subject's diet in the average week (**see Appendix 3**) (Lanigan et al. 2012) and content of any nutritional supplements the subject may be taking. QST testing will be performed. QST data obtained at this session will not be used as pretreatment baseline, but rather to acquaint subjects with the procedure. Pre-treatment baseline QST data will be obtained at Visit Two. The next study visit will be scheduled in two months.

Total volume of blood drawn = 21 milliliters (ml)
Minimum urine volume = 2 ml
Estimated visit duration: 2.5 hours

5.5.3 Visit Two, Initiation of Study Drug (Study Day 0, Month 0)

At Visit 2, the Baseline/Study Initiation Visit, blood will be drawn for CBC, retic, chemistry panel, and female subjects ≥ 9 years of age will receive a urine pregnancy test. Research labs will include a baseline lactate dehydrogenase (LDH), hsCRP, fetal hemoglobin (HbF), inflammatory cytokines, LTB4, cell activation and adhesion markers including L and P selectins, ET-1, VCAM-1, lipidomic analysis, plasma RvD1 and packed blood cell membrane AA, EPA and DHA. Blood will also be collected for analysis of hemostatic markers of thrombin generation (F1.2 and d-dimer) as well as CAT assay. Urine will be collected for RvD1, total protein random urine with creatinine and urine microalbumin/creatinine ratio. The patient and parent/guardian (if applicable) will complete PedsQL ™ SCD Module. Acute care/hospitalization, home pain, and concomitant medication information will be updated. Each subject will have a physical exam. A clinical bleeding assessment will be performed. At either visit One or Visit Two, a registered dietician or research coordinator will administer a validated questionnaire to assess n-3 FA content in the subject's diet in the average week (**see Appendix 3**) (Lanigan et al. 2012) and content of any nutritional supplements the subject may be taking. Baseline QST testing will be performed. The research coordinator will review study procedures with the subject/parent/guardian as well as compliance with pain diary. A 6 week supply of study drug will be dispensed to the subject.

Total volume of blood drawn = 39.5 ml
Minimum urine volume = 21 ml
Estimated visit duration: 2 hours

5.5.4 Visit Three (Month 1)

At Visit 3 the patient will have been receiving the study drug for one month. Baseline safety labs will include: CBC, retic, chemistry panel, UA and pregnancy test for female subjects ≥ 9 years of age. Acute care/hospitalization, home pain, concomitant medication, clinical bleeding assessment and AE information will be collected. Every subject will have a physical exam. Compliance with pain diary will be reviewed. Discontinuation criteria will be checked to make sure the patient is still eligible for the study. All unused study

drug that was distributed at previous Visit 2 will be counted, and documented. An additional 6 week supply of study drug will be dispensed to the subject.

Total volume of blood drawn = 4 ml
Minimum urine volume = 2 ml
Estimated visit duration: 1 hour

5.5.5 Visit Four (Month 2)

At Visit 4 the patient will have been receiving the study drug for two months. Safety assessment labs will be done: CBC and retic, chemistry, and fasting lipid panel. Female subjects ≥ 9 years of age will receive a urine pregnancy test. Research labs include LDH and RBC membrane studies. Acute care/hospitalization, home pain, concomitant medication, clinical bleeding assessment and AE information will be collected. Each subject will have a physical exam. Compliance with pain diary will be reviewed. Discontinuation criteria will be checked to make sure the patient is still eligible for the study. All unused study drug that was distributed at previous visit will be counted, and documented. An additional 6 week supply of study drug will be dispensed to the subject.

Total volume of blood drawn = 9 ml
Minimum urine volume = 1 ml
Estimated visit duration: 1 hour

5.5.6 Visit Five (Month 3)

At Visit 5 the patient will have been receiving the study drug for three months. Blood samples will be drawn and distributed for local and research laboratory analyses: Safety labs will include: CBC, retic, chemistry panel, UA and female subjects ≥ 9 years of age will receive a urine pregnancy test. A physical exam will be performed. Compliance with pain diary will be reviewed. Research labs will include a baseline LDH, hsCRP, HbF, inflammatory cytokines, LTB4, cell activation and adhesion markers including L and P selectins, ET-1, VCAM-1, lipidomic analysis, plasma RvD1 and packed blood cell membrane AA, EPA and DHA. Blood will also be collected for analysis hemostatic markers of thrombin generation (F1.2 and d-dimer) as well as CAT assay. Urine will be collected for RvD1, total protein random urine with creatinine and urine microalbumin/creatinine ratio. The patient and parent/guardian will complete PedsQL ™ SCD Module. QST testing will be performed. Dietary assessment will be performed. Acute care/hospitalization, home pain, concomitant medication, clinical bleeding assessment and AE information will be collected. Discontinuation criteria will be checked to confirm the patient is still eligible for the study. All unused study drug that was distributed at previous visit will be counted, and documented. An additional 6 week supply of study drug will be dispensed to the subject.

Total blood volume drawn = 39.5 ml
Minimum urine volume = 22 ml
Estimated visit duration: 2 hours

5.5.7 Visit Six (Month 4)

At Visit 6 the patient will have been receiving the study drug for four months. Safety assessment labs will be done CBC/retic, chemistries and female subjects ≥9 years of age will receive a urine pregnancy test. A physical exam will be performed. Compliance with pain diary will be reviewed. Acute care/hospitalization, home pain, concomitant medication, clinical bleeding assessment and AE information will be collected. Discontinuation criteria will be checked to confirm the patient is still eligible for the study. All unused study drug that was distributed at previous visit will be counted, and documented. An additional 6 week supply of study drug will be dispensed to the subject.

Total volume of blood drawn = 4 ml
Minimum urine volume = 1 ml
Estimated visit duration: 1 hour

5.5.8 Visit Seven (Month 5)

At Visit 7 the patient will have been receiving study drug for five months. Safety assessment labs will be done CBC/retic, chemistries, and female subjects ≥ 9 years of age will receive a urine pregnancy test. A physical exam will be performed. Compliance with pain diary will be reviewed. Acute care/hospitalization, home pain, concomitant medication, clinical bleeding assessment and AE information will be collected. Discontinuation criteria will be checked to confirm the patient is still eligible for the study. All unused study drug that was distributed at previous visit will be counted, and documented. An additional 6 week supply of study drug will be dispensed to the subject.

Total volume of blood drawn = 4 ml
Minimum urine volume = 1 ml
Estimated visit duration: 1 hour

5.5.9 Visit Eight (Month 6)

Visit 8 will be the last evaluation on study drug. At Visit 8 the patient will have been receiving the study drug for 6 months. Blood samples will be drawn and distributed for local and research laboratory analyses: Safety labs will include: CBC, retic, chemistry panel, UA and female subjects ≥ 9 years of age will receive a urine pregnancy test. A physical exam will be performed. Compliance with pain diary will be reviewed. Research labs will include a baseline LDH, hsCRP, HbF, inflammatory cytokines, LTB4, cell activation and adhesion markers including L and P selectins, ET-1, VCAM-1, lipidomic analysis, plasma RvD1 and packed blood cell membrane AA, EPA and DHA. Blood will also be collected for analysis hemostatic markers of thrombin generation (F1.2 and d-dimer) as well as CAT assay. Urine will be collected for RvD1, , total protein random urine with creatinine and urine microalbumin/creatinine ratio. The patient and parent/guardian (where applicable) will complete PedsQL ™ SCD Module. QST testing will be performed. Dietary assessment will be performed. Acute care/hospitalization, home pain, concomitant medication, clinical bleeding assessment and AE information will be collected. Discontinuation criteria will be checked to confirm the patient is still eligible for the study. All unused study drug that was distributed at previous visit will be counted and documented. Any remaining study drug will be collected and no further study drug will be dispensed.

Total blood volume drawn = 39.5 ml
Minimum urine volume = 22 ml
Estimated visit duration: 2 hours

5.9.10 Visit Nine (Month 7)

Visit 9 will be the final follow-up visit. At this visit, safety data regarding AEs within 4 weeks of coming off study drug will be noted. A physical exam will be performed. Additionally blood will be drawn for the local and research laboratory analyses to include: Safety labs: CBC, chemistry panel and UA. Information about hospitalization, pain and concomitant medications will also be noted.

Total blood volume drawn = 4 ml
Minimum urine volume = 1 ml
Estimated visit duration: 1 hour

Note: A reminder phone call will be made midway between visits 2 and 3 and between visits 4 and 5 by a study team member to each individual subject, related to medication, pain diary, and any interim history. A "reinforcement" discussion related to protocol adherence and date of next scheduled appointment will be included during the telephone call

Note: At each study visit, subject's clinical needs will be assessed and addressed by clinical providers. Adult subjects who do not receive their medical care at Nemours will be directed back to their usual providers for clinical care.

Note: If, due to difficulties with venipuncture or issues with specimen quality, all scheduled research labs are not able to be obtained at a given study visit, subject will be permitted to remain on study and samples will be drawn at next study visit (or sooner if opportunity permits). If such difficulties occur at study visit 2 prior to initiation of study drug, drug initiation may be delayed at discretion of investigator until the baseline research labs can be obtained. A notation of any deviation in timing of specimen collection will be made in subject's CRF.

6.0 Clinical and Laboratory Evaluations

6.1 Efficacy and Safety Laboratory Evaluations

Laboratory evaluations (screening safety) will be performed at month -2, as well as QST. Both safety and research labs will be performed at month 0 just prior to initiation of study drug. Safety and research laboratory tests will be performed over the time of study as appropriate. Specific measurement times for each of the tests are outlined in **Table 6**

Table 6: Study Schedule and Procedures

H & P	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9
Initial Medical History	X								
Update medical history		X	X	X	X	X	X	X	X
Physical exam	X	X	X	X	X	X	X	X	X
BLOODWORK									
CBC/RETIC	X	X	X	X	X	X	X	X	X
Chemistry panel	X	X	X	X	X	X	X	X	X
Fasting Lipid Panel	X			X					
PT/PTT	X								
AA, DHA +EPA in packed blood cells		X		X	X			X	
Lipodomic analysis with plasma	X	X			X			X	
Lipodomic assays with serum	X	X			X			X	
Plasma Resolvin (Rv) D1		X			X			X	
hsCRP	X	X			X			X	
Inflammatory cytokines and LTB4		X			X			X	
Cell activation/adhesion molecules		X			X			X	
ET-1		X			X			X	
Hemostatic markers and CAT		X			X			X	
LDH		X		X	X			X	
Fetal Hemoglobin		X			X			X	
Total Blood Volume (ml)	**21**	**39.5**	**4**	**9**	**39.5**	**4**	**4**	**39.5**	**4**
Clinical Bleeding Assessment	X	X	X	X	X	X	X	X	X
URINE									
Urinalysis	X		X		X			X	X
Urine βHCG (if applicable)	X	X	X	X	X	X	X	X	
Urine for RvD1		X			X			X	
Urine protein/creatinine		X			X			X	
Urine microalbumin/creatinine		X			X			X	
Total Urine Volume (ml)	**2**	**21**	**2**	**1**	**22**	**1**	**1**	**22**	**1**
ASSESSMENTS									
Pain Days	X	X	X	X	X	X	X	X	
Quality of Life Survey		X			X			X	
Sensory Testing	X	X			X			X	
Dietary assessment *Visit 1 or 2	X *	X*			X			X	
Follow-up phone calls		X			X				
Compensation	$100	$100	$100	$100	$100	$100	$100	$100	$250

6.2 Standard Laboratory Evaluations

6.2.1 CBC

CBC differential and retic will be performed in the Clinical Laboratories Improvement Amendments (CLIA) certified clinical lab at NAIDHC and will be measured at each visit. These evaluations will be to monitor safety, to ensure that the hemoglobin level is above the cut-off for exclusion from study.

6.2.2 Chemistry Panel

Chemistry panel will be performed in the CLIA certified clinical lab at NAIDHC. Included in the chemistry panel are creatinine, blood glucose and LFTs. All chemistries will be measured initially at Month -2 prior to randomization, and subsequently at each monthly visit while on study drug to monitor for potential toxicities. Additionally, chemistries will be re-examined at 4 weeks after coming off study drug, to monitor for delayed toxicity. Safety monitoring of fasting lipid panel will be performed as per **Table 6.**

6.2.3 PT and PTT

A screening PT/PTT will be performed in the CLIA certified clinical lab at NAIDHC. This testing is one of the exclusion criteria for the study to ensure that patients are not at excessive risk for bleeding and will be done at Month -2.

6.2.4 Urinalysis, total protein random urine with creatinine, urine microalbumin/creatinine ratio and Urine pregnancy testing

UA, total protein random urine with creatinine, urine microalbumin/creatinine ratio and urine pregnancy test will be performed in the CLIA certified clinical lab at NAIDHC. UA will be performed at baseline/study entry and subsequently at Months 1, 3, and 6 to monitor safety and at 4 weeks after coming off study drug. Urine pregnancy test, when appropriate, will be determined prior to randomization at Month -2, and subsequently at each visit to determine eligibility for continuation on study drug. Urine for total protein random urine with creatinine and urine microalbumin/creatinine ratio will be performed at visits 2, 5 and 7.

6.2.5 Bleeding Assessment

As an added safety measure, a questionnaire to assess bleeding symptoms **(Appendix 2)** will be administered at baseline and at Study visits 2-7. Any increase in bleeding symptoms as identified on this questionnaire will be reported as AEs.

6.2.6 Pain Diary

Subjects with SCD will be asked to keep a daily pain diary to be initiated at Visit 1, two months prior to initiation of study drug and continued until Visit 7, one month post completion of study drug. Apple iPad minis will be distributed to the subjects at the time of enrollment and pain diary entries will be entered into the iPad for the entire 9 months. Subjects are instructed to log in each day and indicate if they have pain. If they report pain, then they are prompted to answer additional questions including duration and location of pain symptoms, type of medication administered, indicate the maximum severity of pain and answer some questions regarding the impact of pain on daily functioning. They must also indicate whether they have taken their study drug that day. See **Appendix 1** for content of diary. The diary is designed so that an assessment can occur only between 6:30 PM and 3 AM each day, after which that day is no longer accessible to the subject. To improve compliance, subjects will be compensated $1 per day for filling out the diary. In addition, participants who complete their diary entries at least 70% of the time will be permitted to keep the iPad after they complete the trial. Estimated time to complete a daily diary entry "with pain" is approximately 5 minutes and an entry "without" pain should be less than 1 minute. Families will not be held financially responsible for lost or stolen iPads.

6.2.7 Varni Pediatric Quality of Life Inventory™ SCD Module

The PedsQL ™ SCD Module Inventory is a brief questionnaire that can be answered by both subject and parent, taking only 5 to 10 minutes to complete. It has been developed for the evaluation of SCD disease-specific HRQoL both in the research and clinical settings. It is a feasible, reliable, and valid tool to measure HRQoL in children with SCD (Panepinto et al. 2008; Panepinto et al. 2013). In the current trial, this inventory will be used as one assessment of baseline disease severity and also to assess for changes over the 6 months of treatment with study drug. For young adult subjects over 18 years, the parent report module will be optional for reasons of feasibility. Institutional licenses for use of the Varni PedsQL ™ scales have been purchased by Nemours. The inventory will be administered and scored according to Scoring Instructions and Administrative Guidelines provided on the Varni PedsQL™ website. http://www.pedsql.org/

6.2.8 Quantitative Sensory Testing:
A detailed description of QST methods is included in **Appendix 4.** QST testing will be performed at study entry (Month -2), Month 0, Month 3 and Month 6.

6.2.9 Research Laboratory Studies
Research laboratory methods are detailed in **Appendix 5**. These investigations will be carried out as noted in **Table 6**.

7.0 SAFETY EVALUATIONS AND REPORTING PLAN

7.1 Safety Assessments Overview and by Study Visits
Subjects will be evaluated every month for the study duration (**see Table 7**). At each visit, subjects and/or their parents will be queried regarding recent medical events or procedures. Specific events will be documented at specific visits to ascertain the nature and treatment of the event, including VOC, episodes of ACS, transfusions, hospital admissions and other interim illnesses and investigative procedures. These reportable events and diagnoses will be followed up by the study staffwho will review medical records document the medical history, physical exam and select lab results of a clinical nature in the EHR and these notes will be printed for the subject's study file.

Table 7: SAFETY MONITORING BY STUDY VISIT

Test	Study Entry Month 1	Randomization Month 0	Month 1	Month 2	Month 3	Month 4	Month 5	Therapy Completion Month 6	Month 7
Medical history/interim history	X	X	X	X	X	X	X	X	X
Physical Exam	X	X	X	X	X	X	X	X	X
Bleeding Assessment	X	X	X	X	X	X	X	X	X
CBC/retic	X	X	X	X	X	X	X	X	X
Chem panel (BILI, ALT, AST, Glucose & Creatinine)	X	X	X	X	X	X	X	X	X
Urinalysis	X		X		X			X	X
Fasting lipid panel	X			X					
ßHCG	X	X	X	X	X	X	X	X	

7.2 Adverse Events
An AE is defined for the purposes of this study as any untoward medical occurrence, that does not necessarily have a causal relationship with treatment, in a subject who is administered a pharmaceutical product. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal product (investigational drug or placebo), whether or not related. AE data are recorded on the case report form (CRF).

Examples of an AE include:

- Exacerbation of a chronic or intermittent pre-existing condition including either an increase in frequency or intensity of the condition.
- Significant or unexpected worsening or exacerbation of the condition under study.
- A new condition detected or diagnosed after study drug administration even though it may have been present prior to the start of the study.

- Signs, symptoms, or clinical sequelae of a suspected overdose of either study drug or a concurrent medication ("overdose" per se, should not be reported as an AE).
- Pre- or post-treatment events that occur as a result of protocol-mandated procedures (e.g., invasive protocol-defined procedures, modification of a subject's previous drug treatment regimen).

An AE does **not** include:

- Medical or surgical procedures (e.g. tonsillectomy, endoscopy). The medical condition that leads to the procedure is an AE.
- Social or convenience hospital admissions where an untoward medical occurrence did not occur.
- Day to day fluctuations of pre-existing disease or conditions present or detected at the start of the study that do not worsen.

7.3 Serious Adverse Events

A serious adverse event (SAE) is defined as any untoward medical occurrence that at any dose:

- Results in death.
- Is life-threatening (i.e., an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe).
- Requires hospitalization or prolongation of existing hospitalization.
- Results in persistent or significant disability/incapacity.
- Results in a congenital anomaly birth defect.
- In the opinion of the investigator, important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the subject or may require intervention to prevent one of the other outcomes listed in the definition above may be considered serious. Examples of such events are intensive treatment in an ER or at home for allergic bronchospasm, blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse.

An **unexpected AE or SAE** refers to an event that is not listed in the investigator's brochure (IB), or if it is listed in the brochure, is not listed at the observed specificity or severity. *Unexpected*, as used in this definition, also refers to adverse events or suspected adverse reactions that the IB mentions as occurring with a class of drugs or is consistent with the pharmacological properties of the drug but is not specifically mentioned as occurring with the particular drug under investigation

Anticipated events include known consequences of the underlying disease or condition under investigation (e.g., symptoms, disease progression, comorbidities) as well as events unlikely to be related to the underlying disease or condition under investigation but common in the study population independent of drug therapy.

Although common side effects associated with other drugs in the same class as SCD-Omegatex™ (ie other n-3 FA ethyl ester products) are well known and outlined in the Investigator's Brochure (IB) accompanying this protocol, SCD-Omegatex™ has not been used previously in human subjects. Therefore as defined by FDA guidelines, all AEs will necessarily be classified as unexpected. As the trial proceeds and experience is gained with the investigational product, the IB will be updated as appropriate, at least on an annual basis. However, since all subjects enrolled in this study have SCD, certain AEs will occur related to the disease process and this should be taken into account when determining whether or not an event is related to the therapy. **Table 8** lists common SCD related events. Events on this list can still be related to therapy if the PI determines that study drug may have triggered the event, made it more severe or occur with unexpectedly high frequency. For painful vasoocclusive events, an AE will be defined as a pain episode resulting in an acute/urgent care visit or hospitalization, or a pain episode that is managed at home that results in a significant change in daily routine such as a missed school or work day.

Particularly close attention will be given to any AEs (even those known to be associated with SCD) that relate to bleeding such as hemorrhagic stroke, retinal hemorrhage or hematuria. These will be carefully examined for causation.

Table 8: List of Adverse Events Related to Sickle Cell Disease

Acute chest syndrome	Hand-foot syndrome	Pulmonary embolism
Anemia	Hematuria	Pulmonary hypertension
Aplastic crisis	Hemiplegia	Pulmonary parenchymal infiltrates
Aplastic crisis/anemia	Hemolysis	on chest x-ray
Arthralgia	Hepatosplenomegaly	Pyelonephritis
Avascular necrosis of joint	Headache	Renal failure
Bone infarction	Hyperplastic bone marrow	Renal insufficiency/albuminuria
Cardiomegaly	Hyposthenuria	Renal papillary necrosis
Cerebrovascular accident	Hypoxemia (PO2 < 65mm Hg)	Reticulocytosis
Cholecystitis,	Infection	Retinal Disease
Hepatic sequestration	Jaundice	Retinal hemorrhage
Cranial nerve palsy	Leukocytosis	Rhabdomyolysis
Decreased kidney function	Meningitis	Sepsis
Decreased lung function	Pain Crisis	Skin ulcers
Delayed growth/puberty	Pain, joint	Splenic sequestration
Dyspepsia	Pain, long bone	Stroke
Elevated urinary urobilinogen	Pain, severe abdominal	Vaso-Occlusive Crisis
Fever	Priapism	

Table 9: Overview of Definitions of Safety Measures

Word or Phrase and (Abbreviation)	Any untoward clinical or medical occurrence.
Adverse event (AE)	Any untoward clinical or medical occurrence.
Serious adverse event (SAE)	Any adverse event that results in any of the following outcomes: Death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect.
Anticipated SAE	An *anticipated SAE* for subjects in this study is an adverse event that is listed in Table 8 and is a SAE, as defined above.
Unanticipated SAE	An unanticipated *SAE* for subjects in this study is any AE that is **not** listed in Table 8 and is a serious adverse event, as defined above.
Adverse Clinical Laboratory Results	Any safety laboratory measurement that is measured beyond the acceptable limits (either high or low) for the population being studied, as described by the investigator.
Suspected Adverse Reaction	Any adverse event for which there is a reasonable possibility that the study drug caused the adverse event
Unexpected Suspected Adverse Reaction	A suspected adverse reaction is considered ‘‘unexpected’’ if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed.
Serious and Unexpected Suspected Adverse Reaction (SUSAR)	A suspected adverse reaction that is both serious and unexpected; an unexpected SAE reasonably possibly related to study drug

7.4 Assessment of AE Severity, Relationship to Treatment, and Expectedness

The principal investigator or co-investigators should the PI be unavailable will grade the severity of identified AEs, which is based on their impact on the patient and will also determine the relationship of the event to the treatment. Where applicable, severity will be assessed on the basis of CTCAE version 4.03. The following scale will be used to "grade" the severity of all AEs:

1. **Mild**. Awareness of sign, symptom, or event, but easily tolerated; does not interfere with usual daily activities or tasks.
2. **Moderate**. Discomfort enough to cause interference with usual daily activity; may warrant therapeutic intervention.
3. **Severe**. Incapacitating; inability to perform usual activities and daily tasks; significantly affects clinical status; requires therapeutic intervention.
4. **Life-threatening**. AE is life-threatening.

5. **Death**. AE causes death.

The standard nomenclature we will use for defining the causal relationship between an AE and the study drug is listed in **Table 10.** The category that overall best "fits" the relationship between the AE and the study drug will be chosen and recorded in the EMR, REDCap™ and MedWatch form as necessary.

Table 10: Relationship between Treatment and AE

Unrelated	• No temporal association to study product. • An alternate etiology has been established or event is consistent with sequelae of sickle cell disease (see Table 8). • The event does not follow the known pattern of response to study product. • The event does not reappear or worsen with rechallenge.
Probably not related / remote	• No temporal association to study product. • Event could readily be produced by clinical state, environmental or other interventions. • The event does not follow the known pattern of response to study product. • The event does not reappear or worsen with rechallenge.
Possibly Related	• Reasonable temporal relationship to study product. • The event is not readily produced by clinical state, environmental, or other interventions. • The event follows a known pattern of response to the study product or as yet unknown pattern of response.
Probably related	• There is a reasonable temporal association with the study product. • The event is not readily produced by clinical state, environmental, or other interventions. • The event follows a known pattern of response to the study product. • The event decreases with de-challenge.
Definitely related	• There is a reasonable temporal relationship to the study product. • The event is not readily produced by clinical state, environmental, or other interventions. • The event follows a known pattern of response to the study product. • The event decreases with de-challenge and recurs with re-challenge.

7.5 Monitoring and Outcome of AEs

Every AE must be followed to a satisfactory outcome or stabilization of the event, even when this requires a time period beyond the scope of the study (this is particularly applicable to SAEs). Outcome includes information on recovery and any sequelae, as well as specific tests and/or treatments that may have been required and their results. All AEs will be collected after the subject signs informed consent and will continue for 30 days after the last dose of study drug. For a fatal outcome, cause of death and a comment on its possible relationship to the suspected reaction should be provided.

The terms used to define outcome are as follows (outcome of reaction/event at the time of last observation):

- Ongoing
- Resolved without sequelae
- Resolved with sequelae

• Death

Actions taken in response to an AE and follow-up results must be recorded in the subject's medical record (this includes follow-up laboratory results). Any treatment administered for the AE must be recorded in the subject's CRF. When subjects are discontinued from the study due to an AE, relevant clinical assessments and laboratory tests will be repeated as necessary until final resolution or stabilization occurs.

SAE reporting will begin with events that arise after the informed consent is signed until 30 days after the last dose of study drug.

7.6 Unexpected SAEs

Within 24 hours of the realization that an unexpected SAE has occurred to a study subject, study personnel must notify the PI (or physician co-investigator if PI is not readily available), who will give an initial report to the Institutional IRB and the Medical Monitor.

This initial report should describe the event as fully as possible and should include the following minimum information: an identifiable subject; study product; an identifiable reporting source; and an event or outcome that can be identified as serious. Supporting documentation (e.g., CRF pages, lab reports, summary notes, autopsy reports) should accompany the report as appropriate. The PI, study coordinator and the Medical Monitor will collaborate to prepare a detailed report of the unexpected SAE.

The Nemours IRB must be notified within 24 hours if an SAE involves death of a subject. Otherwise all unexpected SAEs judged by the PI or co-investigators if the PI is unavailable as possibly, probably or definitely related to treatment will be reported to the Nemours IRB within 5 days. AEs judged as possibly, probably or definitely related to treatment occurring with an unexpected increase in severity or frequency that represent an increased risk of harm will be similarly reported within 5 days. Unexpected fatal or life-threatening AEs possibly, probably or definitely related to the intervention will be reported to the NIH/NIGMS and the FDA within 7 days. Other serious and unexpected AEs possibly, probably or definitely related to the intervention will be reported to the FDA and NIH/NIGMS within 15 days. The exception will be unexpected SAEs which are anticipated as part of the SCD disease process (**Table 8**) and deemed by the PI to be unlikely related to the study drug. These will be reported to the Medical Monitor and Nemours IRB, but will not be reported to NIH/NIGMS or the FDA in an expedited fashion. The PI is responsible for submitting to the NIH/NIGMS and FDA any recommendation of the Medical Monitor to suspend or terminate the study. All SAEs, regardless of expected status, are also recorded in the AE section of the study's CRF. The PI will follow the progress of a subject who experiences an unexpected SAE until the SAE is resolved or considered stable. When the unexpected SAE has not resolved by the report deadline, the PI will make follow-up reports in accordance with directions from the Medical Monitor and/or the Nemours IRB. Note: The PI for this study functioning as Sponsor/Investigator.

7.6.1 Expected SAEs

Study personnel will report all expected SAEs within 3 business days to the PI (or physician co-investigator if PI is not readily available), and Medical Monitor. The PI and the Medical Monitor will collaborate to prepare a report of the expected SAE.

7.6.2 Review of Serious Adverse Events

Three months after the first subject is enrolled in the study, and at the end of each 3-month period thereafter, if any SAEs (expected or unexpected) have been reported in the study during the preceding 3 months, the Medical Monitor and the PI will meet by phone to review. The PI in collaboration with the Medical Monitor will prepare a semi-annual report to the NIGMS Project Officer..

7.6.3 Subject Discontinuation due to AE(s)

The following criteria will be used to determine whether or not subjects exhibit toxicities of the study drug(s) sufficient to require discontinuation from the study.

We will closely monitor signs, symptoms, and laboratory findings to assess for unexpected toxicities (detailed in section 4.7.2). Treatment will be discontinued in any subject who experiences the following:

1. Acute allergic reaction
2. Serious bleeding manifestations
3. Changes in LFTs or creatinine that do not return to baseline on treatment discontinuation or returns to abnormal values on re-challenge
4. Pregnancy
5. Drop in platelet count below 100,000 that fails to recover within 2 weeks or recurs on rechallenge.

Treatment may also be discontinued at the discretion of the PI in the event of any other serious or unanticipated side effects.

7.7 Reporting Requirements:

Nemours IRB

The Nemours IRB must be notified within 24 hours of an unforeseen death of a subject. All unexpected SAEs judged by the PI, or co-investigators if the PI is unavailable, as possibly, probably, or definitely related to treatment will be reported to the Nemours IRB within 5 days as these are assumed to represent an unanticipated problem involving risk to participants. AEs judged as possibly, probably, or definitely related to treatment occurring with an unexpected increase in severity or frequency that represent an increased risk of harm will be similarly reported within 5 days.

External Agencies

Unexpected fatal or life-threatening AE/SAEs possibly, probably, or definitely related to the study intervention will be reported to the NIH/NIGMS within 7 days. As the study intervention involves a drug these events likely also meet the definition of an Unexpected fatal or life-threatening suspected adverse drug reactions, they also will be reported to the FDA within 7 days. Other unexpected SAEs possibly, probably, or definitely related to the intervention will be reported to NIH/NIGMS within 15 days. As these events also meet the definition of Serious unexpected suspected adverse drug reactions, they will also be reported to the FDA within 15 days.

7.7.1 Reporting of Serious Safety Issues; Suspension Guidelines

Serious safety issues that arise in this study will be brought to the attention of PI and Medical Monitor, who will make recommendations to the IRB regarding possible suspension or termination of the study. The PI also will notify the NIH/NIGMS (study funder) and the FDA if the study is temporarily suspended or terminated. The NIH and FDA will consider the recommendations, determine an appropriate action, and notify the PI who will implement the actions directed by NIH and/or FDA.

7.8 Pregnancy Reporting

Because of potential unknown effects on the fetus, pregnant subjects will be excluded from the study. Female subjects who are pubertal will be regularly assessed throughout the study. In the event of a test indicating the study subject is pregnant the subject will be informed of this result and will immediately have study drug discontinued. If female participants acknowledge sexual activity they will be informed that they need to use a reliable form of birth control for the duration of the study.

7.9 Monitoring of Subject Accrual and Compliance

The PI will assess the overall enrollment and compliance with the clinical protocol. The monitoring will focus on data. All protocol violations/deviations will be documented, and will include a description of the event and the corrective action plan.

7.10 Data Collection and Monitoring

7.10.1 Case Report Form (CRF) and Source Documentation

The site study coordinator will complete a CRF or eCRF for each subject. On all other study documents except for the study visit exams which will be documented in the EMR, subjects will be identified by a study subject

number assigned at enrollment, and subject will not be identified by name. Data will be entered into a REDcap™ database. Some source documents such as QoL and dietary assessment will be directly downloaded into REDcap™ via a secure web access. Otherwise data entry into REDcap™ will be done by study team members only.

7.10.2 Data Management

The REDcap™ database will be structured to simplify data entry with rules for restricting data to valid response and ranges (e.g., no negative height values allowed). Random subsets of subjects will be audited periodically for data quality and completeness. If data quality and completeness problems are detected, the audit will be expanded and the entry process will be revised as necessary to avoid the detected problems.
Validation rules will be applied at several points in the data management process. An error correction procedure will be applied to correct data values that fail validation rules.

7.10.3 Staff Training and Data Monitoring

Prior to the onset of enrollment, study personnel will be trained to ensure adherence to the protocol and assure the highest possible data quality. Training will address informed consent procedures, study operations and protocol requirements, maintenance of source documentation, CRF completion and review, routine reporting requirements, and data management. Coordinators will have the responsibility of monitoring CRFs and source documents for accuracy, protocol compliance, subject safety, and adherence to guidelines. As the study progresses, informed consents and completed data forms will be reviewed periodically by the PI and compared to source documentation (medical records) to confirm accuracy.

8.0 Statistical Considerations

8.1 Data Analyses:

8.1.1 General Considerations

Discrete variables will be summarized with frequency counts and percentages. Any continuous variables will be summarized with means and SDs unless there are variables not approximately normally distributed, in which cases we will summarize them with their medians and first and third quartiles. This data will be tabled and plotted to guide decisions on the adequacy of statistical modeling and testing assumptions. All efficacy analysis will be performed on subjects with pre-treatment and post-treatment data. That is, subjects who drop out will be excluded from efficacy analysis. The primary analysis of each outcome will be performed without regard to patient adherence. Secondary analysis will exclude non- adherent patients. Rates of AEs will be estimated for all treated patients. All effect measures estimates for efficacy and safety outcomes will be estimated with appropriate confidence limits. SAS version 9.4 (Cary, NC) will be used for all statistical analyses.

8.1.2 Primary Efficacy End Point

The Phase II portion of the Trial is designed to provide preliminary evidence of efficacy in reducing thermal sensitivity by QST. For each subject at each time, two technical replicate measurements (i.e., duplicates) will be taken for each QST modality (i.e., CS, CP, HS, and HP). A QST thermal composite score (TCS) measure will then be evaluated in these data. It will be based on an aggregation of the absolute values of temperature deviations from the instrument's 32°C baseline level (*d's*) across all eight of the QST measurements at a given visit (i.e., higher values indicating lower sensitivity). A crude estimate of the TCS for the j^{th} participant at the k^{th} visit would be the mean of the measured QST's at that visit,

$$\mathrm{TCS}_{jk} = \frac{1}{8}\sum_{i=1}^{4}\sum_{r=1}^{2} d_{jkir}$$

where *i* is an index for the 4 QST modalities, *r* is an index for the duplicates, and d_{jkir} is the thermal sensitivity deviate measurement. For instance, suppose that for the fifth participant, at the second visit, their heat sensitivity (say, *i* = 3) first duplicate measurement is 25.3°C. Then the d_{5231} = | 25.3°C - 32°C | = 4.7°C.

Changes in these *d*'s over time will be modeled using mixed effects linear regression adjusted for modality. We will treat time as a categorical variable. Correlation among repeated measurements will be modeled using a compound symmetric (i.e., random intercept) or first-order autoregressive within-subject covariance structure. We will examine the distribution of the residuals to assess the normality assumption, and will apply appropriate transformations as needed. Within this model, we will directly estimate parameters for the TCS changes from baseline to all follow-up visit times (after 3 and 6 months of treatment). These results will include an estimate and a two-sided 0.05-level Wald's test of the primary hypothesis of reductions in thermal sensitivity from baseline to 6 months post treatment. We will also evaluate, in these models, potential confounders identified in summary or exploratory analyses and adjust the primary endpoint model for confounding if it becomes a concern.

8.1.3 Secondary End Points

(i) QoL: For the Varni™ Pediatric Quality of Life Inventory™ Sickle Cell Disease Module, pre- and post-treatment values will be summarized using the median and interquartile range. Significance of change in QOL scores will be evaluated using the Wilcoxon signed rank test. We will evaluate association of change in QoL with change in thermal sensitivity and RVD1 using Spearman rank correlation coefficients.
(ii) The number of pain days per month will be modeled using Poisson regression within a generalized estimating equations framework to account for correlation among the repeated measurements.
(iii) Reductions in thermal sensitivity by individual QST parameter measures will be treated and modeled, respectively, using the same mixed modeling approach described above for the primary endpoint analysis.

8.1.4 Exploratory Analyses

(i) Changes in biomarkers, adhesion molecules and lipid metabolites: We will consider changes in biomarkers, adhesion molecules and lipid metabolites using two approaches. First, we will perform paired t-tests for each outcome separately. Second, a longitudinal mixed effects regression modeling approach will be utilized on these biomarker data similarly as for the primary endpoint of thermal sensitivity.
(ii) hsCRP: Values of hsCRP will be log transformed prior to analysis. Efficacy will be measured by calculating the average change in transformed hsCRP values (post minus pretreatment) and reverse transforming this mean change to obtain the Geometric Mean Ratio (GMR). Statistical significance will be evaluated using a one-sided paired t-test with alpha=0.05. The null hypothesis is that there is no change (GMR=1). The alternate hypothesis is that post treatment hsCRP values are not equal to (lower than) pretreatment values (GMR<1). Further analysis of hsCRP will include longitudinal mixed effects regression modeling similarly as for the primary endpoint.
(iii) CAT: Changes in CAT parameters (endogenous thrombin potential or ETP, Lag Time, Peak Thrombin, Time to Peak, and start tail) will be evaluated by paired t-tests separately. Depending on the findings, we may further model these data in a manner similar to the latent process modeling described for changes in biomarkers.

8.1.5 Interim Analysis

No interim analyses will be performed in this study.

9.0 Human Subjects Protection

9.1 Clinical Trial Monitoring

Independent from our study team, we will have a clinical monitor, external to the study, to review study for adherence to regulatory requirement, Good Clinical Practice (GCPs) and the protocol. The clinical monitor will assure that data reporting, including safety reporting, is accurate and complete and that the rights and well-being of human subjects are protected.

9.2 Ethics

9.2.1 Good Clinical Practice and IRB Review

Compliance with GCP guidelines for the conduct and monitoring of this clinical trial will occur through observation of the ethical and regulatory requirements presented in ICH E6, *Good Clinical Practice: Consolidated Guideline*. By signing this protocol, the investigator agrees to adhere to these requirements. The study (protocol and informed consent) will be reviewed and approved by the IRB at NAIDHC. All changes to the protocol will be approved by the Nemours IRB and will also be reported to the NIH/NIGMS Program Director and the FDA. Subjects must sign written informed consent and (when appropriate) assent prior to being screened and before undergoing any study procedures.

9.2.2 Informed Consent

Informed consent is an ongoing process that includes the signing of an informed consent document. The protocol, parental permission form and assent forms will be submitted for approval by the Nemours IRB for full review and approval obtained prior to initiating this study. If it is necessary to amend the protocol and/or consent forms those items will be submitted to the IRB prior to implementation. All staff involved in the study will have completed formal coursework in the protection of human subjects. Study staff will obtain written informed consent, parental consent and age-appropriate child assent prior to conducting any research procedures in accordance with ICH E6; 4.8, “Informed Consent of Trial Subjects.” The informed consent form will adhere to the guidelines in ICHE6: it will contain the elements as specified in section 4.8.10 of that guideline which can be modified to include local IRB required language. Potential subjects will have ample opportunity to ask questions prior to signing consent. They will be informed that they are free to opt out of the study at any time and that declining participation will not in any way influence the care they will receive at NIADHC. One copy of the signed consent will be given to the subject and one will be kept in the study files for documentation. Signed consent will also be scanned into the EMR. Subjects will also be provided with the telephone numbers of study personnel who can assist with their questions and concerns.

9.2.3 Data Safety and Confidentiality

Subject confidentiality will be maintained by the investigator, the investigator’s associates and co-workers, and by all administrators who are part of the project. Confidentiality will be maintained according to ICH E6; 4.8.10, Part O: “Records identifying the subject will be kept confidential and, to the extent permitted by the applicable laws and/or regulations, will not be made publicly available. If the results of the trial are published, the subject’s identity will remain confidential.” All study participants will be assigned a unique study identification number, which will be used on all study documents instead of their name or other identifying information. This number will be used in all data collection and tabulation. Study participants’ protected health information will be linked with their study identification number in a separate computer database that will be password protected. All study computers and databases will be password protected, and study documents will be kept in locked filing cabinets or computers in the offices of project staff at NAIDHC. Study visit exams will be documented in the EMR.

9.2.4 Disclosure of Data

The PI or her staff and associates, and the appropriate regulatory agencies may use the information included in this protocol as necessary for the conduct of the trial and the safety of subjects. Data from the trial are confidential and may not be disclosed without the written permission of the NIH.

9.2.5 Publication of Research Findings

Manuscript(s) and abstract(s) prepared from the data collected during this trial can be prepared by the study investigators with the subjects’ identity remaining confidential.

9.3 Discontinuation of Study

Safety will be monitored by the PI, Medical Monitor and Nemours IRB, who can recommend suspension or termination of the trial for safety reasons at any time. The PI, Medical Monitor and IRB will receive expedited reports for deaths or unexpected SAEs and regular summaries as described earlier under AEs. Particular attention will be paid to specific SAEs such as death of a participant, stroke or cerebral hemorrhage, and any

grade 3-4 bleeding events. Subjects can discontinue for any reason at any time during the trial, with no consequences to the quality of their treatment. This study will be stopped prior to its completion if: 1) the intervention is associated with AEs that call into question the safety of the study drug, 2) difficulty in study recruitment or retention will significant impact the ability to evaluate the study endpoints, 3) any new information becomes available during the trial that necessitates stopping the trial; or 4) other situations occur that might warrant stopping the trial.

10.0 Subject Compensation

Subjects will be compensated at the time of each study visit as reimbursement for their time and for parking fees. For visits 1 through 8, subjects will receive $25 to cover transportation expenses and $75 compensation. At visit 9, subjects who remain in the study and have completed all study visits will receive $250. In addition, subjects will receive $1 for each daily entry into their pain diary. Subjects who are compliant with pain diary entries at least 70% of the time will be permitted to keep their iPad mini at the conclusion of the study.

Bill S.139 Ensuring Access to Clinical Trials Act of 2015 permanently allows an exclusion under the Supplemental Security Income program and the Medicaid program for compensation provided to individuals who participate in clinical trials for rare diseases or conditions of which SCD is included. Therefore, compensation up to $2000.00 annually, in this clinical trial, will not negatively impact the SSI and Medicaid Income received by study participants.

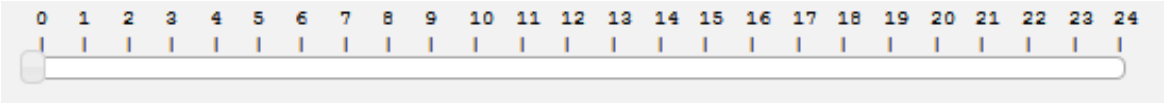
Appendix 1: Sickle Cell Pain Survey

Participant ID:
Today's Date: _____

Did you take your study medication today? O Yes O No

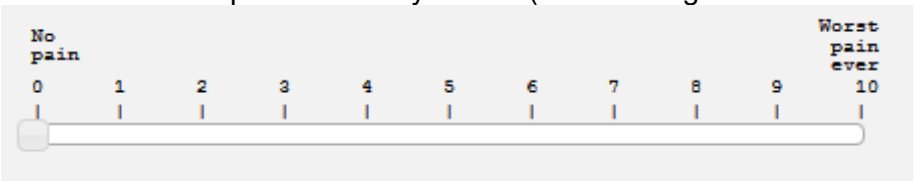
Did you have any pain today: O Yes O No
If yes, please continue.
If no, you are finished and don't have to complete any more questions.

About how many hours did you have pain since the last time you filled out diary? (Circle a single number on the line.)

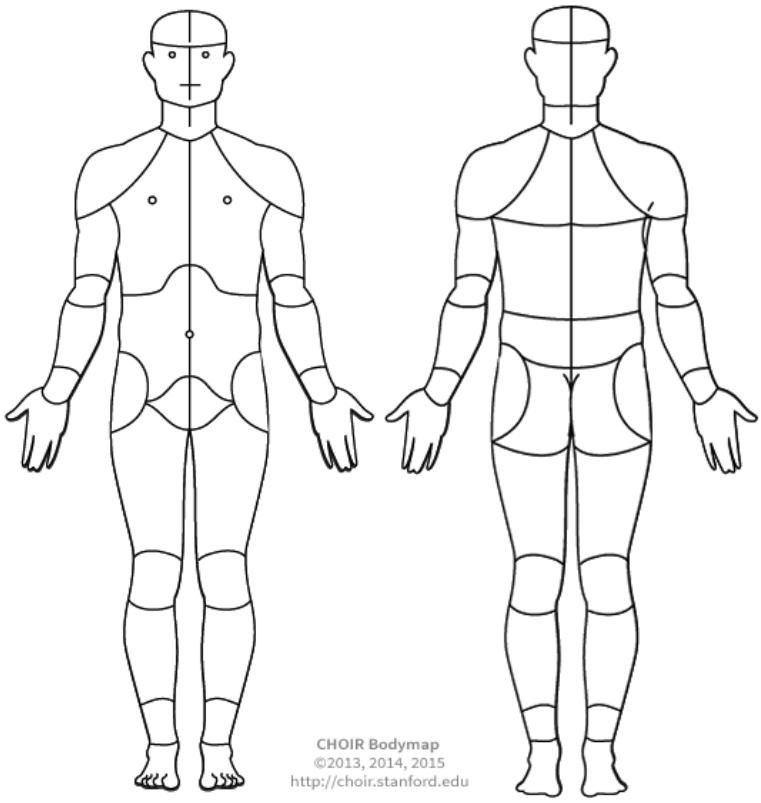


If you circled 0 or one, you are finished and don't have to complete any more questions

How bad was the pain at it's very worst? (Circle a single number on the line.)



Please color in all of the body parts where you felt pain.



Did you take any medicine for your pain? O Yes O No

If you took medicine for your pain, what medications did you take for your pain (check all that you took)
☐ Tylenol (acetaminophen)
☐ Ibuprofen (motrin)
☐ Tylenol with codeine
☐ Percocet
☐ Hycet
☐ Oxycodone
☐ Morphine
☐ Dilaudid

Was today a school day? O Yes O No
If yes, did you miss school today because you had pain? O Yes O No

Did you have trouble sleeping last night? O Yes O No
If yes, was it because of pain? O Yes O No

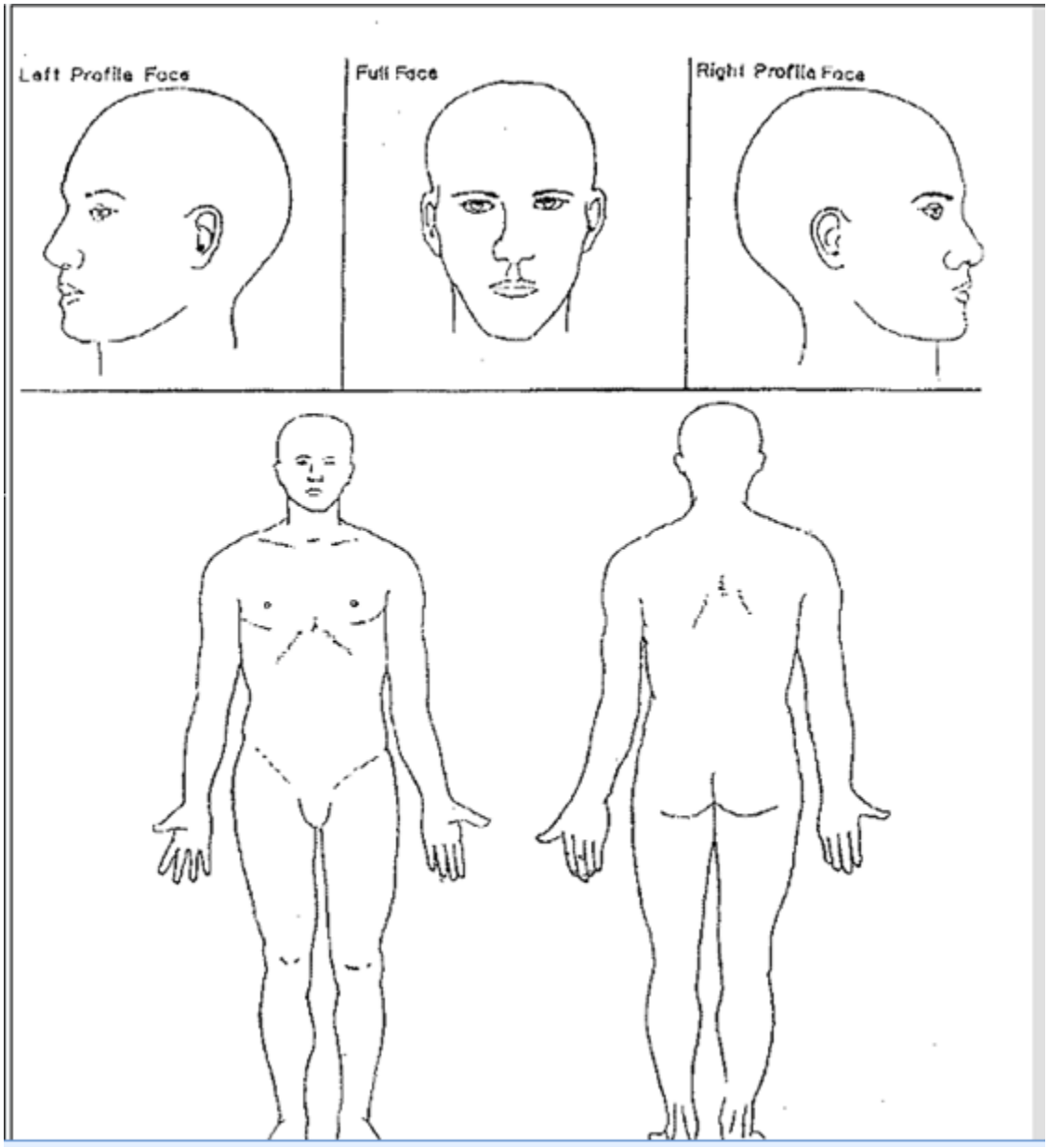
Appendix 2: Bleeding Questionnaire

Please fill in Yes or No to each of the following questions:

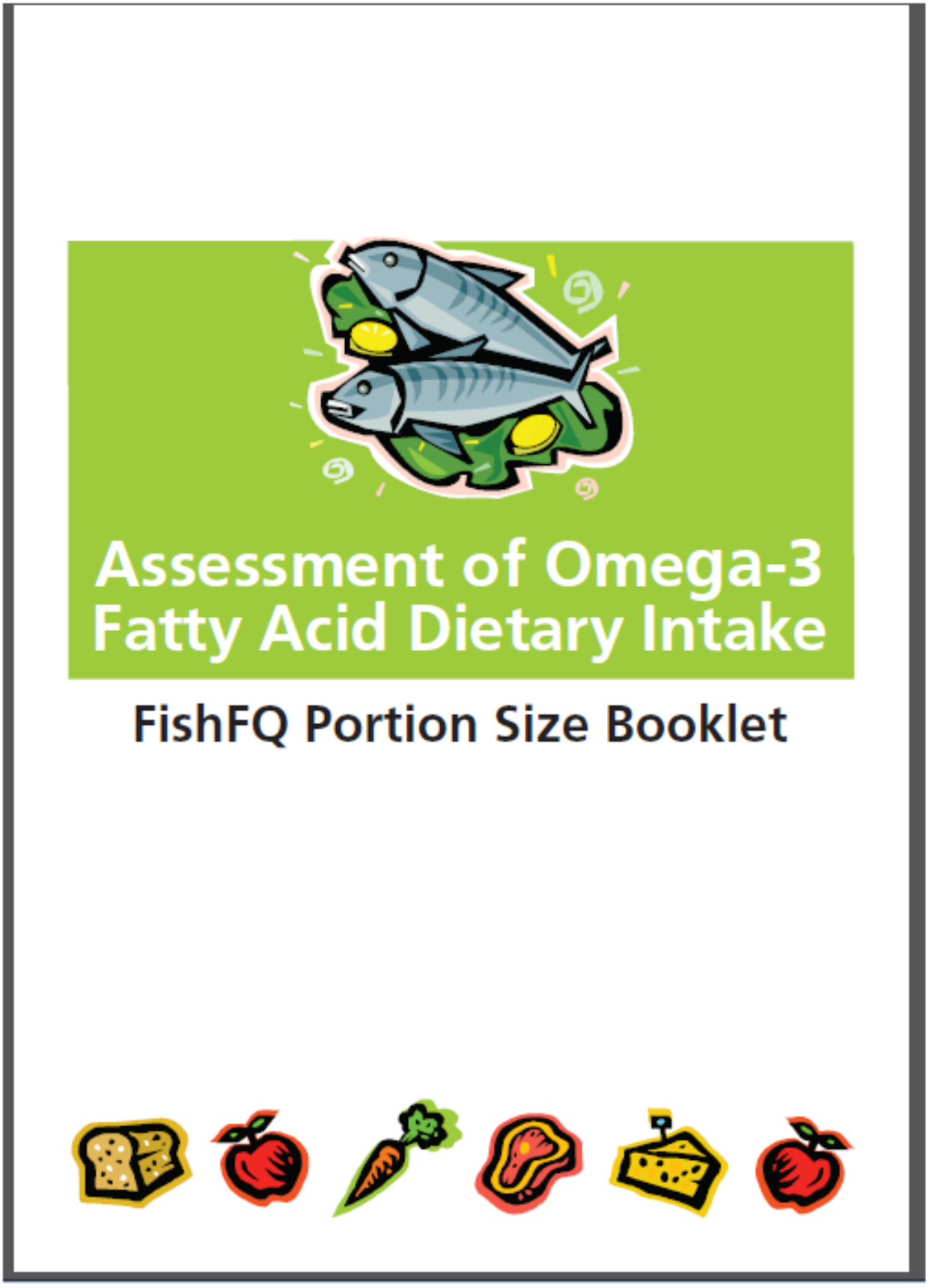
Symptom	Yes	No
Bruising		
<1cm		
2-5cm		
>5cm		
Spontaneous		
Minimal Trauma		
Oral Cavity Bleeding		
Spontaneous		
After Brushing		
Nose Bleeds		
1-5 min		
>5 min		
Medical Attention		
Bleeding from Minor Cuts		
Medical Attention		
GI Bleeding		
Medical Attention		
Increase in Average Duration of Menstruation		

Note: All areas of bruising are to be marked on body map

Body Map:



Appendix 3: Dietary Questionnaire



FishFQ

MRC Childhood Nutrition Research Centre
Fish Frequency Questionnaire

The enclosed food frequency questionnaire has been designed to give us information about your usual dietary intake of the omega-3 fatty acids that are found mainly in certain varieties of fish and meat.

On the Fish Frequency Questionnaire (FishFQ) please mark the box that indicates how often you eat each food.

Example:

White Fish e.g. Cod, Haddock, Plaice, Sole, Halibut (coated, fried or grilled)

	Never	1-3/month	1/week	2-4/week	5-6/week	1/day	2-3/day	4-5/day	5-6/day
Cod	□	□	■	□	□	□	□	□	□
Haddock	■	□	□	□	□	□	□	□	□
Plaice	■	□	□	□	□	□	□	□	□
Sole	■	□	□	□	□	□	□	□	□
Halibut	■	□	□	□	□	□	□	□	□

Please make sure you mark a box in every line so that we can be sure whether you have this food or not.

The example above shows clearly that this person eats cod once a week but never eats any other type of white fish.

How to describe Omega-3 portion sizes using this booklet

The pictures on Chart 1 of this booklet show portions of fresh or tinned fish, fish dishes and meats, which are good sources of Omega-3, on standard plates.

Please look at the pictures carefully and decide which serving size is closest to the one you would usually eat.

Please enter the serving size number that is closest to what you usually eat when you have this food in the portion column of the questionnaire.

Example:

If picture 2 looks most like your usual portion size of white fish enter the picture number as shown below:

Portion (chart 1)

0	2

Chart 2 lists other foods that may contain Omega-3 fatty acids. Some fat spreads, milk, yoghurt, and eggs have Omega-3 fats added.

Please indicate on the FishFQ whether you eat any of these products by entering the code from Chart 2 in the brand column and where applicable enter your usual portion size.

Example:

If you drink about 1 cup/glass of Omega-3 enriched milk, fill in the table like this:

0	1

2	0	0

Chart 1

The following pictures show portions of fresh fish on a standard dinner plate. Please enter the serving size number that is closest to what you usually eat when you have this food in the portion column of the questionnaire.

White Fish e.g. Cod. Haddock, Plaice, Sole, Halibut

Only 1 type of fish is shown here (cod) because all white fish look and weigh very similar.

Cod (fresh, raw)

Oily Fish

Herring (canned, cooked in tomato sauce)

Mackerel (smoked)

Kipper Fillet (smoked)

The following pictures show portions of fresh fish on a standard dinner plate. Please enter the serving size number that is closest to what you usually eat when you have this food in the portion column of the questionnaire.

Smoked Salmon Slices


13


14

Salmon Steaks (fresh, raw)


15


16

Trout (fresh, raw)

17

18

Tuna (fresh, raw)


19


20


21

The following pictures show portions of tinned fish and other foods on a standard side plate. Please enter the serving size number that is closest to what you usually eat when you have this food in the portion column of the questionnaire.

Oily Fish (canned, cooked)

Salmon


22


23

Pilchards


24


25

Sardines


26


27


28

Skippers (Sprats/Brisling)


29


30

The following pictures show portions of shellfish on a standard side plate. Please enter the serving size number that is closest to what you usually eat when you have this food in the portion column of the questionnaire.

Shellfish

Crab (canned, cooked)

Prawns (fresh, cooked)

Mussels (pre-packed, cooked)

The following pictures show portions of fish dishes on a standard side plate. Please enter the serving size number that is closest to what you usually eat when you have this food in the portion column of the questionnaire.

Fish Dishes

Fish finger/cakes (cooked)

Enter the number of fish fingers or cakes you would usually eat in the portion column.

Meats

Chicken/Turkey (cooked)

Liver/Kidney (cooked)

Chart 2

If you regularly eat omega-3 enriched foods please indicate the food you eat by entering the number in the "Brand" column on the questionnaire. Indicate your usual portion size by entering the amount in grams in the portion column.

Omega 3 Enriched Foods

Milk

In "Brand" column enter **01** for Full Cream Milk or **02** for semi-skimmed.

In portion column enter the amount usually taken in mls

Standard glass = 200 mls. In tea/coffee = 15-20mls. On cereal = 125mls

Drinks

In "Brand" column enter **03** if taken.

One carton = 150g. In "portion" column enter amount usually eaten.

Yogurts

In "Brand" column enter **04** if taken.

One carton = 150g. In "portion" column enter amount usually eaten.

Eggs

In "Brand" column enter **05** if taken.

In "portion" column enter number of eggs usually eaten.

Spread

In "Brand" column enter **06** if taken.

In "portion" column enter amount in grams usually eaten.

Spread thinly = 5; medium = 7; thickly = 10

Appendix 4: Quantitative Sensory Testing Methods

Testing will take place in a dedicated, quiet room with controlled air temperature (68-72 ^{o}C). Testing will be performed without a parent, guardian or observer in the room as presence of mother during testing has been shown to influence results (Zohsel et al. 2006). However, in the presence of the subject, parent/guardian will be permitted to undergo a test trial of the planned thermal and mechanical stimuli to reduce concern that child will experience unacceptable discomfort. Parent/guardian will be permitted to observe testing procedures via video monitoring from an adjacent room. Subjects will be given 10 minutes to acclimate to the room temperature. Testing will be performed by a trained research team member who will provide very clear and concise instructions on testing procedures to subjects. Subjects will be seated in a comfortable chair, and positioned so they cannot see monitors.

Thermal sensory equipment has been purchased from, Medoc U.S.A, Compass Medical Technologies, Inc.

Neurosensory Analyzer Model TSA-II

The TSA II is a computerized device designed for both clinical and advanced research applications of the quantitative assessment of small nerve fiber dysfunctions. It measures sensory thresholds such as the sensation of warmth, cold, heat-induced pain and cold-induced pain. This device has FDA clearance via a 510K. The TSA II measures thresholds for four sensory sub-modalities:

- Warm sensation (WS), a C fiber mediated sensation.
- Cold sensation (CS), mediated by A-delta fibers.
- Heat induced pain (HP), a mostly C fiber mediated sensation, with some involvement of A-delta fibers.
- Cold induced pain (CP), at about 10°C. Mediated by a combination of both C and A-delta fibers.

Thermal testing:
Thermal testing will be performed using the TSA II Neurosensory Analyzer TSA-II (Medoc Ltd, Ramat Yishai, Israel) at non-tissue damaging temperatures using Peltier-type contact thermal stimulators. Testing sites will be the volar surface of the forearm and the thenar eminence bilaterally. These sites were chosen based on previous studies which have consistently shown that lower extremity thresholds are consistently higher than upper (Meier et al. 2001). A 9 cm^2 thermode will be used. The baseline temperature will be 32 ^{o}C with stimulus range between 0-50 ^{o}C. Thermal thresholds will be determined using the Method of Limits where subjects are instructed to press a button or tell the tester to stop when the sensation in question is first perceived. This method has been used in other pediatric QST studies in SCD (Brandow et al. 2013; O'Leary et al. 2014) as well as adult SCD studies (Campbell et al. 2016) and requires the shortest testing duration of all methods (desirable for a pediatric study). The order of testing will be as follows (Brandow et al. 2013; Heldestad et al. 2010):

1) Cold detection threshold
2) Warm detection threshold
3) Cold pain threshold
4) Heat pain threshold
5) Heat pain tolerance

Cold and Heat Detection Thresholds:
The probe temperatures will be increased/decreased from baseline at increments of 0.5 ^{o}C /sec Subject will be asked to press a button or acknowledge verbally when they first perceive a sensation of heat or cold. This will be defined as the cold/heat detection threshold. Temperature will then be returned to baseline.. This will be repeated twice at each site with an interstimulus interval designated by the participant to signal when they are ready for a following stimulus.

Cold and Heat Pain Thresholds:

The probe temperatures will be increased/decreased from baseline at increments of 0.5 ºC. Subject will be asked to press a button when they first perceive the stimulus to produce pain. This will be defined as the cold/heat pain threshold. This will be repeated twice at each site with an interstimulus interval designated by the participant.

Heat Pain Tolerance:
The probe temperatures will be increased from baseline at increments of 0.5 ºC. Subject will be asked to press a button when they first perceive the stimulus to produce pain which they find to be intolerable. This will be defined as the heat pain tolerance threshold. Temperature will then be returned to baseline.. This will be repeated once on each forearm with an interstimulus interval designated by the participant. Fifteen seconds after they press the button to stop the test, subjects will be asked to rate the pain that they still feel at that time on a scale of 1 to 10 to test for after sensations.

Pain perception and Anxiety
Unpleasantness and anticipatory anxiety will be assessed using a 10 point scale , with 0 indicating the lowest level and 10 indicating the highest level. Before testing, subjects will be instructed on use of the scale. Prior to each testing session, each participant will be asked to identify using the scale “how nervous, afraid or worried” they are about the upcoming task to indicate level of anxiety. For “unpleasantness”, subjects will be asked at the end of the testing session how unpleasant the experience was for them.

Appendix 5: Research Laboratory Methods

Assay of Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA) and Arachidonic Acid (AA) in Circulating Blood Cells to Monitor Study Drug Compliance:

Omega-3 index (O3I), defined as the sum of EPA and DHA as a percentage of total FAs, has been used clinically to monitor the beneficial effects of dietary supplements of n-3 FAs or the diets enriched in these FAs (Harris 2008; Superko et al. 2013). To assess the intake of these FAs, the levels of EPA and DHA have been measured in phospholipids or total lipids from several blood fractions and tissues including adipose tissue, plasma, serum, whole blood, platelets and erythrocytes (Bell et al. 2010; Katan et al. 1997; Marangoni et al. 1993). Measurement of n-3 FAs in the erythrocyte membranes, however, has been regarded as the gold standard, since erythrocytes have a long lifespan of approximately 120 days which could reflect long term intake of n-3 FAs (Harris and Von Schacky 2004; Poppitt et al. 2005). In contrast to erythrocytes, other blood cells are short lived with a half life of ~10 days for platelets and a few hours for WBCs. RBCs from subjects with SCD are also short lived with an average lifespan of less than 20 days and are fragile (McCurdy and Sherman 1978). In addition, measurement of n-3FAs in the erythrocyte phospholipids involves the isolation of erythrocytes from whole blood and separation of phospholipid fraction from other lipid components. Because of the fragile nature, older sickle RBCs could be lost during in vitro manipulation resulting in a non-representative and skewed red cell population. While washed red cell preparation involves multiple washing/centrifugation steps, packed total blood cells can be obtained with minimal in vitro manipulation of whole blood with a single centrifugation step. Moreover, packed total blood cells are mainly erythrocytes with minimal number of WBCs and platelets. To test whether packed total blood cells could be used to assess O3I in our clinical trial patients (so that their blood samples would be minimally subjected to in vitro manipulation), in preliminary experiments using methods detailed below and blood from control volunteers, we measured EPA, DHA and AA levels in packed total blood cells, determined their O3I index values and FA ratios (between AA and EPA plus DHA), and compared the values with those generated with erythrocyte phospholipids from the same donors. Preliminary results from a representative control presented in the Table below demonstrate that levels of AA, DHA, EPA, O3I index, and fatty acid ratios measured in packed total blood cells and erythrocyte phospholipids are similar (**Table-1**). These results demonstrate that packed total blood cells can be used to monitor fatty acid intake and **we will use packed total blood cell assay to determine O3I index in our clinical trial patient cohort.**

Table-1: Fatty Acid levels and Omega-3-Index values from Erythrocyte Phospholipids and Total Blood Cells

Fatty Acid (% of total)	Red blood Cells	Total Blood Cells
Arachidonic Acid (AA)	9.51	9.63
Eicosapentaenoic acid (EPA)	1	1.02
Docosahexaenoic acid (DHA)	4.43	4.2
Omega-3-Index (O3I)	5.43	5.22
Fatty Acid Ratio (AA to (EPA + DHA))	1.75	1.85

Blood cells from EDTA anticoagulated whole blood (100 microliters (µl)) will be pelleted by centrifugation at 1500 x gravity (xg) for 10 minutes. Packed cells prepared (in 2-ml microfuge tubes) shortly after blood collection (30-45 min) will be saved immediately at -80ºC until further analysis. At the time of analysis, cell pellets will be thawed on ice, lysed in 0.5 ml water and transferred to extraction tubes. Samples will be spiked with 25 micrograms (µg) 1,2,3-tri-heptadecanoyl-glycerol (a representative synthetic triglyceride) and 25 µg 1,2-dipentadecanoyl-sn-glycero-3-phosphoethanolamine (a representative synthetic phospho-lipid). These synthetic lipids will be added to monitor extraction recovery of blood lipids and their conversion to Fatty Acid Methyl Esters (FAMEs). Total lipids from lysed blood cells will be extracted as described by Folch *et al* (Folch, Lees, and Sloane Stanley 1957) using a mixture of chloroform and methanol (2:1) in the presence of 0.01% butylated hydroxy-toluene (BHT). BHT will be provided as an antioxidant to protect polyunsaturated FAs

(PUFAs) from oxidative degradation. BHT will also be present during subsequent lipid analysis procedures including thin layer chromatography (TLC). FAMEs will be prepared by heating lipid extracts in 250 µl of anhydrous toluene (added to dissolve all neutral lipids including triglyceride and cholesterol esters) and 1.5 ml of anhydrous methanolic-HCl (0.5 normal solution, a transmethylating reagent) for 90 minutes at 80°C in sealed glass tubes (Blau and Halket 1993). Under these reaction conditions, which were established in preliminary experiments, both esterified and non-esterified FAs in lipid extracts (from up to 200 µl whole blood) are completely converted to FAMEs. Following dilution of the reaction mixture with 1.5 ml water, FAMEs will be extracted with 2.0 ml hexane, separated from other interfering components (such as free cholesterol, which can co-extract with FAMEs) by TLC (Dodge and Phillips 1967; Mangold 1965) and analyzed using an Agilent gas chromatograph (6850 GC System). Analysis will be performed using a polyethyleneglycol capillary column (30m x 250 µm x 0.25 µm, HP-INNOWax) and a temperature gradient between 150°C and 240°C (David, Sandra, and Wylie 2001). The injector and detector temperatures will be maintained at 240°C and 250°C, respectively. Helium will be used as a carrier gas. FAMEs will be identified by comparing their retention times with authentic standards. The gas chromatography (GC) conditions employed resolve all n-3 and n-6 FAs with chain lengths between C18 and C22 with a base-to-base resolution of all FAMEs as shown in **Figure A** below. Peak areas will be integrated using Agilent ChemStation Software, and quantitated using a standard curve set up with methyl heptadecanoate (a C17 synthetic FA methyl ester). Assay conditions [transmethylation, and GC parameters including detector response and reproducibility (intra- and inter-assay variability)] have been set up in initial experiments employing either synthetic lipid standards or whole blood lipid extracts. The detector response is linear between 0.25 and 6.0 µg of FA with an r^2-value of 0.995. The variability between injections is <3% and between experiments is <4%. The recovery of added lipid standards through GC was >80%.

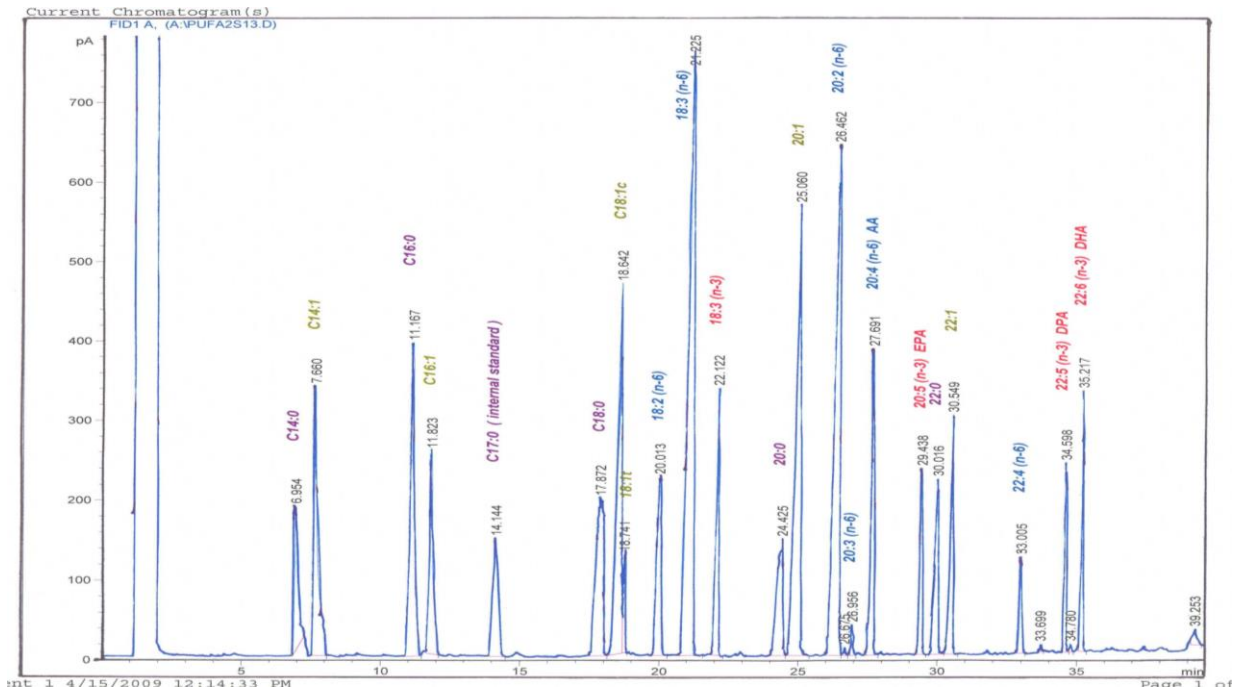


Figure A: Representative GC profile of standard fatty acid methyl esters.

Peaks: Myrisitic acid (C14:0), myristoleic acid (C14:1), palmitic acid (C16:0), palmitoleic acid (C16:1), Heptadecanoic acid (C17:0 internal standard), Stearic acid (C18:0), Oleic acid (C18:1c), elaidic acid (C18:1t), linoleic acid (C18:2 n-6), γ-linolenic acid (C18:3 n-6), α-linolenic acid (C18:3 n-3), arachidic acid (C20:0), eicosaenoic acid (C20:1), eicosadienoic acid (20:2 n-6), homo-γ-linolenic acid (20:3 n-6), arachidonic acid (C20:4 n-6, AA), eicosapentanoic acid (C20:5 n-3, EPA), behenic acid (C22:0), docosaenoic acid (C22:1),

docosatetraenoic acid (C22:4 n-6), docosapentaenoic acid (C22:5 n-3, DPA) and docosahexaenoic acid (C22:6 n-3, DHA).

Analysis of Plasma Lipid Mediators including Resolvin (Rv) D1 and Leukotriene B4 (LT) B4 :

RvD1, LTB4 and other plasma lipid mediators [eicosanoids (oxygenated metabolites of AA and EPA) and docosanoids (oxygenated metabolites of DHA)] will be analyzed, in initial experiments, by Liquid chromatography–mass spectrometry (LC-MS)-based targeted lipidomics as described previously (Maddipati and Zhou 2011; Maddipati et al. 2014; Markworth et al. 2013) in collaboration with Dr Maddipati (Director, Lipidomics Core Facility, Bioactive Lipids Research Program, Department of Pathology, Wayne State University, Detroit, MI). Lipidomics studies have used either serum or plasmas prepared in the presence of various anticoagulants. Previous studies, however, have shown that the levels of eicosanoids and docosanoids measured in serum are different when compared to those levels measured in plasma. For example, serum levels of thromboxane B2 (TxB2) and 12-hydroxyeicosatetraenoic acid (12-HETE) are increased, while the levels of 18-hydroxyeicosapentaenoic acid (18-HEPE) and 17-hydroxydocosahexaenoic acid (17-HDHA) are decreased (Mas et al. 2012). These differences in mediator levels could be due to an increased synthesis of some (TxB2 and 12-HETE) and possibly due to metabolic conversion of others (for example, 18-HEPE and 17-HDHA are the upstream precursors for the synthesis of E- and D- series resolvins, respectively). While plasma levels reflect a more accurate measure of circulating eicosanoids and docosanoids, serum levels may indicate maximal eicosanoid and docosanoid production by activated cellular elements of blood. We will therefore assay both plasma and serum samples in our lipidomic assays using LC-MS/MS. Studies have shown that the levels of resolvin are not significantly different between plasmas collected into different anticoagulants (Mas et al. 2012). LTB4 levels measured in EDTA plasma, however, are significantly lower compared to those measured in plasmas prepared with other anticoagulants (Parameter LTB4 Assay Kit from R&D Systems), which appears to be due to prevention by EDTA of ex-vivo conversion of LTA4 to LTB4 during blood collection and processing. We will therefore use EDTA plasmas in lipidomic assays and in all our biomarker assays with the exception of assays involving hemostatic biomarkers and CAT assays, for which we will use citrate-anticoagulated plasmas. While measurable levels of RvD1, RvD2, 17R-RvD1 and RvE1 have been reported in control plasmas, presence of other SPMs including Protectin D1 (PD1), Maresin 1 (MaR1), RvE2, RvE3, and 18R-RvE3 have been documented to be present in plasmas from both control and inflammatory arthritic patients who were on n-3FA therapy (Colas 2014; Mas et al. 2016; Psychogios et al. 2011). In addition, a significant increase in plasma RvD1 has been noted in subjects after n-3 FA supplementation (Mas et al. 2016). Median levels of these plasma mediators range between 17 pg/ml and 774 pg/ml (Barden et al. 2016; Mas et al. 2016). In addition, 17-HDHA and 18-HEPE, the upstream precursors of D- and E- series resolvins, respectively, are also present in plasma at concentrations ranging between 63.6 and 235.3 pg/ml and their levels increase by 2 to 4-fold following n-3FA therapy (Mas et al. 2016). The increase in 17-HDHA following fatty acid therapy may be clinically relevant as it can affect several inflammatory responses including promotion of phagocytosis (Kohnke et al. 2013) and suppression of the synthesis of pro-inflammatory cytokines including TNF-α (Gonzalez-Periz et al. 2006). In our clinical trial patients, we will quantitate all of the specialized pro-resolving mediators (SPMs) and their upstream precursors documented to be present in plasmas from individuals pre- and post- n-3 FA therapy. Analysis will also include any novel biologically relevant docosanoid and eicosanoid identified in the future. Using appropriate statistical tests (Pearson and/or Spearman Correlation tests) we will evaluate whether there is any relationship exist between the levels of SPMs that are elevated following FA therapy and the levels of pro-inflammatory cytokines including IL-1β and TNF-α to assess whether FA therapy provided protection against inflammation and also to identify the SPM(s) mediating the anti-inflammatory response.

If lipidomic assay delivers results comparable to those measured by ELISA for LTB4 and RvD1 with acceptable intra- and inter- assay variabilities, the ELISA assays will be used in the Clinical Trial. ELISA kits for both RvD1 (Cayman Chemicals) and LTB4 (R&D Systems) are commercially available. We have previously assessed LTB4 levels in plasma using an immunoassay kit following extraction and HPLC separation with appropriate correction for recovery (Setty and Stuart 2002). LTB4 levels in race-matched controls and children with HbSC and HbSS disease were 136 ± 32 (mean ± SD), 177 ± 83 and 207 ± 64 femtomole (fmol)/ml, respectively (Setty and Stuart 2002). The plasma LTB4 levels measured in our laboratory are comparable to those reported in serum

by LC-MS/MS (Markworth et al. 2013) [ranged between 28 and 72 picogram (pg)/ml in the placebo group equivalent of 89 to 214 fmol/ml]. Plasma LTB4 levels in our study population will be measured using a Parameter LTB4 Assay Kit from R&D Systems. The R&D kit can detect LTB4 in the range of 3.7 to 10.9 pg/ml with a mean minimum detectable level of 8.2 pg/ml. The assay range is between 10.3 and 2500 pg/ml. RvD1 levels in controls, assayed with LC-MS/MS, ranged between 3.7 and 17.09 pg/ml (Colas 2014; Psychogios et al. 2011). The Cayman RvD1 assay kit can detect RvD1 at concentration as low as 10-20 pg/ml and the assay is linear between 50 and 100 pg/ml. Assuming a plasma RvD1 concentration of 10 pg/ml, a 5 ml plasma sample provides sufficient amount of RvD1 for quantitation by ELISA. We will therefore employ RvD1 ELISA kit to assess plasma RvD1 levels. The lipid mediators in plasma will be extracted using C18 extraction columns as previously described (Maddipati and Zhou 2011), the column eluate will be dried under a stream of nitrogen, reconstituted in ELISA assay buffer and quantitated following Manufacturer's instructions. We have previously used immunoassays to quantitate multiple eicosanoids in plasma and urine samples from patients with SCD and controls following extraction of lipid mediators using C-18 cartridges (Setty, Chen, and Stuart 1995; Setty et al. 1998; Setty and Stuart 2002).

Analysis of Urinary Resolvin D1:

A recent study, using an LC/MS/MS assay, has documented the presence in human urine of docosanoids including RvD1 which is present at mean levels of 71.4 ± 53.3 and 72.8 ± 48.3 pg per mg creatinine in non-smoking men (n=13) and women (n=10), respectively (Sasaki et al. 2015). Based on the urinary creatinine levels (500 to 2000 mg/day) and urine volume (800 to 2000 ml/day), a 5 ml urine sample provides sufficient amount of RvD1 for quantitation by ELISA. We will therefore employ RvD1 ELISA kit (Cayman) to assess urinary RvD1 levels. Fresh urine samples will be centrifuged at 2900 xg for 5 min at room temperature and supernatant saved at -80ºC until assayed. At the time of the assay urine samples will be diluted with water to adjust creatinine levels to 0.6 mg/ml. RvD1 will be extracted from 5 ml diluted urine samples using C-18 cartridges, extracts evaporated to dryness, re-constituted in ELISA assay buffer and quantitated following Manufacturer's instructions.

General ELISA Quality Control Measures:

Plasma will be prepared shortly after blood collection (30-45 min), and aliquots (50 to 250 µl) saved immediately at -80ºC until further analysis. Multiple freeze-thaw of frozen plasmas will be avoided. For biomarker analysis, batched plasma samples will be thawed completely on ice, and mixed well prior to analysis. Plasma will be centrifuged if large amount of particulate material is present in the sample, and this sample treatment will be recorded. Severely hemolyzed and lipemic plasmas will not be included in the analysis when possible. The bio-markers: CRP, cytokines, soluble cell adhesion molecules, ET-1 and hemostatic markers will be assayed employing commercial ELISA assay kits. Kit manufacturers' provide all reagents and buffers needed to perform the assay. These kit reagents and buffers will be stored and used according to the manufacturer instructions. All reagents, buffers and assay strips will be warmed to recommended temperature before use. Reagents from different kits will not be mixed. Kits will not be used after their expiration date. All standards, controls and samples will be run in duplicate. In addition to kit controls, in-house controls will be included with every assay to cover low-end, mid-range, and high-end of the standard curve. If control values fall outside pre-established ranges, the assay will be repeated after appropriate instrument maintenance procedure. Assays with standard curves with an r^2 value <0.98 will be repeated. Samples with values outside the pre-set calibration range will be diluted and repeated. Other general quality control measures include pipette calibration to meet American Association for Clinical Chemistry (AACC), International Organization for Standardization (ISO), Good Laboratory Practice (GLP) / Good Manufacturing Practice (GMP), National Committee for Clinical Laboratory Standards (NCLLS) and College of American Pathologists (CAP) guidelines with dated logs for each pipette used in the assay, and individual biomarker analyses using assay kits from the same lot number when possible. We have used age-matched African American controls with an n-value ranging from 10 to 29 subjects for biomarker controls in our labs. Mean intra- and inter-assay variability are <5%, and <8%, respectively. Control marker levels reported from our laboratories are comparable to those reported in the literature. In addition, our research laboratory served as a Core Biomarker Facility between 2005 and 2009 for the National

Comprehensive Sickle Cell Centers' Clinical Trial of Dexamethasone in Acute Chest Syndrome (Quinn et al. 2011).

Assessment for C Reactive Protein (CRP):

CRP in plasma will be assayed using ALPCO hs-CRP Immunoassay kit. The standard curve spans a range of 1900-150000 pg/ml, with a minimum detection limit of 921pg/ml. Median (25^{th} and 75^{th} percentile) CRP level in our African-American controls is 0.1 (0.05, 0.5) mg/liter (l) (Krishnan et al. 2010) and is similar to National Health and Nutrition Examination Survey (NHANES) control values in age and race-matched children.

Analysis of Pro- and Anti-Inflammatory Cytokines:

Representative pro- (IL-1β, IL-6, IL-8 and TNF-α) and anti- (IL-4 and IL-10) inflammatory cytokines that have been documented to be abnormal in patients with SCD (Bowers et al. 2013; Hyacinth et al. 2014; Keikhaei et al. 2013; Raghupathy et al. 2000; Setty et al. 2012) will be evaluated in EDTA plasma using commercially available ELISA kits (R&D Systems). Assay range, minimum detection level, and reported control levels (from our laboratories and others) for the test cytokines are shown in **Table 2** below. No matrix interference was noted with plasma/serum at dilution between 1:2 and 1:16.

Table 2: Cytokine assay Parameters

Cytokine	Assay Range (Standard Curve)	Minimum Detection Level	Reported Control Plasma Range
IL-1β	0.125 to 8 pg/ml	0.057 pg/ml	0.00 - 0.452 pg/ml
IL-4	7.8 to 500 pg/ml	<2 pg/ml	15 pg/ml (mean)
IL-6	0.156 to 10 pg/ml	0.039 pg/ml	0.435 - 9.57 pg/ml
IL-8	1.0 to 64 pg/ml	0.4 pg/ml	2.27 – 11.1 pg/ml
IL-10	0.78 to 50 pg/ml	0.09 pg/ml	<0.78 pg/ml
TNF-α	0.5 to 32 pg/ml	0.106 pg/ml	0.00 - 1.195 pg/ml

Soluble Cell Activation Markers: Soluble VCAM-1 (endothelial activation), soluble P-selectin (platelet activation) and soluble L-selectin (neutrophil activation) will be evaluated using ELISA kits from R&D Systems. Reported plasma levels in pediatric controls for sVCAM-1, sP-selectin and sL-selectin from our laboratories were 536 ± 105 nanograms (ng)/ml, 38.1 ± 8.5 ng/ml, and 1321± 418 ng/ml, respectively (Setty and Stuart 2002; Setty et al. 2012). These levels were comparable to those reported by other investigators.

Endothelin-1: Endothelin-1 levels in plasma will be assessed using an ELISA assay kit from R&D Systems. The assay is linear between 0.4 and 25 pg/ml with a minimum detection limit of 0.207 pg/ml. Plasma levels in controls range between 0.58 and 1.96 pg/ml with a mean level of 1.17 pg/ml (n=35). No significant interference was noted with big endothelin-1 and -2 or endothelin-2.

Standard Hemostatic Markers: Hemostatic markers, including the prothrombin fragment F1.2 and D-dimer will be evaluated by ELISA as previously described (Krishnan et al. 2010; Setty et al. 2012).

Novel Hemostatic Markers: Calibrated Automated Thrombograpy (CAT) is a novel assay of in vitro thrombin generation, which is employed to evaluate several CAT parameters including endogenous thrombin potential (ETP), peak thrombin, lagtime, time to peak, and start tail (Hemker et al. 2003). CAT parameters will be measured on platelet poor plasma in a 96-well plate fluorometer (Fluoroskan Ascent, Thermo Electron Corp.) with 390/460 nanometers (nm) excitation/emission filters and a dispenser to dispense the substrate. For each experiment, 2 sets of readings will be taken: one from a well in which thrombin generation (TG well) takes place and a second from a well in which calibrator (CL well) has been added. In the TG well, 20 µl trigger (consisting of desired concentrations of tissue factor and phospholipids) is added followed by 80 µl plasma sample. In the CL well, 20 µl of alpha2-Macroglobulin-Thrombin complex solution is added followed by 80 µl plasma. The plate will be warmed at 37°C for 10 min in the instrument, 20 µl fluorogenic substrate dispensed to all the wells, zero time registered, and reading initiated at a predetermined sampling rate for 45-60 minutes. All experiments will

be carried out in triplicate. A dedicated software program (Diagnostica Stago) will generate the thrombogram and calculate thrombin concentration and other CAT parameters.

Using the assay described above, we have previously measured CAT parameters in 23 individuals with Sickle Cell Disease with SS or Sβ°thal genotype (18 to 53 years old) and 6 age matched controls (Betal et al. 2009). Significant differences were noted in the SCD compared to the controls with shortened Lag Times [1.9 ± 0.39 (mean ± SD) vs 2.39 ± 0.42 minutes, $p<0.001$] and time to peak (3.72 ± 0.55 vs 4.77 ± 0.49 min, $p<0.001$). However, there was overall a lower and less sustained thrombin generation with SCD subjects demonstrating an early start tail (16.76 ± 2.29 vs 20.33 ± 1.71 min, $p<0.01$) and less total ETP (1377 ± 360 nM vs 1629 ± 212 nM, $p<0.01$) when compared to the controls. Peak thrombin levels were similar in both groups at 354 and 336 nM, respectively. Thus the subject with SCD has a significant early upsurge in thrombin generation followed by the phenomenon of a rapid attenuation.

While the anti-thrombotic mechanism ascribed to n-3 FAs relates to in vitro anti-platelet aggregation effect, limited information is available about its effect on the plasmatic or fluid phase of coagulation. This study therefore provides an opportunity for some exploratory analyses on the effects of n-3 FA on thrombin generation as a novel measure of coagulation activation which we will contrast with standard measures of thrombin generation (F1.2) and fibrin dissolution (D-Dimer). These data could provide both a qualitative measure of efficacy (suppressing ancillary pathogenicity related to coagulation activation) and potential safety measure (suppression of thrombin generation as an indicator of potential additive bleeding risk in these patients).

Lactate Dehydrogenase (LDH): Plasma LDH levels will be evaluated using an LDH assay kit (TOX07, Sigma). The enzyme activity international units (iu)/l) will be read from a calibration curve generated using an LDH standard (Sigma) as previously described (Krishnan et al. 2010).

Fetal Hemoglobin (HbF): HbF levels will be determined using cation exchange-high performance liquid chromatography on 50 to 100 µl of stored frozen red cell pellets. We will use the services available at the Mayo Medical Laboratories, Mayo Clinic for HbF analysis (Krishnan et al. 2010).

Other Relevant Inflammatory Biomarkers: Please NOTE that no extra blood samples are collected for future studies. We will save any unthawed remaining plasma and urine aliquots for evaluation of novel and relevant biomarkers identified in the future.

Appendix 6
Dose Calculator for SCD-Omegatex™

Dosage:

Please note: all dosing is based on total omega-3 fatty acid content of capsules (combined DHA plus EPA). Dosing will be based on a per capsule combined DHA plus EPA content of 336 mg.
Doses will be rounded to capsule size. Doses that calculate to ≥ 0.5 capsule will be rounded up while doses calculating to < 0.5 capsule will be rounded down for capsule size

Lower dose range is 25 mg/kg/day
Higher dose range is 37.5 mg/kg/day
Maximum total daily dose is 4 grams

Dose calculation in number of capsules for 25 mg/kg/day dose level

Weight in kg	Number of capsules per day
15-20	1
21-33	2
34-47	3
48-60	4
61-73	5
74-87	6
88-100	7
101-107	8

Dose calculation in number of capsules per day for 37.5 mg/kg/day dose level

Weight in kg	Number of capsules per day
15-22	2
23-31	3
32-40	4
41-49	5
50-58	6
59-67	7
68-76	8
77-85	9
86-94	10
95-103	11
104-111	12

Sample Calculation:

For a 35 kg child:
At 25 mg/kg dose range child will receive 25 mg x 35 kg = 875 mg divided by 336 mg EPA+DHA per capsule = 2.6 capsules, round up to 3 capsules.
At 37.5 mg/kg dose range child will receive 37.5 mg x 35 kg = 1312.5 mg divided by 336 mg EPA+DHA per capsule = 3.9 capsules, round up to 4 capsules.

Maximum total daily dose is 12 capsules =3624mg DHA plus 408 mg EPA in a 9:1 ratio (4032 mg omega-3 fatty acid total, combined DHA plus EPA).

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