Title: Topical Sodium Nitrite in Sickle Cell Disease and Leg Ulcers
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Protocol Title: **Phase II trial of topical sodium nitrite in patients with sickle cell disease and leg ulcers**

Abbreviated Title: Nitrite cream for leg ulcers

Identifying words: Sickle cell disease, anemia, sodium nitrite, wound healing.

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Duration of Study: 48 months

Number and Type of Subjects: Screen and enroll up to 50 subjects to get completed studies for 40 male or female, age 18 and older

Product Uses Ionizing Radiation: No

Project Uses IND/IDE: Yes

If yes, list:	Name of Drug:	Sodium Nitrite Cream
	IND #:	110420
	IND Sponsor:	Einstein College of Medicine
	IND Holder:	Caterina P Minniti, MD
	Manufacturer:	Pine Pharmaceutical, New York

Project Uses "Durable Power of Attorney": No

Multi-Institutional Project: Yes

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1. Precis

Leg ulcerations have long been identified as a serious and debilitating complication of SCD; indeed, even the first SCD patient described in North America in 1910 had leg ulcerations. The prevalence varies – being low before 10 years of age and in genotypes other than SS. Moreover, the prevalence of leg ulcerations is influenced by geographical location, with an occurrence as high as 75% among SS patients in Jamaica, and 8-10% in North America. The etiology of chronic ulcers in SCD and other hemolytic disorders is unknown, but mechanical obstruction by dense sickled red cell, increased venous pressure, bacterial infections, abnormal autonomic control with excessive vasoconstriction when in the dependent position, and degree of anemia with decreased oxygen delivery capacity have all been proposed as potential contributing factors.

Morbidity from chronic leg ulcers remains a substantial clinical burden in patients with sickle cell disease, despite advances in care – including the use of disease-modifying agents such as hydroxyurea and blood transfusions – and improved supportive care. Patients with sickle cell disease and leg ulcers have biomarkers of more severe hemolytic anemia, a state associated with low bioavailability of nitric oxide. Existing therapeutic approaches for sickle cell disease ulcers are unsatisfactory and are mostly based on treatments for venous and arterial ulcers in the general population. A recent Cochrane review identified only six prospective randomized therapeutic trials in sickle cell disease leg ulcers in the past 30 years – four in Jamaica and two in the USA. The results were mixed, with statistically significant increases in wound closure reported with topical Arg-Gly-Asp (RGD) peptide and intravenous arginine butyrate. These agents remain in early-phase drug development, and patients have few therapeutic options available. We selected sodium nitrite for clinical development on the basis of the extensive published literature about its safety profile when administered intravenously and orally, its vasodilating properties, and preliminary reports of acidified nitrite in other patient populations with chronic skin ulcers.

In animals, sodium nitrite has been shown to promote revascularization of ischemic limbs; to protect against ischemic infarction of the heart, liver, and brain; and to have a protective effect against cardiac arrest-mediated heart and brain injury. The nitrite anion acts as a vasodilator *in vivo* by generating nitric oxide in tissues with low oxygen tension and pH, which are conditions that are likely to be present in chronic wounds. The mechanism involves an oxygen- and pH-dependent nitrite reductase activity of hemoproteins or xanthine oxidoreductase.

Experimental models have suggested beneficial effects of nitric oxide in both the early and late phases of wound healing, including increased extracellular matrix production, immune response modulation, and stimulation of keratinocyte cell proliferation, angiogenesis, and bactericidal properties. Nitric oxide mediates essential vascular homoeostasis, including vasodilation, as well as antiplatelet activity, and affects several growth factors involved in endothelial homoeostasis.

We have completed a dose escalation, safety and tolerability phase 1 study in 18 adult patients with sickle cell anemia and leg ulcers. No grade 3-4 adverse events were observed, and there were no serious adverse events or dose-limiting side-effects. Pharmacokinetic analysis showed low systemic absorption of sodium nitrite. Application of topical sodium nitrite was associated with a significant increase in periwound cutaneous blood flow measured by laser speckle contrast imaging, and increased periwound skin temperature by infrared thermography. Most interestingly, there was a dose-dependent decrease in leg ulcer size ($p=0.0012$) and ulcer-associated pain ($p<0.0001$). Ulcers healed completely in three patients who received the highest concentrations of topical sodium nitrite (the 1.8% and 2.0% cream). In our post-hoc analysis of pain, brief pain inventory scores improved in pain severity ($p=0.0048$) and pain interference ($p=0.0013$).

On the basis of these results, we propose a ten-week, placebo-controlled, double-blinded phase II study of 2.0% topical cream containing sodium nitrite vs. placebo in patients with sickle cell and leg ulcers.

The **primary objective** is to determine the safety of prolonged (10 weeks), twice a week application of topical sodium nitrite in patients with sickle cell disease and leg ulcers. We will do this by accomplishing the following specific aims: 1)To determine if topical sodium nitrite accelerates wound healing as defined by >25% improvement over placebo arm; 2) To determine if topical sodium nitrite decreases pain at the wound site>20% over placebo; 3) Secondary aims: a) To evaluate the effect of Hydroxyurea on leg ulcer's healing in combination with topical sodium nitrite or placebo; b) To assess changes in ulcer microbiome after application of sodium nitrite or placebo and how they may relate to healing; c) To evaluate the dermal composition and microvascular structure in the ulcer's biopsy of subjects enrolled on this study.

Significance

This phase II study is the next step in the drug development plan, as we move from a phase I dose-finding study to a more prolonged safety and tolerability study at a fixed dose, comparing to placebo.

The second aim is to develop data to indicate a preliminary signal for efficacy. We can be encouraged in this regard by the signs of an efficacy signal in the uncontrolled phase I study, comparing low dose to higher dose in patients with sickle cell disease and chronic leg ulcers.

The successful completion of this trial would allow us to define the effect size that would be utilized to plan for the next appropriately powered phase III study, a crucial step towards development of an effective therapy for this debilitating complication that affects patients with SCD worldwide.

From a practical standpoint, obtaining these data would be very helpful to attracting industry or venture capital or SBIR funding to ensure further drug development.

Research Strategy

2.1 Background

Chronic ulcerations of the legs are frequently encountered in clinical practice, but little information is available about their prevalence and natural history, and no consensus has been reached on best practice management. Data obtained from population-based studies suggest that about 1% of the general population have leg ulcers at some point in their lives[1,2]. Baker et al, 1991, reported that 57% of all patients with a chronic leg ulcer also had a venous abnormality, with a prevalence of 0.62 per 1,000 in the population. There was an increased prevalence with age and more women were affected than men, although the disparity between men and women was not apparent when adjusted for age-related prevalence. An abnormal refilling time was the only abnormality in 64% of ulcers; additional etiologic factors were diabetes, rheumatoid arthritis, and arterial ischemia. A history of deep venous thrombosis was present in 17% of the patients, but most of the others had predisposing factors, such as bone fractures, pregnancy, general anesthesia, or crush injury. Interestingly, the left leg was more affected than the right, consistent with the increased frequency of deep venous thrombosis of the left side[3].

Over the last several years, the treatment of chronic leg ulcers has developed from a merely symptomatic to a differentiated therapy. Although more than 90% of these ulcers are of vascular origin, many other causes are possible, including hematological, neuropathic, infectious, malignant, and chemical or physical insult.

Leg ulcerations can be divided into three broad and overlapping categories: arterial, venous, and neuropathic. Three main processes are at play: peripheral vascular occlusive disease (PVOD), chronic venous insufficiency (CVI), and vasculitis. The classic treatment of elevation, ambulation, and compression for venous disease remains unchallenged, with great room for improvement. Two groups of vasculitic disorders that share varying degrees of vascular inflammation and necrosis are arteritis (lupus, erythematosus, periarteritis nodosa, dermatomyositis) and blood dyscrasias (sickle cell disease, thalassemia). Leg ulcers associated with vasculitis are due to inadequate tissue oxygenation at the local level, are typically chronic and slow to heal, and commonly recur.

Sickle cell disease (SCD) is an autosomal recessive disorder and the most common genetic disease affecting African-Americans. Approximately 0.15% of African-Americans are homozygous for sickle cell disease, and 8% have sickle cell trait (SCT). As per the CDC webpage, the exact number of people living with SCD in the US is not known. However, it is estimated that:

- SCD affects 90,000 to 100,000 Americans.
- SCD occurs among 1 out of 500 Blacks or African-American births.
- SCD occurs among 1 out of 36,000 Hispanic-American births.
- SCT occurs among 1:12 Blacks or African-Americans.

Hemoglobin S polymerization leads to red cell rigidity, microvascular obstruction, inflammation, and end-organ ischemia-reperfusion injury and infarction. Previous data indicate that up to 50% of sickle cell patients have endothelial dysfunction due to impaired bioavailability of endogenous nitric oxide, due in large part to scavenging of nitric oxide by cell-free plasma hemoglobin. These data suggest that therapies directed at restoring NO bioavailability might prove beneficial. Between 8 and 20% of patients with sickle cell disease develop painful, disfiguring, and indolent leg ulcers, but higher rates of more than 50% have been reported[4,5]. The ulcers usually appear between the ages of 10 and 50 years and have been reported to be more frequent in males than in females in some but not all studies. Leg ulcers are more common in homozygous S disease (HbSS) and less frequent in double heterozygous with HbC and beta

thalassemia (HbSC, HbS/Beta thal). The etiology of leg ulcers is unclear. A review was published recently by Dr. Minniti and colleagues that found that trauma, infection, severe anemia, and warmer temperature predispose to ulcer formation, and healed ulcers often recur[6]. Recently, sickle cell ulcers have been recognized to be associated with other complications, such as pulmonary hypertension, priapism, and renal dysfunction, constituting a subphenotype of SCA in which intravascular hemolysis and endothelial dysfunction play a key role[7]. Indeed, in SCA, high levels of endothelin-1 and impaired bioavailability of nitric oxide exist, and lead to vasoconstriction and proliferative vasculopathy[8-10].

Current treatment options for leg ulcerations, including antibiotics, compression bandages, dressing changes, Unna boot compression dressing, silver and zinc oxide gauze, and maggot therapy, rely on stimulation of granulation formation and biofilm control in the wound. They are uniformly not very effective, and often ulcers persist and/or recur for up to 20 years, some never healing. Pathological changes in the microcirculation associated with ulceration are not addressed. We recently published a survey of the status of sickle cell wound care in North America[11], which depicted a lack of a coordinated approach to the care of these patients. Most recently, we have published the most authoritative clinical approach to ulcers in SCD[12].

The etiology of SCD-related ulcers is not completely understood, and many factors likely contribute to their occurrence, related to both the host and the environment. Evidence is mounting that "chronic microbial colonization," long considered a harmless feature of chronic wounds, may contribute to a chronic inflammatory process that impedes healing. In SCD in particular, recent literature from Kato[13] and others reveals that abnormal heme and iron turnover primes the TLR4-inflammasome pathway in the SCD innate immune system, potentially drastically sensitizing the immune system to activation of minute amounts of lipopolysaccharide, the natural ligand of TLR4. Data from Frenette's group[14] indicates that alterations in intestinal flora affect systemic activation of immune system-endothelial adhesion function, and we hypothesize that SCD wound colonization follows an analogous pathway. This emphasizes the importance of investigating and hypothetically intervening in bacterial colonization of chronic non-healing SCD leg ulcers.

Researchers no longer rely solely on culturing for microbial identification, but instead use sophisticated sequencing technologies to characterize the full diversity of microbial communities throughout the human body and the environment. Of interest is an understanding of the microbial diversity in a healthy individual and in disease. Microbiome research will shift the ways scientists and physicians think about human health and disease, translating the findings into new interventions in clinical practice and public health.

Dr. Minniti has been collaborating for several years with Vence Bonham, an investigator at NHGRI, to evaluate the impact of the microbiome of skin and ulcers on ulcer formation and delayed healing in patients with SCD (**Insights into Microbiome and Environmental Contributions to Sickle Cell Disease and Leg Ulcers Study (INSIGHTS Study)** ClinicalTrial.gov# NCT02156102). Several reports from the 1980s, using culture-based studies, have described an increase in the abundance of specific bacterial microflora[15-17]. Interestingly, *Staphylococcus*, *Pseudomonas,* and *Streptococcus* were also commonly identified in leg ulcer cultures. In these studies, individuals with infections responded well to topical antibiotic treatment. This would suggest that microbes, if not responsible, are associated with the progression of this condition and likely impede the healing process similar to the speculated role of microbes in non-healing diabetic foot ulcers. A recent study characterized the microbiome of diabetic foot ulcers in 52 patients using two approaches, high-throughput sequencing and culturing. The most abundant sequenced genera in the diabetic foot ulcers were *Staphylococcus* (49/52 samples), *Streptococcus* (15/52), and *Lactococcus* (38/52)[18]. Although culturing was only conducted in the HbSS leg ulcer studies from the 1980s, similar bacterial isolates were also recovered in the diabetic microbial foot survey. Gardner et al. suggest that in order to reveal informative relationships between microbiome changes and disease state (i.e. leg ulcers),

microbiome studies must also consider the clinical metadata for each individual patient[18].

Nitrite and its product nitric oxide are conceptually attractive for intervening against microbial inhibitors of wound healing. Nitric oxide mediates essential biological processes, including vasodilatation, antimicrobial activity, and wound healing[19-21]. NO is manufactured on epithelial surfaces, such as the mouth and stomach, and on the skin surface in humans by sequential reduction of nitrate and nitrite[22]. This relies on the synthesis of nitrite by the bacterial reduction of inorganic nitrate present in saliva or sweat. Since antiquity, nitrite has been added as a curing agent to meat, in which likely the myoglobin and hemoglobin present in the meat reduce the nitrite to nitric oxide (which binds to iron in the globins, giving the characteristic pink color), which then exerts a broad antimicrobial effect.

Most chronic wounds are characterized by the presence of a polymicrobial community which develops without causing a clinically detectable host response. This community can thrive for months, but at some time an event occurs that causes this group of organisms to become virulent and cause the typical clinical presentation of an acute wound infection. The event or events that trigger this response is unknown. It could be caused by changes in the environment, the host response or a combination of both. The effects of nitric oxide (NO) in wound healing could be associated with a response of this biofilm community to NO. The effects of NO on certain bacterial species may allow a host response to normalize the acute inflammatory response and resume the subsequent stages of wound healing.

2.2 Nitric Oxide and Vascular Function

NO is a soluble gas with a half-life of seconds, continuously synthesized in endothelial cells from the amino acid L-arginine by the nitric oxide synthase enzyme. In their seminal experiment, Furchgott and Zawadzki found that strips of rabbit aorta with intact endothelium relaxed in response to acetylcholine but constricted in response to the same agonist when the endothelium had been rubbed off[23]. The substance responsible for acetylcholine-stimulated relaxation was initially called endothelium-derived relaxant factor, but was subsequently found to be NO. NO released from the endothelium as a gas or attached to transport molecules activates soluble guanylyl cyclase in smooth muscle after binding to its heme group, resulting in increased cyclic GMP. Cyclic GMP activates GMP-dependent kinases that decrease intracellular calcium concentration in smooth muscle, producing relaxation.

The Gladwin group recently discovered that the nitrite anion acts as a vasodilator *in vivo* by generating nitric oxide (NO) in tissues with lower oxygen tension and pH[24]. The mechanism involves a novel physiological function of human hemoglobin as an oxygen- and pH-dependent nitrite reductase. Therefore, nitrite provides the ideal substrate for NO generation along the physiological oxygen gradient, potentially providing a novel mechanism for hypoxic vasodilation.

2.3 Topical Sodium Nitrite Therapy

The nitrite anion acts as a vasodilator *in vivo* by generating nitric oxide (NO) in tissues with lower oxygen tension and pH[24]. The mechanism involves a novel physiological function of human hemoglobin as an oxygen- and pH-dependent nitrite reductase. Therefore, nitrite provides the ideal substrate for NO generation along the physiological oxygen gradient, potentially providing a novel mechanism for hypoxic vasodilation. We hypothesized that therapeutic application of sodium nitrite should provide selective vasodilation to hypoxemic tissue and could be used to treat diseases associated with ischemic tissue and hemolytic conditions such as sickle cell disease, where free hemoglobin released during hemolysis scavenges NO and disrupts NO-dependent vascular function. Available data indicates that sodium nitrite will not only inhibit the ability of free hemoglobin (by oxidizing it to methemoglobin) to scavenge NO, but will actually generate NO in tissue beds with low oxygen tension[25].

Recent data indicates that nitrite also favorably alters mitochondrial respiration in a manner that closely mimics ischemic preconditioning[26]. This process of providing small ischemic exposures

to tissue confers a marked degree of protection against infarction of the tissue upon more prolonged subsequent tissue ischemia. The effect of nitrite on mitochondrial respiration may provide the mechanism for a significant component of the anti-infarctive properties of nitrite[27].

Thirty-seven adult patients with a clinical diagnosis of *Mycobacterium ulcerans* disease (Buruli ulcer) were randomly assigned to be treated with either active creams or placebo in a study published by Benjamin and his colleagues. The active creams contained sodium nitrite (6%, wt/wt) and citric acid monohydrate (9%, wt/wt), while the placebo creams contained only the aqueous cream base[28]. Creams were applied daily for 6 weeks to group one. After the 6 weeks, both groups received the active creams for 6 more weeks. Patients on sodium nitrite and citric acid showed a significantly greater decrease in ulcer size compared to the placebo group during the first 6 weeks. In the second six weeks, both groups had similar rates of healing. The only side effect was a yellow pigmentation of the skin, which disappeared 3 days after the treatment was stopped.

Another study was performed in patients with Raynaud's Syndrome, using a nitric oxide-generating gel, prepared by mixing two viscous solutions, one with Analar grade sodium nitrite ranging from 1% to 15% (weight/volume) and the other with ascorbic acid at 1% to 15% (weight/volume). The two solutions were mixed on the patient's skin to generate active nitric oxide[29]. This study evaluated skin microcirculation response to local NO formation with infrared photoplethysmography and flux by laser Doppler fluxometry. Twenty patients with severe Raynaud's syndrome and 10 healthy volunteers were studied and both groups experienced a significant increase in microcirculatory volume and flux. Side effects were limited to stinging, if applied to broken skin.

2.4 Preliminary Data

We recently completed a phase 1 study of escalating doses of topical sodium nitrite in 18 subjects with sickle cell disease and chronic leg ulcers.[30] The trial consisted in twice a week applications of increasing concentrations of topical sodium nitrite for 4 weeks total to adults with sickle cell disease.

No SAE's occurred during this trial. The most frequent reported adverse event was a grade 1 decrease in diastolic blood pressure below 50 mmHg, judged as possibly related to study drug. This event occurred 16 times in 4 participants. Of the four participants, two enrolled in Cohort 4, the highest concentration of the drug (2.0% sodium nitrate cream dosage), and the other two enrolled into Cohort 3.a (1.8% sodium nitrate cream dosage). For Cohort 4, the event occurred 5 times in one participant and 3 times in another participant. For Cohort 3.a, the event occurred 6 times in one participant, and 2 times in another participant. The participants had fluctuating diastolic blood pressure measurements that occasionally reached below 50 mmHg even **before study cream application**; therefore, the association with study cream application is unclear. All events resolved spontaneously within an hour (i.e., at the next BP check).

Results: Tolerability was excellent, with short-lived stinging at the site of application reported by three subjects, which did not need any intervention, and resolved spontaneously. Methemoglobin levels did not exceed pre-established safety thresholds (max of 4.1 % in one subject in cohort 3). Pharmacokinetics of plasma nitrite and nitrate indicated minimal systemic absorption of topically applied sodium nitrite (median plasma nitrite AUC: 0.311 (0.169 – 0.659) umol*h/L/umol nitrite dose), with high interpatient variability. There was no evidence of plasma nitrite, nitrate, or methemoglobin accumulation during the 4-week study trial. All but one subject

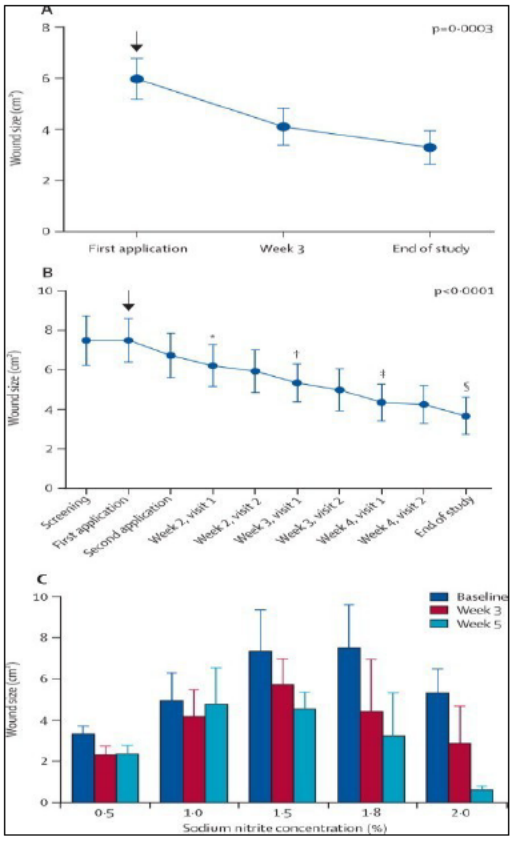

Figure 1: Changes in leg ulcer surface area before and after application of sodium nitrite cream (A) Wound size measured by digital planimetry. (B) Wound sizes measured manually (longest length and widest width). (C) Changes in wound size according to sodium nitrite concentration. Arrows in (A) and (B) represent first application of cream. Plotted points or bars are mean values and error bars are standard error of the mean. *p=0·0058 compared with first application. †p<0·0001. ‡p=0·0001. §p=0·0008. from *Lancet Haematology* **1**, e95-e103. PMC4415859 (Figure 1, PI is first author)

experienced a decrease in leg ulcer surface area, fig 1 (pretreatment 4.65 vs. post-treatment 2.78 cm^2, $p < 0.001$), and improvement in pain scores at the ulcer site (pretreatment VAS score of 4.87 vs. post treatment of 2.91, p=0.024). Changes in pain score were not statistically significant at the ulcers that were not treated with study cream, p=0.96 (50% of subjects had more than one ulcer and only one was treated with study cream). Healing was dependent on sodium nitrite concentration, and it was most marked in cohort 4 (2.0%), the one treated with the highest strength of sodium nitrite cream, with 2 of 3 subjects experiencing complete closure of the ulcer (89% decrease in surface area), then cohort 3a (1.8%), with a 68.5% healing rate and one out of 4 experiencing complete closure, then cohorts 1, 2 and 3 with healing rates of 29, 3.8 and 33%, respectively. See Table 1 below.

Table 1 : Percent of ulcer's surface healed from beginning of therapy		
Cohort/concentration	Week 3	End of study
1/0.5%	29.9%	28.8%
2/1.0%	15.8%	3.8% (one subject progressed)
3/1.5 %	22.5%	32.9%
3a/1.8%	56.0%	68.5% (one of four ulcers closed completely)
4/2.0%	55.1%	88.6% (two of three ulcers closed completely)

Application of topical sodium nitrite was associated with a significant increase in periwound cutaneous blood flow measured by laser speckle contrast imaging and increased periwound skin temperature by infrared thermography (fig 3).

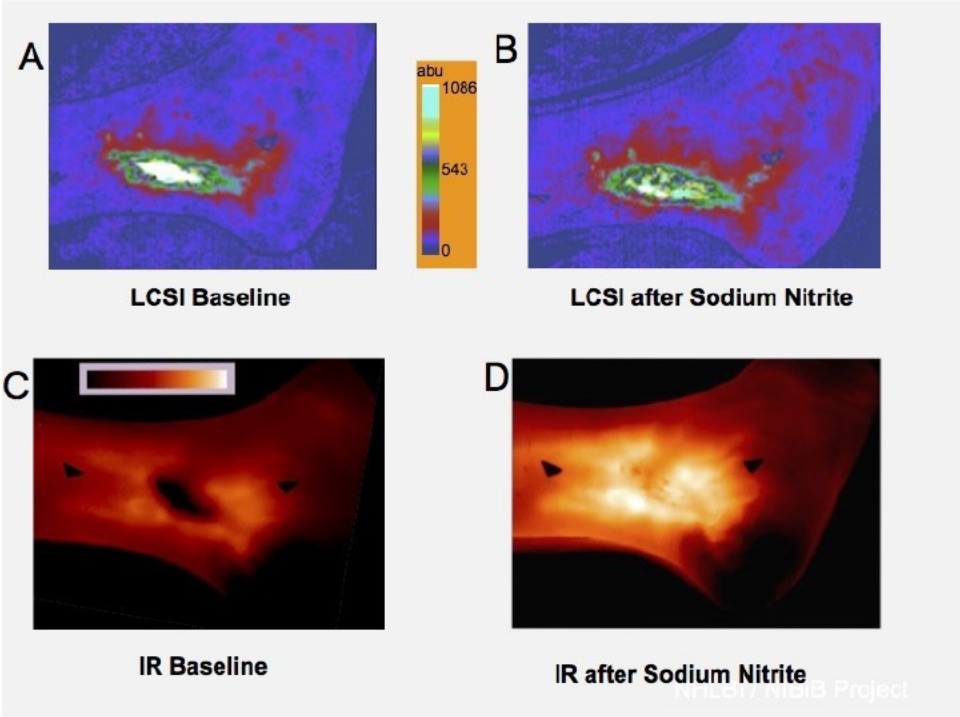

Figure 2 legend: Top panels: Laser speckle analysis of blood flow before (A) and after (B) application of sodium nitrite
Lower panels: Infrared photography of an individual subject before application (C) of topical baseline) and immediately after (D).

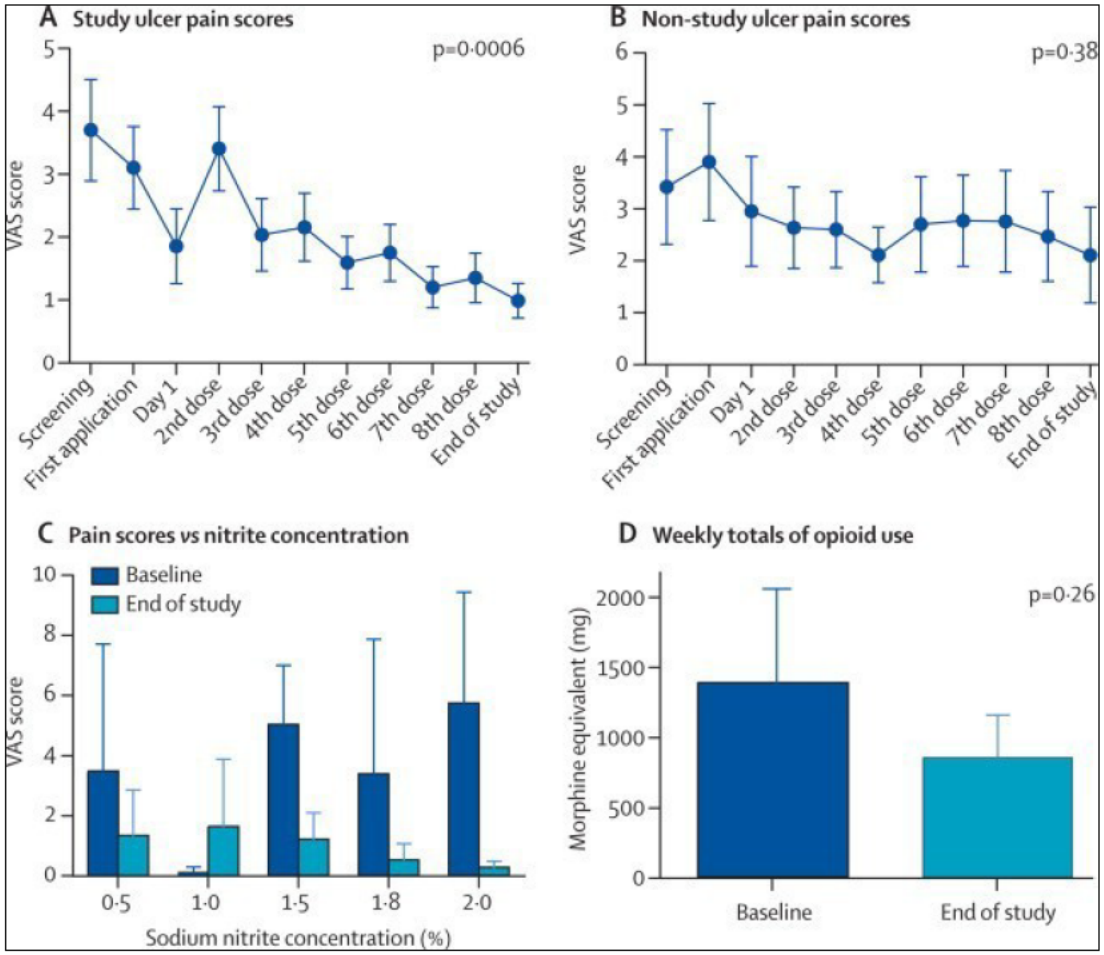

Figure 3: Changes in leg ulcer pain during the topical sodium nitrite treatment period
Patients were asked to score pain from the ulcer treated with topical sodium nitrite and any additional ulcers not treated with topical sodium nitrite. (A) Changes in VAS pain scores for the ulcer treated with topical sodium nitrite at all doses, from screening to the end of the study. (B) Changes in VAS score for additional ulcers not treated with topical sodium nitrite (n=9). (C) Changes in VAS scores for the study ulcer treated with sodium nitrite cream at different concentrations. (D) Mean weekly use of opioid analgesics (total morphine equivalents) by all patients at the beginning of treatment compared with the end of treatment. Error bars are standard deviations.
VAS=visual analogue scale. From *Lancet Haematology* **1**, e95-e103. PMC4415859 (Figure 2, PI is first author)

2.4.1 Microbiome Preliminary Data

In collaboration with Elizabeth Grice, Ph.D. at the University of Pennsylvania and postdoctoral fellow, Keisha Findley, Ph.D., we sequenced 16S rRNA bacterial sequences from the skin of the first 12 (five with active ulcers) study participants using the Illumina MiSeq Technology. Approximately 1.3 million sequences were taxonomically classified using the online molecular ecology tool, mothur, and the RDP classifier and training set (version 9).

The relative abundance of bacteria on the ankles of SCD participants with and without leg ulcers is distinct. Those with leg ulcers have lower bacterial diversity and an abundance of the skin commensal, *Staphylococcus,* while those without leg ulcers display higher bacterial diversity. This finding supports the claim that microbial diversity is higher in a healthy state and is, conversely, lower in a disease state. Additionally, we conducted Principle Coordinate Analysis (PCoA) using thetayc distances comparing bacterial community structure (similarity) in participants with and without leg ulcers. This analysis confirmed that the two communities differ, and we speculate that this variation could potentially explain the differences in the skin microbiome of individuals with SCD who develop leg ulcers over their life course. We are currently processing microbiome samples as they are collected and will complete the sequence analysis when the data is available.

Figure 4: Bacterial relative abundance plots in participants living with sickle cell disease leg ulcers.

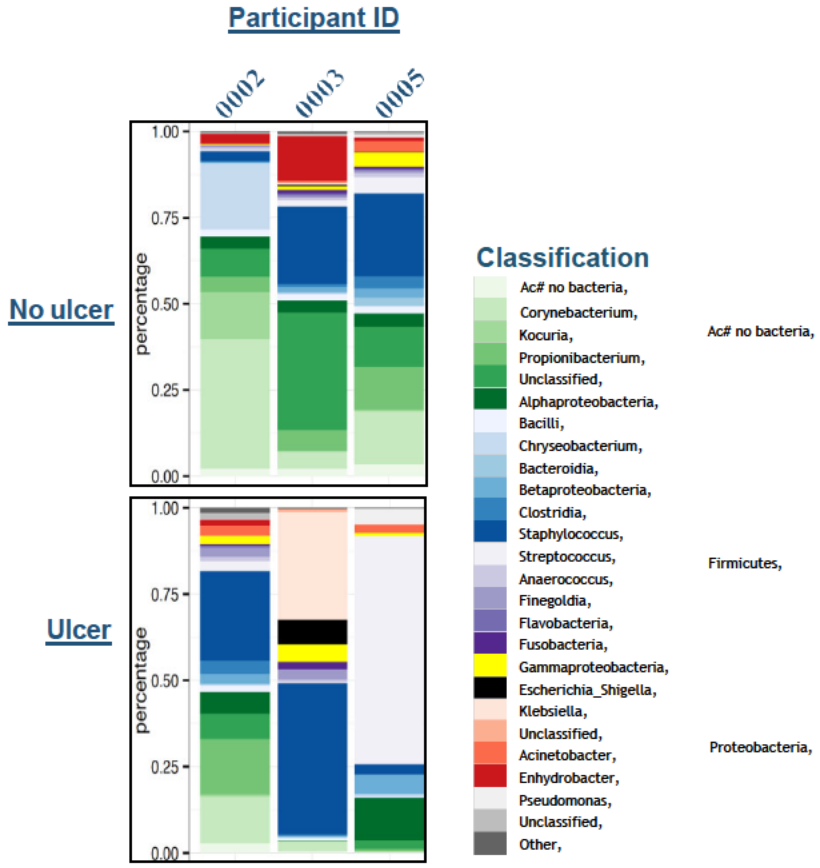

Figure 4 Legend: Approximately 1.3 million 16S rRNA bacterial sequences were taxonomically classified using the online molecular ecology tool, mothur, and the RDP classifier and training set (version 9). Participants #0002, 0003, and 0005 are shown. Swab samples were collected from an ulcer and the contralateral leg without an ulcer, and samples were sequenced.

2.4.2 Potential Toxicity

Prior to our phase 1 trial, most of the data available on the use of topical sodium nitrite in humans involved the use of a mixture of sodium nitrite and either citric acid or ascorbic acid. Acidification of nitrites produces nitrous acid, which rapidly decomposes to form oxides of nitrogen, one of which is nitric oxide.

The 30 patients with *Mycobacterium ulcerans* (Buruli Ulcer) treated with a mixture of sodium nitrite (6% wt/wt) and citric acid monohydrate (9% wt/wt) tolerated the acidified nitrite cream well, and developed a yellow pigmentation at the ulcer site that disappeared when the treatment was discontinued[28]. Another frequent side effect of the application of topical mixtures containing nitric oxide and citric or ascorbic acid is stinging and burning of broken skin[29]. Since we plan to apply it over leg ulcers, it is possible that there will be pain at the site of application. In order to minimize this pain, the NIH pharmacy will be preparing a cream that contains no ascorbic or citric acid.

Moreover, patients will be receiving oral or parenteral analgesics, as appropriate for the level of pain. Most sickle cell patients with leg ulcers already take opioids for their chronic pain, therefore this is not considered a significant potential for toxicity. We hypothesize that the conversion of nitrite to NO will occur along the hypoxic gradient and therefore be more gradual and targeted than the conversion from an added acidic source. The conversion in the latter case is rapid and NO becomes dispersed within minutes.

There were case reports of methemoglobinemia resulting from percutaneous absorption of sodium nitrite in solution. The first case involves a 3 and 1/2 years old boy with first-, second-, and third-degree burns involving an estimated 45 – 55% of the body area[31]. The burns were covered initially with petrolatum dressings, which were subsequently changed on days 8 and 14. On both days when the dressings were changed, the burned areas were debrided and irrigated thoroughly with 1:1000 benzalkonium chloride solution. On day 14, the patient was noted to be cyanotic while the petrolatum pressure dressings were being applied. It was later found that the benzalkonium chloride solution was contaminated with 0.5 g of sodium nitrite. The patient was given methylene blue and the cyanosis was gone within 20 minutes. The concentration of sodium nitrite in the irrigation solution was 125 mg/L.

The second case involved the use of liniment solutions containing sodium nitrite in concentrations of 30g/L (liniment solution A) and 140 g/L (liniment solution B)[32]. The liniment solution A was applied all over the body of a 4-year-old boy a few days prior to his death. The boy became listless and had one episode of vomiting after the application of solution A. The stronger liniment solution B was then applied all over the boy's body a few days later. Immediately after his second treatment, the boy gradually went into shock with signs of severe cyanosis. He was hospitalized immediately and was on intensive care support for 2 hours before being declared death. The concentration of nitrite anion in serum was found to be 1 mg/L (i.e. 21.7 µmol/L).

In our phase 1 study of a non-acidified sodium nitrite cream preparation, we did not observe significant elevation of methemoglobin in any of the 18 subjects enrolled, over the course of one month after bi-weekly therapy.

Another potential side effect of topical sodium nitrite is the absorption from the skin into the systemic circulation, with subsequent decrease in systemic blood pressure. A clinical trial performed at the NIH by Dr. Kato studied the consequences of the administration of intravascular sodium nitrite infusions to 14 subjects with sickle cell disease, assessing changes in regional blood flow[33]. In these subjects, they found evidence that sodium nitrite induces vasodilatation and improves regional blood flow without inducing hypotension, nor other detectable adverse effects. In a dose dependent manner, sodium nitrite infusion rates of 0.4, 4, and 40 µmol/min into the brachial artery augmented mean venous plasma nitrite concentrations ($p<0.0001$) and stimulated forearm blood flow up to 77 ± 11% above baseline ($p<0.0001$), measured by venous occlusion strain gauge plethysmography. Sodium nitrite infusions were well tolerated without hypotension, clinically significant methemoglobinemia, or other untoward events.

During the abovementioned phase 1 trial of topical sodium nitrite, we observed no SAE's. We documented a grade 1 decrease in diastolic blood pressure below 50 mmHg, judged as possibly related to study drug 16 times in 4 participants. Of the four participants, two enrolled in Cohort 4, the highest concentration of the drug (2.0% sodium nitrite cream) and the other two enrolled into Cohort 3.a (1.8% sodium nitrite cream). The participants had fluctuating diastolic blood pressure measurements that occasionally reached below 50 mmHg even **before study cream application**; therefore, the association with study cream application is unclear. All events resolved spontaneously within an hour (i.e., at the next BP check).

Experiments in rats showed that methemoglobin levels peaked at 45 – 50 min after application of 30 g/L sodium nitrite solutions on abraded skin[32]. Percutaneous absorption of a solution differs from the absorption rate from Aquaphor (the vehicle in our current preparation) and it seems to be slower than that from solution. There was no evidence of accumulation either, as predicted by our pharmacokinetic models[34,35].

In the proposed phase 2 trial we plan to answer this toxicity question, since we will be treating patients blindly with a placebo preparation and we will be able to ascertain whether the fluctuations in BP are intrinsic to this patient population, their frequent use of opioids, and/or chronic wide pulse pressure.

3.0 Specific Aims

Hypotheses:

- Topical sodium nitrite cream, at 2% concentration, is safe and well tolerated when applied for an extended period of time (10 weeks) in patients with sickle cell disease and leg ulcers.

- Topical sodium nitrite accelerates healing of skin ulcers and decreases ulcer related pain compared to placebo in patients with sickle cell disease and leg ulcers.

Specific Aims:

- **To determine the safety and tolerability of prolonged (10 weeks), twice a week application of topical sodium nitrite in patients with sickle cell disease and leg ulcers**
- **To determine if topical sodium nitrite accelerates wound healing as defined by >25% improvement over placebo arm**
- **To determine if topical sodium nitrite decreases pain at the wound site >20% over placebo**

Secondary Objectives:

- To evaluate the effect of hydroxyurea on leg ulcer's healing in combination with topical sodium nitrite or placebo
- To assess changes in ulcer microbiome after application of sodium nitrite or placebo and how they may relate to rate of healing
- To evaluate the dermal composition and microvascular structure in the ulcer's biopsy of subjects enrolled on this study.

4.1 Inclusion and Exclusion Criteria

4.2 Eligibility

Subjects must be at least 18 years of age and have provided informed, written consent for participation in this study.

Each subject must meet all of the following inclusion criteria during the screening process in order to participate in the study:

- Subjects must have a diagnosis of sickle cell disease (SS, SC, Sß-thalassemia, SD, SO^{Arab}).
- Have one or more ulcers in one or both legs and/or feet.

- Leg ulcers that will receive treatment must be no larger than 100 cm^2.
- No history of congenital methemoglobinemia.
- Have documented normal G6PD activity.

4.3 Exclusion Criteria

Subjects meeting any of the following criteria during baseline evaluation will be excluded from entry into the study:

- Exposure to therapeutic nitric oxide, L-arginine, nitroprusside, or nitroglycerine within the past 1 week.
- Subjects presenting with clinically-diagnosed bacterial infection (e.g., osteomyelitis, pneumonia, sepsis, or meningitis).
- Subjects who have a pre-existing methemoglobinemia (more than 3.5% on two different occasions).
- Patients who are currently enrolled in any other investigational drug study (this does not include observational or natural history protocols).
- Use of PDE5 inhibitors, such as sildenafil, 4 days prior to screening.
- Pregnant women (urine or serum HCG +) or nursing mothers.
- The following list of drugs and agents may cause methemoglobinemia and should be avoided while on this study:

  Anesthetics (local): Benzocaine, procaine, prilocaine, Anbesol, Orajel
  Antimalarials: chloroquine, primaquine, quinacrine
  Aniline dyes
  Chlorates
  Dapsone
  Diarylsulfonylureas
  Doxorubicin
  Metoclopramide
  Nitric and nitrous oxide
  Nitrobenzenes (shoe and floor polish and in paint solvents)
  Nitroethane (artificial nail remover, propellant, fuel additive)
  Nitrofurantoin (furadantin)
  Pyridium (phenazopyridine)
  Phenacetin
  Phenylhydrazine
  Rasburicase
  Sulfonamides (sulfacetamide, sulfamethoxazole, sulfanilamide, sulfapyridine)

* If a patient does not qualify because he/she has an infection at the time of enrollment or at run-in, he/she can initiate the protocol two weeks after the infection has been cleared.

5.1 Study Design and Methods

5.2 Overview

This will be randomized, placebo controlled, double blinded study

We plan to enroll fifty subjects with homozygous sickle cell disease (HbSS), sickle-thalassemia (Hb S-ß thalassemia), HbSC, HbSD, or $HbSO^{Arab}$, in order to have 40 subjects complete at least 8 weeks of treatment, after an expected 20% dropout.

Subjects will receive 2% topical sodium nitrite cream twice a week for 10 weeks or placebo with vehicle only.

Subjects will have 0.1 cm of 2% sodium nitrite cream or placebo cream applied per 1 cm^2 of the total ulcerated area. If a subject has more than one ulcer, all of them will be treated with either "study cream" or placebo. Areas will be rounded to the nearest 1 cm^2. If a subject has more than one ulcer and the total sum of all of them is above 100 cm^2, then one of the ulcers can be enrolled

in the study, as long as it does not exceed the 100 cm^2 size. The other wounds will continue standard of care only. The total amount of sodium nitrite administered to each subject will vary depending upon the size of the ulcer but the treated total surface area will not exceed 100 cm^2 (i.e., subjects will receive a maximum of 10 cm of sodium nitrite cream).

Administration of study cream or placebo will be double blinded. The pharmacist will not be blinded, so he/she will be aware of who is receiving the investigational drug for safety purposes.

Randomization:
Randomization will be stratified by use of hydroxyurea to minimize its potential confounding effect on the study outcomes; we expect that 50% of participants use hydroxyurea. In addition, within each stratum (use or non-use), random treatment assignment will be balanced, i.e., 1:1 allocation, within blocks. This type of stratified block-randomized allocations will be generated by the study statistician applying a computer algorithm. The randomized allocation will occur once a subject is determined to be eligible for participation after the screening visit and the run in phase, but prior to the first cream application.

Un-blinding Procedures:
The medical monitor may request unblinding to attribute toxicities if a serious adverse event occurs to determine attribution, or if a dose limiting toxicity occurs to determine if placebo or active treatment was used.

A run in period of at least 2 weeks will be conducted, during which patients will receive/optimize wound care at the Center that will enroll the subjects, following standard of care, routinely available therapies. Patients that experience a reduction of their wound size by >25% during this time will be excluded from the trial, as this suggests an effect secondary to improved wound care only. These patients will remain on protocol and their wound will be measured monthly for up to 6 months or until closure, whichever comes first. If a subject develops an acute vaso-occlusive episode and is hospitalized during the run in period, he/she will have the run in period extended in order to allow the subject to be safely discharged from the hospital and return to outpatient care.

Close monitoring of VSS, especially BP, will be done, to assess changes in patients receiving study cream, compared to placebo.

Measurements of all leg ulcers and photographs will be obtained at the following points:
At Baseline
After the 2 weeks run in period, to determine eligibility for study cream application
At mid-point: after 10 applications of study cream, to determine the need for drug modification
At end of study: after the last application of the study cream

An accurate assessment of pain will be obtained each time that size measurements are obtained.

Quality of life questionnaire (ASCQ-Me) will be given before initial application and at the end of protocol or when the ulcer(s) is (are) healed, whatever comes first.

We will monitor metHb level as a safety measure, despite the fact that the phase 1 study did not show significant elevations of metHb. **Teratogenicity** is not a consideration, since the phase 1 study demonstrated minimal systemic absorption of nitrite and nitrate, therefore the risk for the gonads and potential offspring is limited to nonexistent.

Among the entry studies to be obtained, a **3-4 mm skin optional biopsy** may be performed at the edge of the ulcerated area. If the patient has multiple ulcers, the surgeon/wound care expert will decide which one is the safest to biopsy. This will be done

in order to assess cutaneous vascularization (i.e., angiogenesis), inflammation (tissue Il-6, TGF-□, etc.), and markers of neurogenic inflammation[36].

At the end of 10 weeks of treatment, subjects that still have an open ulcer will be offered continuation of care at Montefiore or University of Pittsburg or referred back to the community.

Subjects will be followed off treatment but on study with monthly visits for an additional six months to evaluate the long term effect of topical sodium nitrite on the host and the ulcer (how many ulcers re-open or close). These visits will consist of measurement of all wounds by light photography and a detailed history of intervening ulcer treatments. If patients cannot return to the hospital or have moved away, we will call them and obtain the information on the phone and ask for a photograph. The photo can be obtained by the patient him or herself. Thereafter, they will be followed up with phone calls once a year for a total of 2 years, to establish whether this therapy is changing the natural history of ulcers in this population.

Rationale for the selection of subjects' number and end points
Sample size: We plan to randomize a total of 50 subjects to sample size of 20 completers per arm (40 subjects total) after an anticipated 20% dropout rate is taken into account. This sample size is determined based on feasibility of recruitment within the budget constraint and time frame of the study; we anticipate enrolling 1~2 eligible subjects per month. Although the focus of analysis will be more on estimation of treatment effect sizes and their 95% confidence intervals, the anticipated sample size is adequate to detect 88% vs. 48% response rate, as defined by ≥25% reduction of ulcer size from baseline, between treatment and control groups, with >80% at a two-sided significance level of 0.05 (see table 1). We note that in the Phase 1 study we observed an 88% response rate. Therefore, our study will be adequately powered if response rate in the placebo group is smaller than 48%.

Rationale for end point of decrease in ulcer size: A decrease of 25% from baseline was used as a significant end point in Perrine's study of arginine butyrate in SCD leg ulcers[37]. The FDA's own guidelines for research in cutaneous ulcers and burns specify: "*Measurement of **partial wound healing in early phase clinical trials,** if prospectively defined, may indicate relevant biological activity and help **guide subsequent trial design***". Therefore, since this is a phase II trial, I think it is prudent to be conservative and reserve the complete closure end point for the phase III trial. Recently Dr. Kirsner and other dermatologists have challenged the idea of complete closure as the only end point for skin ulceration as being counterproductive for the development of new therapies, and significantly different from standards used in cancer trials, where a 10-20% response rate is often sufficient to claim success of a drug or device[38].

Pain threshold: A decrease of 20% was selected based on the accepted change of pain of **minimum 2 point** decrease on the VAS scale being considered as significant in clinical end point in research settings[39]. The VAS scale ranges from 0-10, so 20% change is 2 points. Moreover, the finding of improvement in pain at the treated ulcer site was very interesting and needs further investigation.

In the phase 1 study[40] we conducted, we observed an increase in blood flow in the skin of individuals with chronic ulceration compared to an area that was not ulcerated. The lack of controls without ulcers prevented us from concluding that the high blood flow had a causative effect on the ulcer formation and delayed healing. Furthermore, it was not clear whether the high blood flow observed at baseline, i.e., before cream application, was different than what we would expect in patients with SCD.

Secondary end points were chosen in order to further our knowledge of the etiology of poor healing in SCD ulcers, based on the results from the prior study.

Rationale for the study of changes in microbiome. One of the most studied effects of NO is its bactericidal and general antimicrobial activity. NO exhibits nitrosative and oxidative action alone and after reacting with oxygen, such as peroxy-nitrite, RSNOs, and nitrogen dioxide[41]. These species affect microbial proteins, DNA, and metabolic enzymes, disrupting cellular walls and functions. NO acts on both planktonic bacteria and biofilm communities[42]. It is likely that a significant portion of the wound healing properties of NO are mediated through its bactericidal properties, through a reduction in biofilm and inflammation. We propose to examine changes in the microbiome.

Rationale for the sodium nitrite dose: The completed phase I, dose-escalating study identified the 2% concentration as the most effective for wound healing. Two of the three subjects in this cohort experienced complete closure, for a healing rate of 86%, higher than any of the other cream concentrations. The maximum sodium nitrite dose that will be administered to patients at this dose level is 55 mg per dose. This amount of sodium nitrite is 18% of the total sodium nitrite dose that is approved by the FDA for use in the emergency treatment of cyanide poisoning (i.e. 300 mg), and is predicted to produce a peak whole blood sodium nitrite concentration of approximately 29 µmol/L if given as an intravenous bolus or 5 µmol/L if infused over 15 minutes. These levels of systemic nitrite should be safe, as similar nitrite concentrations were observed and well tolerated in one of our healthy volunteer studies (05-H-0088). A median (range) nitrite peak concentration of 20 µmol/L and a steady state level of 8 (4 – 12) µmol/L were attained during the phase I study, significantly lower than what was previously reported to be safe. This indicates that sodium nitrite is minimally absorbed when applied topically, even on abraded skin. Methemoglobin reached 4.1% at its highest level in one subject, while did not change from baseline in any of the other 17 subjects. Mean arterial blood pressure did not change, while diastolic blood pressure decreased below a predetermined threshold of 50 mmHg in 2/3 subjects at this dose level. There was no correlation between nitrite and nitrate levels and changes in BP.

5.3 Recruitment

Potential subjects will be identified from the more than 800 patients in the Sickle Cell database at Montefiore Medical Center and the ~300 at the University of Pittsburgh. Referrals will be solicited from the New York Metropolitan area and Pennsylvania. We anticipate enrolling 35 subjects at Montefiore and 5 in Pittsburg. Dr. Minniti has initiated a multidisciplinary SCD/wound care clinic at Montefiore, in collaboration with Dr. Giacomo Vinces, an associate investigator in this study and a wound expert, which sees about 4 patients per week and draws referrals from many of New York Hospitals. Entry into the study will begin when the subject meets all inclusion criteria and signs an informed consent document to confirm their willingness to participate.

Screening: The subjects will be initially evaluated during a routine clinical visit to determine eligibility, obtain informed consent, have a detailed medical history and physical examination, and have clinical labs that include a hemoglobin electrophoresis for genotype confirmation, unless one is available in the chart within the previous 3 months. Potential subjects will have a detailed assessment of all of their wounds. Methemoglobin level will be drawn to exclude congenital methemoglobinemia. As methemoglobin levels vary with the state of oxygenation and the severity of the anemia and hemolysis, patients who have at least two documented metHb levels below the 3.5% threshold will continue the study even if the meth Hb levels rise above 3.5% during the weeks leading to the first application, as long as they remain below 4.5%. A pregnancy test will be performed for women in reproductive age.

The subject that is deemed eligible will then be scheduled for routine wound care, as determined by the WONC and the PI, of two weeks' time, called the "Run In Period". At the end of the two weeks, the ulcer areas will be measured. Patients whose ulcer total surface area decreases by >25% at the end of the run in period will be excluded from the therapeutic portion

of the trial.

Standard of care for wounds includes the following:

- Cleanse leg with skin cleanser
- Cleanse ulcer with normal saline & gauze removing any debris from woundbed.
- Apply petrolatum-based ointment around the edge of the wound
- Cover ulcer with non-adhering foam pad if the ulcer size is $< 2cm^2$
- Cover ulcer with Calcium Alginate dressing if the ulcer size is $> 2cm^2$
- Apply 2 layer compression wrap to leg (depending on the condition of the ulcer)
- Use of topical or systemic antimicrobials

All participants will be requested to complete the Brief Pain Inventory (BPI) prior to initiation of the therapeutic portion of the trial.

Baseline visit: The following tests will be performed:
1. Baseline labs, which include CBC, reticulocyte count, CRP, ESR, LDH, hepatic panel, basic metabolic panel, ferritin, Pro-B Natriuretic Peptide (BNP), urinalysis, urine microalbumin, uric acid, urine pregnancy test (female at reproductive age)
2. Research labs: Pax gene, and stored 5-10 cc blood samples
3. Saliva for DNA analysis
4. The surface area of ulcer(s) will be measured and photographed with defined lighting, distance, exposure and camera type
5. A pain assessment tool (VAS assessment)
6. Brief Pain Inventory (BPI), ASCQ-ME, PROMIS surveys, Beck Depression Inventory

Screening visit and baseline visits can be combined in one appointment.

After at least two weeks of "run in period", where the patient is treated with standard of care therapies only, the total ulcer surface will be measured and, if there isn't a reduction of >25%, the patient will be randomized to receive sodium nitrite cream or placebo.

A biopsy of the ulcer will be obtained in patients that consent during the run in period.

Week 1/Visit 3: First application of "study cream". After cleaning the wound, the wound will be measured by light photography and manually by wound nurse, a microbiome sample will be obtained, and the "study cream" will be applied to all the ulcerated surfaces. Vital signs will be obtained before application to establish baseline MAP and post-application at hours 1, 2, 3, 4, 5. If no significant decrease in MAP pressure from baseline is detected (clinically significant hypotension, or symptomatic dizziness, tachycardia, or hypoxia as per the judgment of the PI), the patient will be discharged and instructed to return for a second application two to three days later. Methemoglobin will be obtained one hour ± 30 min after cream application.

Week 2-10/ Visits 4-22*:* The subjects will be seen twice a week for cream application, wound care and monitoring of side effects. The wound will be measured once a week manually by the wound care nurse and measured by photography after 10±2 applications. Blood pressure and vital signs will be measured upon arrival to outpatient unit and 30 minutes±10 minutes after the application of the study cream, unless clinically indicated. If the blood pressure is stable after cream application for all visit 4, 5, 6, post cream application blood pressure monitoring wound not be needed for future study visits. The wound will be cleaned and study cream will be applied.

Routine bloods will be drawn as clinically indicated. A research methemoglobin level will be obtained every other week.

A repeat pregnancy test will be performed at week 4 in women of reproductive age. Pain assessment: a VAS scale will be obtained with each application of the study cream. If there is more than one ulcer, individual VAS scale per ulcer will be obtained.

After the first three subjects have completed the first application of the study drug, if there are no significant side effects, especially significant changes in BP, patients will be offered the option of applying the second weekly dose of the cream at home, by themselves or by a home health nurse. We will dispense the exact amount of cream to be applied each week.
This will reduce the number of visits to the wound care / hospital location to once a week.

End of study: Subjects will return the week after the last application to have the study cream removed and for end of study procedures. Blood pressure and vital signs will be measured with a pulse oximeter. The surface area of all ulcerated surfaces will be measured and photographed with defined lighting, distance, exposure, and camera. The wound doctor or nurse will remove the "study cream" and obtain a repeat microbiome sample. A VAS scale will be obtained to assess pain. If there is more than one ulcer, individual VAS scales will be obtained per ulcer. ASCQ-ME, PROMIS, BDI, and BPI surveys will be distributed to the patient. Study labs will be obtained (see Appendix 1). Patients who discontinue the study will still receive end of study procedures.

5.4 Dose Adjustment

Sodium nitrite dose will be modified according to this scheme:

a) If a subject's ulcer surface decreases by more than 50% of the original area, as measured after 10 applications, a 50% reduction in the dose of sodium nitrite will take effect for the remaining applications.
b) If a subject's ulcer(s) increase by 50% of the original area, as measured after 10 applications, a 50% increase in the dose of sodium nitrite will take effect for the remaining applications.

Dose-limiting toxicities are defined as:

- Severe stinging pain or burning sensation, requiring a 50% increase in opioid use.
- Peripheral edema causing significant discomfort by patient subjective report.
- Clinically significant hypotension or symptomatic dizziness, tachycardia, or hypoxia as per the judgment of the PI
- Methemoglobinemia >7%

In the event that a subject experiences dose limiting toxicity, he/she will discontinue participation in the protocol.

5.5 Preparation of Sodium Nitrite Cream and Placebo

Sodium nitrite cream for use in humans will be provided by Pine pharmaceuticals and dispensed by the local hospital pharmacy. A complete description of the sodium nitrite cream preparation and properties is in Section 10.

5.6 Quality of Life and VAS Scale

A quality of life assessment using ASCQ-me will be completed by the subject before initial cream application and at the end of study. The VAS scale will be administered at each patient encounter.

5.7 Tissue sampling - Skin Biopsy

A 3-4 mm punch biopsy of the ulcer edge will be performed with local anesthesia at the edge of one of the ulcers in patients with ulcers.

Biopsy material will be divided in two: a portion will be frozen, or immediately homogenized for further study; another portion will be fixed in 10% formalin and analyzed as follows. The skin biopsy will be fixed in 10% neutral buffered formalin and routinely processed and sectioned for histological examination by our collaborator Dr. Yanhua Wang, a pathologist at Montefiore Medical Center. Histological examination will focus on the assessment of dermal blood vessels, dermal stromal cells (especially the fibroblasts), and inflammatory infiltrate.
Immunohistochemistry will be performed to further characterize the skin biopsy: (1) for the assessment of vascular density, immunohistochemistry staining using antibodies to CD31 (endothelial cell marker), CD34 (endothelial cell marker), and SMA (vascular smooth muscle cell marker), (2) plasma cell infiltration by CD138 (plasma cell marker), and (3) the number of activated fibroblasts by Yes-associated protein (YAP) and phosphorylated YAP (pYAP). The number of vessels, CD138+ plasma cells, and YAP+ fibroblasts will be counted for 10 high power fields. Special stains for microorganisms will also be performed on the tissue sections to exclude the possibility of infection: PAS-D (fungal stain), GMS (fungal stain), AFB-FITE (acid fast bacteria), and B&H (bacteria).

Immunofluorescent staining, collagen formation, and microvessel density.

Five-micron-thick sections of formalin fixed scars will be stained with hematoxylin and eosin (H&E) and evaluated for the following parameters: epidermal and dermal regeneration, granulation tissue formation, presence or absence of edema, congestion, hemorrhage, thrombosis, and intravascular or intervascular fibrin formation. Thickness of granulation tissue and epidermal regeneration will be scored as previously described[43]. Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angiogenesis[43].

6.1 Clinical and Laboratory Methods

Routine laboratory studies will be obtained in clinic prior to the initiation of therapy and during follow up visits as deemed necessary by the clinical team. They may include CBC with differential, reticulocyte count, basic metabolic panel, uric acid, hepatic panel, LDH, pro-BNP, hemoglobin electrophoresis, microalbuminuria, urinalysis, serum ferritin, erythropoietin, and G6PD status.

Methemoglobin analysis will be performed by the clinical lab.

Three 5-10 cc serum, EDTA, and sodium heparin samples will be stored for future testing. These tubes will be spun at 3000 RPM, and plasma, serum, RBC will be aliquoted into freezer vials. The freezer tubes used are Sarstedt No./REF 72.694.006 (clear) for the plasma, and Sarstedt No./REF 72.694.034 (brown) for the RBC. They are 2mL micro tubes. The tubes will be barcoded and placed in boxes in the -80 freezer.
A Pax gene and a saliva sample will be collected at baseline.

All samples will be sent to Einstein College of Medicine bio-repository facility.

6.2 Specimen Storage, Tracking and Disposition

INTENDED USE OF SAMPLES/SPECIMENS/DATA
Samples and data collected under this protocol may be used for future studies of biomarkers (for example, soluble cell adhesion molecule (CAM), nitric oxide consumption, amino acid levels, cytokines, chemokines, endothelin, nitrate/nitrite levels, and other biochemical markers that may indicate vascular dysfunction, cell proliferation or wound healing.). Stored plasma will be reviewed for applicable future biochemical marker assays that become available in the future.

The coded results of clinical and research tests and samples obtained during participation in this study may be combined with other related research studies performed at Einstein College of Medicine. This will help to increase the amount we can learn from the information obtained and reduce the number of duplicate tests that are performed for research. Coded samples may also be sent to our collaborators for analysis after a MTA or other appropriate documentation is in place.

Sample, Specimen, and Data Storage
Access to research samples will be limited. The samples will be placed in the freezer. Samples and data will be stored using codes assigned by the investigators or their designee(s). Data will be kept in password-protected computers. Only investigators or their designee(s) will have access to the samples and data.

All samples stored will be barcoded for a complete inventory. Only investigators (or their designee(s)) will have access to the samples and data.

Sample, Specimen, and Data Tracking
Research samples acquired from all time points will be labeled and tracked utilizing the BSI II Barcoding system. All samples will be stored in a -80 freezer, off site, in a secured repository. They can only be accessed by approved users.

Samples, Specimens, and Data at Completion of Protocol
In the future, other investigators may wish to study these samples and/or data. In that case, IRB approval must be sought prior to any sharing of samples. Any clinical information shared about the sample with or without patient identifiers would similarly require prior IRB approval.

7.1 Monitoring of Subjects and Criteria for Withdrawalof Subjects

Monitoring: The safety of topical sodium nitrite will be closely monitored. Vital signs will be obtained before application and post-application at hours 1, 2, 3, 4, 5. If no decrease in systolic blood pressure is detected (clinically significant hypotension: see Section 7.1), patients will be discharged home.

The primary expected toxicity is low grade, asymptomatic methemoglobinemia. Therefore, a blood sample for methemoglobin safety measurement will be obtained after the first application, then weekly.

7.2 Stopping Rules
The study medication will be stopped if the subject experiences healing of the ulcer.

Subjects will be removed from therapy should one or more of the following occur:

- If any methemoglobin reading is >7.5%, the research study cream will be discontinued.
- Investigator or patient considers discontinuation of the study to be in the patient's best interest.
- Clinically significant hypotension (decrease in the systolic BP >20mmHg from the baseline systolic BP and of 10mmHg from the baseline diastolic BP application, and symptomatic dizziness, tachycardia, or hypoxia as per the judgment of the PI).
    - A dose will be skipped if the subject has a systolic blood pressure of ≤80mmHg and/or a diastolic blood pressure of ≤50mmHg, once the blood pressure has been checked with a repeat blood pressure check. If three doses are skipped in a row for this reason, the subject will be removed from therapy.
- Sustained pulse oxygen saturation below 85% for >15 minutes while on supplemental oxygen (up to 4L by NC).

- Increased ulcer pain requiring increased opioid use by 50%.
- Development of osteomyelitis or other systemic infection while on therapy.
- Lack of compliance, as measured by missing more than two sequential cream applications and/or at the PI's discretion.

Rules for Stopping the Study:

- Occurrence of severe toxicities:
  - Three subjects requiring sodium nitrite cream discontinuation
  - Death while on protocol, if it is attributable to the sodium nitrite cream

8. Analysis of the Study

We will first review and summarize data using descriptive summaries and graphical analyses to ensure recorded values are within appropriate ranges, in order to check for outliers and abnormal values. We will explore the success of randomization by comparing groups on potential confounding variables, and will include variables that are not equally distributed between groups in multivariable models. For primary analyses, we will adhere to the intent-to-treat analysis principle that assigns groups as randomized as opposed to actually treated. For sensitivity analyses, we will conduct as-treated analysis and use incomplete data as well after imputation of missing data due to early dropouts; imputation methods such as last-observation-carried-forward or MCMC will be conducted and results from thereof will be compared.

8.1 Primary Endpoints and Analysis

The primary endpoint of this phase II study will be the assessment of tolerability and occurrence of adverse effects of topical sodium nitrite compared to placebo.

8.1.1 Frequency of each adverse event will also be tabulated. To compare the safety of prolonged (10 weeks) treatments between the experimental/treatment and usual care/placebo groups, we will apply a non-parametric Mann-Whitney test on the number of adverse events.

8.1.2 Effect sizes of the treatment on the ulcer size reduction will be determined by differences in response rates or odds-ratios, and the 95% CI's will accordingly be estimated. To test if topical sodium nitrite accelerates wound healing, as defined by >25% improvement over placebo arm, we will apply a Chi-square test. Logistic regression models will be applied if inclusion of confounding variables deems necessary. We will also test if declines of weekly-measured absolute ulcer size on a continuous scale over the study period will be different between the two groups by applying mixed effects linear models for analysis of repeated-measure outcomes.

8.1.3 Effect sizes of the treatment on the pain reduction will be determined by differences in response rates or odds-ratios, and the 95% CI's will accordingly be estimated. To test if topical sodium nitrite decreases pain at the wound site >20% over placebo, we will apply a Chi-square test. Logistic regression models will be applied if inclusion of confounding variables deems necessary. We will also test if declines of weekly-measured absolute pain VAS scores on a continuous scale over the study period will be different between the two groups by applying mixed effects linear models for analysis of repeated-measure outcomes.

8.2 Secondary Endpoints and Analysis

8.2.1 Hydroxyurea effect: We will compare the study outcomes between subjects with use and non-use of hydroxyurea using Chi-square, Mann-Whitney and mixed-effect linear models depending on the outcome scales and the format of the data (cross-sectional vs. longitudinal). In addition, we will also assess if the effects of hydroxyurea on the outcomes will be different between treatment arms by modeling interaction effects in pertinent statistical models.

8.2.2 Microbiome analysis: This analysis will be performed by commercial labs. Our hypothesis suggests that specific microbes are predominant in leg ulcers of SCD patients. To identify these microbes, we will build on our previous characterization of the microbiome of HbSS leg ulcers compared to non-ulcers during the INSIGHTS study. The presence of such isolates may impede the healing process, worsening the condition and increasing healing time. To address this hypothesis, we will process and prepare samples for DNA extraction and sequencing from all study participants. Samples will then be sent off for sequencing using the MiSeq platform. We will first check for chimeras and, if any are identified, remove them from the sequence file. Then we will align and taxonomically classify our sequences using online microbial ecology tools like Qiime (or mothur) and a 16S (bacterial) and/or ITS (fungal) reference database to characterize the microbial communities present on the foot of HbSS patients with and without leg ulcers[44,45]. Using the same bioinformatics tools, we will assign the amplicon sequences to operational taxonomic units using a percent similarity cutoff to further bin/group sequences. This step is important for the analysis employing diversity-based metrics to highlight community richness (Chao1) and microbial community structure (Theta Index) and membership (Jaccard Index). We will also infer phylogenetic relationships using the same tools (UniFrac) mentioned above. To visualize these relationships among microbial communities, we may also wish to conduct principal coordinates analyses (PCoA) to show similarities or differences between two environments (i.e. disease vs. healthy states). If we decide to sequence the fungal community present in the leg ulcers, we will follow the procedure described above with minor modifications, using CD-HIT for sequence clustering[46].

We will use the statistical software, R (http://www.r-project.org/), to represent visually the taxonomic classification of the sickle cell leg ulcer and diabetic foot ulcer data. UniFrac will require phylogenetic information generated in earlier steps of the analysis to compare both leg ulcer microbial community datasets[46]. We will also employ Spearman's correlation to identify significant abundance relationships accounting for variation in each sample.

8.2.3 Microvascular structure analysis

Analysis of skin biopsies will be mostly descriptive as an exploratory analysis aimed at understanding the pathophysiology of ulceration in this population and at building a tissue repository analysis.

9. Human Subjects Protection

9.1 Inclusion of Women and Minorities

Because sickle cell disease primarily affects African-Americans and Latinos or Hispanics, we expect that most, if not all, the patients will be from these ethnic groups. However, no one will be excluded based on ethnicity. Because sickle cell disease is autosomally inherited, we expect to recruit an equal number of males and females. Criteria for exclusion or withdrawal from the study are based on the presence of other disease processes that may interfere with the interpretation of our results or situations that may be harmful to the healthy subjects.

9.2 Inclusion of Children

As this is a pilot study of an un-licensed medication that has not yet been tested in adults, children under the age of 18 will be excluded from this study.

9.3 Evaluation of Benefits and Risks/Discomforts

The level of risk to the adult research participants is greater than minimal risk with a prospect of direct benefit. This assessment is in accordance with 45 CFR 46.102 (h)(i).

Risks:

A literature search was performed using PubMed back to 1965 with the following search words: nitrite, sodium nitrite, toxicity, methemoglobinemia, humans. Literature searches were also performed by Caterina Minniti, MD, principal investigator updating through 2015, using the following search terms: nitrite, sodium nitrite, toxicity, poisoning, adverse effects, human.

Sodium Nitrite: Sodium nitrite has been used commercially as a food preservative, an anti-corrosive agent, a coloring agent, and an anti-angina agent, with additional uses in laxatives, burn ointments, and liniments. Amyl nitrite and isobutyl alcohol have been inhaled or ingested as euphoric stimulants. Nitrite has also been found as a contaminant in well water. The literature searches generated case reports of nausea, vomiting, abdominal pain, dizziness, headache, flushing, cyanosis, tachypnea, dyspnea, hypotension, and death attributed to excess nitrite (high dose) exposure from these sources as a consequence of methemoglobinemia due to oxidation of heme-iron in oxyhemoglobin. If levels of methemoglobin rise above approximately 30% of total hemoglobin, a subject may appear cyanotic and experience dyspnea, due to the reduced oxygen carrying capacity of hemoglobin (methemoglobin cannot bind oxygen). Levels above 50% can cause seizures, hypotension, coma, and death.

Nitrite in the form of sodium nitrite for parenteral administration is currently available and accepted by the FDA for use in the emergency treatment of cyanide poisoning. Nitrite administration at this dosage causes methemoglobinemia, a desirable effect in the treatment of cyanide poisoning, as methemoglobin binds to cyanide, thus protecting cellular mitochondria. Sodium nitrite (4 mg/kg, approximately 300 mg) was infused intravenously over 10 minutes in eight healthy volunteer subjects[48]. Methemoglobin levels in venous blood samples rose from 0.02% at baseline to 5.7% of total hemoglobin following infusion of sodium nitrite, with no subject experiencing adverse events.

Sodium nitrite cream in association with citric acid has been used as a topical agent for the therapy of chronic leg ulcers in non-SCD patients. In these human studies, no systemic adverse events were noted. This reflects the prompt production of NO when nitrite is mixed with citric acid. The phase I study of topical sodium nitrite cream without an acid that we just completed did not cause significant elevation of methemoglobin. An oral formulation of sodium nitrite did not cause elevation on metHb, but caused modest decreases in blood pressure.[49]

Blood Drawing: Some patients may experience localized bruising at the site where blood is drawn. Routine blood drawing protocol will be followed to minimize this risk.

Skin Biopsy: There is a small risk of bleeding and infection at the site of biopsy. There is a small but real risk of increase in localized pain for a short period of time. Lastly, we do not know if patients that have a tendency to develop leg ulcers, may develop a chronic ulcer after a biopsy.

ASCQ-me, PROMIS, Beck Depression Inventory: There are no risks or discomforts from taking part in the ASCQ-me, PROMIS, and Beck Depression Inventory Surveys other than possibly getting tired from answering the questions.

Brief Pain Inventory: There are no risks or discomforts associated to completing the Brief Pain Inventory other than possibly "test fatigue" -- getting tired of answering questions.

9.4 Protocol Consent Processes and Documents

Each subject will receive an oral and written explanation of the purposes, procedures, and risks of this study. The original signed consent form will be placed in the subject's medical record and a copy will be given to the subject. A member of the protocol team will be available to answer questions about the study to be performed.

All medical information collected from study participants will be kept in a locked file at the Montefiore Medical Center or The Heart, Lung, Blood, and Vascular Medicine Institute of University of Pittsburgh. Unique patient identifiers will be used to label all data. Strict standards of confidentiality will be upheld at all times.

The following is a list of Associate Investigators who are authorized to obtain informed consent:

Caterina Minniti, MD, Laura Decastro, MD, Giacomo Vinces, DO, Anna Flattau, MD.research nurse Elly Jinok Kim, NP

10. Pharmaceutical Information

Supply: Sodium nitrite 2% cream and placebo will be compounded and provided by Pine Pharmaceuticals, New York.

Product description: The 2% cream consist of 0.02 g of sodium nitrite, respectively, in 1 g of aquaphor ointment and will be dispensed in 30 g tubes. The amount of sodium nitrite contained in 1 cm 2% cream is 5.50 mg.

Storage: Store at 2^0C – 8^0C in the refrigerator.

Stability: Stable for 36 months at 4^0C.

Route of administration: Apply topically to the ulcerated area and cover with dressing two times a week.

Toxicities: Stinging and burning sensation, yellow pigmentation at the application site, and vasodilatation.

Placebo

Supply: Placebo cream will be provided by the Pine Pharmaceuticals, New York.

Product description: The placebo will be made from aquaphor ointment.

Storage: Store at 2^0C – 8^0C in the refrigerator.

Stability: Stable for 36 months at 4^0C.

Route of administration: Apply topically to the ulcerated area and cover with dressing two times a week.

11. Conflict of Interest

There is no real or apparent conflict of interest. However, in the interest of full disclosure, the patent application filed by the NIH Office of Technology: U.S. Provisional Patent Application No.

62/077,622, filed on November 10, 2014, entitled "TOPICAL SODIUM NITRITE FORMULATION," and PCT Patent Application No. PCT/US2015/060015, filed on November 10, 2015, entitled "TOPICAL SODIUM NITRITE FORMULATION," has six inventors: Gregory J. Kato, Caterina P. Minniti, Haksong Jin, George Grimes, Deborah Sperling, and Gopal Petti, who were all NIH employees at the time of invention.

- Dr. Gregory J. Kato is presently employed on the faculty of the University of Pittsburgh.
- Dr. Caterina P. Minniti is presently employed on the faculty of the Albert Einstein College of Medicine.

12 Adverse Events Reporting

The Principal Investigator (or a physician member of the research team) and the study coordinator will assess each participant for any new or continuing adverse events. An adverse event is defined as any untoward medical occurrence in a subject of a clinical investigation that involves the administration of a pharmaceutical product. The event need not to have a causal relationship with the treatment. This includes any events that are not seen at baseline or, if present at baseline, have worsened in severity. These adverse events will only be collected during study treatment time. The severity and drug relationship will be determined, and any management required will be recorded. The investigator will review the clinical laboratory test results in a timely fashion. Only those results qualifying as adverse events, as defined above, will be recorded as an adverse event.

Table 2. Expected Adverse Events Possibly Related to Study Drug
Severe stinging and burning sensation, requiring an increase in narcotic use.
Peripheral vasodilation with hypotension (BP below 85/50, or symptomatic dizziness, tachycardia, or hypoxia as per the judgment of the PI)
Methemoglobinemia >7.5%

Table 3. Expected Adverse Events Related to Sickle Cell Disease	
Acute chest syndrome	Leukocytosis
Angioedema	Meningitis
Aplastic crisis	Metabolic acidosis
Aplastic crisis/anemia	Osteomyelitis
Arthralgia	Pain
Appendicitis	Pain, joint
Bone Infarction	Pain, severe abdominal
Cardiomegaly	Priapisim
Cerebrovascular accident	Pulmonary embolism
Cholecystitis, hepatic sequestration	Pulmonary hypertension (worsening)
Cranial nerve palsy	Pulmonary parenchymal infiltrates on x-ray
Decreased kidney function	Pyleonephritis
Decreased lung function	Renal Failure
Fever	Renal Insufficiency/albuminuria
Headache	Renal papillary necrosis
Hematuria	Reticulocytosis (10%-20%)
Hemiplegia	Retinal disease
Hemolysis	Retinal hemorrhage
Hepatosplenomegaly	Skin ulcers
Hyperplastic bone marrow	Splenic sequestration
Hyposthenuria	Sepsis
Hyperkalemia	Upper respiratory infection
Increase Jaundice	Urinary tract infection

12.1 Serious Adverse Events

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

- 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 inpatient 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, is an important medical event 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, or that may be considered serious. Examples of such events are intensive treatment in an emergency department or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse.

12.2 Study Drug Relationship

The Principal Investigator and the Study Sponsor are responsible for assessing the causal relationship between any events and the study treatment (table 3). Additionally, the investigator is responsible for providing appropriate treatment for the event and for adequately following the event until resolution. The principal investigator and study sponsor should determine the study drug relationship using the following definitions:

Table 4. Criteria for attributing relationship of AE and SAE to study drug	
Unrelated	No temporal association to study product. An alternate etiology has been established. The event does not follow the known pattern of response to study product. The event does not reappear or worsen with re-challenge.
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 re-challenge.
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.

Temporal sequence is defined as an association between the suspect drug and the observed reaction or event in which the suspect drug was present prior to the reaction or event as defined by history or blood level of drug.

Study drug(s) includes the drug(s) under evaluation, the reference drug(s), placebo, or any other drug(s) required by the protocol.

Severity of an adverse event will be defined from the qualitative assessment of the degree of intensity of the event as determined by the principal investigator or as reported to him/her by the subject.

12.3 Guidelines for Serious Adverse Events Reporting

Serious adverse events are defined by the FDA as any untoward medical occurrences that result in death, are life-threatening, require or prolong hospitalization, cause persistent or significant disability or incapacity, result in congenital anomalies or birth defects, or are other conditions which in the opinion of the investigator represents significant hazards.

Any and all serious adverse events relating to the acquisition of blood samples, and the application of the study cream, will be reported in writing to the chair of the IRB. The written report of the serious adverse event (e.g., death or life-threatening adverse event) will be sent within 5 days if the serious adverse event is unexpected and possibly, probably, or definitely related to participation in the study. In the event that an adverse event or deviation occurs that could present an immediate or urgent danger to subjects, the IRB chair will be contacted immediately.

All serious adverse events and unexpected non-serious adverse events relating to the acquisition of blood will be summarized and reported at the time of the continuing review.

Vaso-occlusive crisis occurs frequently and at unpredictable intervals in subjects with sickle cell disease, often requiring hospitalization. These events are expected and they will be summarized and reported at the time of the continuing review.

13. Data and Safety Monitoring Plan

The PI will assemble an independent Data Safety Monitoring Board, composed by 4 individuals appointed from within and outside the institution to ensure safe and effective conduct of the trial. The DSMB may recommend early termination of the study for considerations of safety and efficacy. A progress report will be forwarded to the DSMB at these times. The DSMB will meet with the Principal Investigator annually to perform the following activities:

a. Assess participant safety, including:
   i. All protocol deviations and adverse events
   ii. All scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the study.
   iii. Benefit/risk ratio of procedures and participant burden
b. Evaluate trial progress, including:
   i. Quality, accuracy, and timeliness of collected data and statistical analysis
   ii. Selection, recruitment, and retention of participants
   iii. Confidentiality of trial data
   iv. Efficacy of the study intervention
c. Performance of clinical research sites, including:
   i. Performance of clinical research pharmacy and the core lab
   ii. Adherence to protocol requirements
d. Make recommendations to the IRB and investigators, including:
   i. Continuation or conclusion of the trial
   ii. Amendments to the study protocol and consent forms

Safety Monitoring:

Principal Investigator: Accrual and safety data will be monitored by the PI. The protocol will be continuously evaluated for any unusual or unpredicted complications with the sodium nitrite cream.
Accrual and safety data will be monitored and reviewed annually by the Institutional Review Board (IRB). Prior to implementation of this study, the protocol and the proposed informed consent and assent documents will be reviewed and approved by the properly constituted Institutional Review Board (IRB) operating according to the 45 CFR 46 Code of Federal regulations. This committee must approve all amendments to the protocol or informed consent, and conduct continuing annual review so long as the protocol is open to accrual, open for follow up of subjects or open for data analysis and sharing.

Data Management. The PI will be responsible for overseeing entry of data into an in-house password protected electronic system (RedCap web application) and ensuring data accuracy, consistency and timeliness. The principal investigator, associate investigators, research nurses and/or a data manager will assist with the data management efforts. RedCap will be the main electronic system for data collection while paper CRFs will serve as a backup. Data will be entered into RedCap in real time whenever feasible. However,data can also be abstracted from Clinical Center progress notes as well as intake form and the other paper based case report forms. For data not entered in real time, source documents will be kept in the subject binder and entered at a later time. Date of entry will be automatically recorded.Data will be stored in locked cabinets and in a password protected database until it is no longer of scientific value.

Loss or Destruction of Data: Should we become aware that a major breech in our plan to protect patient confidentiality and trial data has occurred, the IRB will be notified.

Table 5: Sodium Nitrite Study Visit Schedule

	Screening[€]	Baseline	Run-in	Double-Blinded Study Treatment Phase										EOS
Visit Number	0	1	2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23
Week on Study Intervention		Week ~-2	Week ~-1	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
Assessment Window	-	-	-	± 3 d	± 3 d	± 3 d	± 3 d	± 3 d	± 3 d	± 3 d	± 3 d	± 3 d	± 3 d	± 3 d
Study Procedure														
Informed Consent	x													
Basic Contact Info	x													
Demographics	x													
Medical History	x													
Beck Depression Inventory		x												x
ASCQ-Me		x												x
PROMIS		x												x
Brief Pain Inventory		x	x											x
Concomitant Medications	x	x		xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	x
Vital Signs	x	x	x	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	x
Baseline Physical Exam		x												
Non-Baseline Physical Examination	x			v3				x						x
MetHb	x			v3				x				x		
G6PD	x[a]													
Hb Electrophoresis	x[b]													x
Baseline and EOS Labs		x												x
Monitoring Labs				x				x				x		
Research Labs (3 tubes)		x												x
Saliva for DNA Analysis		x												
Urine Pregnancy Test*		x						x						x
Randomization				v3										
Application of Cream				xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	
Wound Care		x	x	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	x
Ulcer History	x													
Ulcer Assessment	x	x	x	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	x
Ulcer Photography		x	x	x					v13					x
Ulcer Biopsy			x											
Ulcer Microbiome			v2											x
Dose Modification									v13					

*: For women of childbearing potential only

xx: Twice per week assessment

a: If the subject had documented normal G6PD and metHb lab results, then no need to repeat

b: Only if no results available within 3 months

€: Screening and baseline can be combined into one visit

After the first three subjects have completed the study, if there are no significant side effects, especially significant changes in BP, patients will be offered the option of applying the second weekly dose of the cream at home, by themselves or by a home health nurse. Dose modification and ulcer photography for visit 13 can be done anytime between visit 11-15.

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