Published in final edited form as:

Nitric Oxide. 2020 January 01; 94: 79–91. doi:10.1016/j.niox.2019.10.011.

Sickle cell disease subjects and mouse models have elevated nitrite and cGMP levels in blood compartments

Luis E. F. Almeida^a, Sayuri Kamimura^a, Celia M. de Souza Batista^b, Nicholas Spornick^a, Margaret Y. Nettleton^a, Elizabeth Walek^c, Meghann L. Smith^a, Julia Finkel^c, Deepika Darbari^d, Paul Wakim^e, Zenaide M.N. Quezado^{a,1}

^aDepartment of Perioperative Medicine, National Institutes of Health Clinical Center, National Institutes of Health, Bethesda, MD 20892, USA.

^bDepartment of Nutritional Sciences, Howard University, Washington, DC 20059, USA.

^cThe Sheikh Zayed Institute for Pediatric Surgical Innovation, Children's Research Institute, Children's National Health System, School of Medicine and Health Sciences, George Washington University, Washington, DC 20010, USA.

^dDivision of Hematology, Center for Cancer and Blood Disorders, Children's National Health System, Department of Pediatrics, George Washington University School of Medicine, Washington, DC 20010, USA.

^eBiostatistics and Clinical Epidemiology Service, National Institutes of Health Clinical Center, Bethesda, MD, USA, 20892.

Abstract

The hypothesis of decreased nitric oxide (NO) bioavailability in sickle cell disease (SCD) proposes that multiple factors leading to decreased NO production and increased consumption contributes to vaso-occlusion, pulmonary hypertension, and pain. The anion nitrite is central to NO physiology as it is an end product of NO metabolism and serves as a reservoir for NO formation. However, there is little data on nitrite levels in SCD patients and its relationship to pain phenotype. We measured nitrite in SCD subjects and examined its relationship to SCD pain. In SCD subjects, median whole blood, red blood cell and plasma nitrite levels were higher than in controls, and were not associated with pain burden. Similarly, Townes and BERK homozygous SCD mice had elevated blood nitrite. Additionally, in red blood cells and plasma from SCD

¹**Corresponding Author:** Zena M.N. Quezado, MD, Department of Perioperative Medicine, NIH Clinical Center, National Institutes of Health, Bethesda, MD 20892, zquezado@nih.gov.

AUTHORSHIP CONTRIBUTIONS

LEFA and ZMNQ planned the experiments and wrote the paper
LEFA, PW, ZMNQ analyzed and interpret the data
LEFA, SK, CMSB, NS, EW and MN performed the experiments
NS, EW, MN, JF, DD and ZNMQ recruited human volunteers
All authors reviewed the final version of the manuscript

CONFLICT OF INTEREST DISCLOSURES

The authors declare no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

subjects and in blood and kidney from Townes homozygous mice, levels of cyclic guanosine monophosphate (cGMP) were higher compared to controls. *In vitro*, hemoglobin concentration, rather than sickle hemoglobin, was responsible for nitrite metabolism rate. *In vivo*, inhibition of NO synthases and xanthine oxidoreductase decreased nitrite levels in homozygotes but not in control mice. Long-term nitrite treatment in SCD mice further elevated blood nitrite and cGMP, worsened anemia, decreased platelets, and did not change pain response. These data suggest that SCD in humans and animals is associated with increased nitrite/NO availability, which is unrelated to pain phenotype. These findings might explain why multiple clinical trials aimed at increasing NO availability in SCD patients failed to improve pain outcomes.

Keywords

Sickle cell disease; nitrite; cGMP; hemoglobin; kidney; nitric oxide

INTRODUCTION

Pre-clinical studies have generated the hypothesis that hemolytic anemias including sickle cell disease (SCD) are associated with decreased nitric oxide (NO) bioavailability [1; 2; 3]. In SCD, according to this hypothesis, repeated cycles of sickle hemoglobin polymerization, hemolysis, and release of free hemoglobin and arginase in plasma contribute to increased consumption and decreased production of NO. Free hemoglobin reacting with and scavenging NO [4; 5] together with arginase competing with NO synthases (NOS) for their substrate, arginine, limit NO production by the endothelium [1; 3]. In turn, NO depletion in the circulation, possibly by inducing endothelial injury, leukocyte adhesion, platelet activation and production of oxygen free radicals, would induce vasoconstriction and contribute to vaso-occlusion, the hallmark of SCD [1; 3; 6; 7; 8; 9]. However, conclusive evidence that NO bioavailability is decreased in SCD is lacking and the hypothesis that a NO deficit contributes to SCD pathobiology has been challenged [10; 11]. In fact, results of clinical trials of therapeutic interventions aimed at increasing NO bioavailability (arginine administration, inhaled NO or administration of the phosphodiesterase-5 inhibitor sildenafil) have failed to provide reproducible clinical improvements in cardiovascular and pulmonary performances and SCD-related pain. Therefore these clinical trial findings, question the validity of the NO-deficit hypothesis in SCD [12].

Amid this controversy, some argue that rather than using strategies aimed at acute or chronic administration of agents that increase NO availability/activity, a better alternative would be nitrite supplementation [13; 14]. Nitrite can serve as a storage pool for NO activity as it can be reduced into NO by deoxyhemoglobin, xanthine oxidoreductase (XOR), and NO synthase (NOS), thus enabling the delivery of NO and increased NO activity directly to hypoxic areas [15; 16; 17; 18; 19]. In humans, nitrite-generated NO has been shown to improve blood flow, especially under hypoxic conditions [16; 18; 20] and in subjects with SCD, intravascular nitrite infusions were shown to improve regional blood flow without complications [21]. In humanized SCD mice, nitrite supplementation was shown to decrease hemolysis and inflammation-induced leukocyte and platelet adhesion [13] and to improve grip force, a marker for muscle hyperalgesia [22]. Importantly, these reported anti-adhesion and

vasodilatory properties under physiological hypoxic conditions are thought to be desirable in SCD.

In SCD patients, acute pain crises are the main reason for emergency room visits and hospitalizations [23; 24; 25]. While the triggers and mechanisms underlying SCD acute pain crises are incompletely understood, many believe that these crises result from tissue ischemia and reperfusion caused by vaso-occlusion and obstruction of the microvascular bed [26]. Therefore, the putative NO bioavailability deficit in SCD could be a contributing factor for vaso-occlusion, tissue ischemia, and pain. In turn, therapeutic strategies capable of increasing vascular NO levels in hypoxic areas could have a role in preventing and/or treating SCD pain crises. Here we investigated the association between nitrite, NO availability, and pain phenotype in SCD subjects and humanized SCD mouse models [27; 28].

MATERIALS AND METHODS

Study Participants

Volunteers were recruited at Children's National Health System (CNHS) after Institutional Review Board approval () and informed consent/assent was obtained. Subjects with SCD (HbSS, HbSC, HbS β^0 thalassemia, or HbSO-Arab), who were at clinical steady state (no admission for vaso-occlusive episodes for three weeks and no blood transfusions for three months preceding enrollment), were recruited during routine clinic visits.

Hydroxycarbamide use was annotated from patient files and confirmed verbally with each patient. Compliance was inferred from elevated HbF levels. Pain burden was determined as previously described [29]. Participants with a history of ≥ 3 pain-related hospitalizations per year over the last two years were included in the high pain burden group and those with a history of ≤ 2 severe pain episodes for the last two years in the low pain burden group [29]. Healthy control subjects included African-American peers, unaffected family members of SCD participants, and Caucasian subjects.

SCD Mouse Models

Institutional Animal Care and Use Committees from CNHS and the National Institutes of Health Clinical Center approved animal studies. Townes [28; 30] and BERK [27] SCD mouse models were used. Both models carry knockout mutations of mouse hemoglobin genes and knock-in mutations of human hemoglobin genes. Townes controls, heterozygotes and homozygotes have similar genetic background and replacement of human β for β^S gene (heterozygotes and homozygotes). BERK mice express human β^S genes (homozygotes), and those that also express a mouse β gene (hemizygotes) have an attenuated phenotype. We used C57BL/6J animals as controls for the BERK strain as they share >50% genetic identity with BERK mice [27]. Detailed mouse breeding and genotyping have been described [31]. Animals had free access to nitrite-free water and to mouse chow (Teklad LM485, Envigo) containing 1.49 ± 0.08 nmols nitrite/g.

Blood Collection and Processing

Human blood samples were collected from non-fasting subjects and processed avoiding nitrite contamination as described [32]. Hemoglobin, hematocrit and white cell count were measured in a clinical laboratory. Mouse blood was collected via cardiac puncture from anesthetized animals [33] and complete blood counts were obtained using an automated cell counter (Hemavet, Drew Scientific). A whole blood aliquot (300µl) was mixed with nitrite stabilizing solution to preserve nitrite levels [34].

Nitrite Assay

We measured nitrite in blood compartments using the acetic and ascorbic acids method, which is selective for nitrite and is linear from 1–300 pmol nitrite per injection [33]. Briefly, sample aliquots (usually 50µl) were injected into a reaction chamber connected to a Sievers 280i NO analyzer (GE Instruments). Sample nitrite was quantified by measuring the area under the curve using LabView (National Instruments) and interpolating into a curve obtained by injecting 30, 20 and 10 pmols nitrite from a freshly prepared nitrite standard solution. Nitrite measurements from whole blood samples were normalized to hemoglobin concentration given that the blood compartment containing the most nitrite are red blood cells [35; 36]. In order to measure nitrite in red blood cells, we removed plasma from each whole blood sample from all genotypes and diluted the packed red cells to the same hemoglobin concentrations with PBS before the assays were carried out.

For nitrite metabolism experiments, after careful separation (1000g, 10min, 4°C), plasma (125µl) or red blood cell aliquots (50µl) diluted with phosphate buffered saline with added calcium and magnesium (final concentration 1.3mM and 0.5mM, respectively) to a hemoglobin concentration of 2–3 g/dL were removed, mixed with nitrite preserving solution and frozen for later processing [34].

To investigate nitrite or nitrate metabolism, we used a randomized block design and added 10µM or 100µM, respectively and incubated randomly chosen samples at 37°C in a dry heat block while others were incubated at 4°C to examine the role of temperature. At predetermined intervals (1, 5, 10, 15, 30, 60, and 120min), an aliquot (300µl for whole blood and red blood cells and 125µl for plasma) was pipetted, mixed with stabilizing solution and frozen for later processing. In vitro experiments investigating nitrite metabolism in the presence of N ω -Nitro-L-arginine methyl (L-NAME, 100µM), allopurinol (10µM), xanthine (10µM) and/or β -nicotinamide adenine dinucleotide (NAD, 5mM) followed the same routine but these drugs were added 5min before nitrite. To carry out these experiments, we interrupted nitrite metabolism after 30min of adding nitrite so that a “floor” or “ceiling” effect would be avoided. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and tested for nitrite contamination.

We also investigated the potential role of NOS and XOR on nitrite levels *in vivo*. The selective inducible NOS antagonist L-N⁶-(1-iminoethyl)lysine (L-NIL, 5mg/Kg), the non-specific NOS antagonist N ω -Nitro-L-arginine methyl ester (L-NAME, 30mg/Kg) or the XOR-antagonist allopurinol (30mg/Kg) were diluted in PBS and administered ip at a volume

of 10 μ l/g. After 1h, animals were anesthetized and blood was collected and processed for nitrite assay as described above.

Cyclic guanosine monophosphate (cGMP) Elisa Assay

Immediately upon collection, 0.5mM 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor) was added (from a 40mM IBMX stock; final DMSO = 1.25%) to samples used for cGMP assay (Cayman Chemicals). Whole blood, plasma or red blood cell samples (diluted to Hb = 4–5 g/dL with IBMX-containing Elisa buffer) were acetylated by adding (to 500 μ l sample) 300 μ l 4M KOH followed immediately by 75 μ l of acetic anhydride. Samples were vortexed for 1 min followed by 4 min rest; reaction was terminated by adding 75 μ l 4M KOH. Acetylated samples were clarified by centrifugation (15,000g, 10min, and 4°C) and the supernatant was pipetted and frozen until assayed. Standards of known cGMP concentrations were also acetylated as described above. We also measured tissue cGMP in mouse heart, lungs, liver and kidney. Tissue samples (100mg) were cut, weighted, mixed with 1ml Elisa buffer (with 0.5mM IBMX) and dissociated by rapid agitation in a FastPrep-24 5G (MP Biomedical). Organ homogenates were clarified by centrifugation) and the supernatant was acetylated as described above. Preliminary experiments were carried out to document the best sample dilution (if needed) so that results were within the linear segment of the standard curve (usually 3.0 – 0.18 pmols/ml). In whole blood and red blood cell samples, cGMP levels were normalized to hemoglobin content and in organs per tissue weight.

Fetal hemoglobin assay

Fetal hemoglobin (HbF) fraction was measured by capillary zone electrophoresis in a MINICAP™ (Sebia) according with manufacturer's recommendations [37]. Red blood cell hemolysates from SCD subjects were subjected to high voltage protein separation and hemoglobins were detected at 415nm. HbF levels are expressed as percentage of total hemoglobin.

Bleeding time

Immediately after scalpel amputation of 0.5 cm of the distal tail of isoflurane-anesthetized mice, bleeding times were measured every 10–15 sec (using cotton pads) until it stopped. To investigate the effect of NO signaling in bleeding time, animals received a single ip injection of vehicle (PBS), nitrite (1mg/Kg) or the NO-chelator 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium (carboxy-PTIO potassium salt, 1g/Kg). All drugs were diluted in PBS and administered at 10 μ l/g. Bleeding times were assessed 1hour after injections.

Nitrite Supplementation

In different cohorts, we examined the effects of chronic nitrite supplementation on nocifensive behavior phenotype, plasma nitrite levels, and hematologic profile in Townes and BERK mice. Age-matched (8–12 weeks) male and female SCD mice from each genotype were treated with nitrite in drinking water or vehicle (distilled water) for four weeks. Behavior outcome measurements were obtained before and after completion of the

four-week treatment while animals were receiving the experimental treatment. All other outcome measurements were obtained after treatment only. In BERKs, we examined the effect of four-week treatment with 0.05, or 1g/L and in Townes only with 1g/L of nitrite in drinking water or vehicle [33]. Investigators, who treated animals, collected behavior outcomes, and procured blood and organs, were blinded to animals' genotype and treatment assignments. Daily nitrite consumption for each mouse model and genotype has been published[22].

Behavior Studies

The same investigator conducted behavior studies in animals from all experimental groups between 9 AM and 2 PM. We examined nocifensive response to thermal (hotplate and cold plate sensitivity) and electrical stimuli (sensory fiber interrogation) [37; 38; 39; 40; 41]. Only one behavior-testing paradigm was conducted in a given day to avoid animal stress.

Nocifensive response to thermal stimulation

To evaluate nocifensive response to noxious heat, mice were placed on a hotplate (Harvard Apparatus, Holliston, MA) and the latency to display pain-avoiding behaviors (jumping, stomping or repeated lifting or licking of hind or front paws) was measured [41]. The hotplate temperature was set (55°C) in accordance with previous SCD studies [37; 38; 42]. Animals were allowed to stay on the hotplate for a maximum of 30 seconds to avoid injury.

Cold plate sensitivity

We used a Peltier cooler to generate a cold surface maintained at 2°C (Harvard Apparatus) to examine cold sensitivity. Mice were placed on the cold plate for 5 minutes, as their behavior was recorded on video. We measured the total time animals spent withdrawing from the cold plate (lifting front or hind paws, rubbing of front paws) during the observation period, which we interpreted as cold sensitivity[43].

Nocifensive response to sine wave electrical stimulation of nerve fibers

We evaluated specific somatosensory fibers using sine-wave electrical stimulation using three frequencies: 5, 250 and 2000 Hz, which have been shown to stimulate C, A δ , and A β fibers respectively as described [37; 38; 40; 42; 44]. Briefly, electrical stimuli generated by a neurostimulator (Neurotron, Inc, Baltimore, MD) were delivered to the mouse tail. Stimuli at each frequency (5, 250 and 2000 Hz) were delivered at increasing intensities, lasted one second and were set on a 50% duty cycle (each 1s stimulus was followed by a 1s stimulus-free interval). Between stimulations at different frequencies, animals rested for one-minute. The nocifensive behavior outcome was vocalization and its occurrence terminated the stimulus. The electrical stimulus amperage that elicited audible vocalization or the maximum amperage delivered were defined as the current threshold [39; 40; 45]. Current thresholds for each frequency were the average of five measurements. The current threshold was measured in units (U), which corresponds to 100 times the amperage that elicited audible vocalization [39].

Statistical methods

For data recorded at one time point, which were not normally distributed, Kruskal-Wallis or Chi-square tests were used for analysis. For data with repeated measures (nitrite metabolism experiments) a repeated-measures ANOVA with interaction terms (genotype by time) was used. Model fit diagnostics were examined to check whether model assumptions were satisfied. When appropriate, measured outcomes were transformed to the natural logarithmic scale to meet model assumptions. For nociception behavior measurements, we analyzed thermosensory response and current vocalization thresholds differences between after treatment (post) and baseline measurements (before). Pearson correlation between nitrite levels in various blood compartments were examined. As we report p-values unadjusted for multiple comparisons [46; 47], the reader is encouraged to keep that in mind when interpreting reported p-values, particularly those close to 0.05. All analyses were conducted using SigmaStat 13.0 (Systat Software, Inc., San Jose, CA) or the statistical software SAS, version 9.4 (SAS Institute, NC, USA).

RESULTS

Participants

Participants' demographics and laboratory values are shown in the supplemental Table. Twenty-six participants with SCD (13 with low and 13 with high pain burden) and nine healthy subjects (five African Americans and four Caucasians) were enrolled. In the control group, African Americans participants had similar age [19 (19–21) years, median (interquartile range)] as SCD subjects, whereas Caucasians were older (supplemental Table).

SCD subjects have elevated nitrite and cGMP levels in blood compartments

We first measured absolute nitrite content in whole blood of sickle cell disease subjects and controls (supplemental Fig 1A). Compared to controls, SCD participants with low- (n=13, p=0.022) and high-pain (n=13, p=0.007) burden had significantly higher whole blood nitrite content (supplemental Fig 1A). Given that the blood compartment that contains the most nitrite are red blood cells [35; 36], and that subjects with SCD are profoundly anemic (supplemental Fig 1B), in all experiments we normalized the sample nitrite content to hemoglobin concentration.

Nitrite levels in all blood compartments from African-American and Caucasian control subjects were similar (p=1.00 for whole blood and red blood cell, and p=0.190 for plasma). In turn, data were combined and are shown as the control group (Fig 1). Among SCD participants, subjects with high and low pain burden had similar nitrite levels in whole blood [9.4 (3.1–24.0) (median, 25% and 75% interquartile range) and 5.2 (3.0–15.0) nmols/g of Hb, high and low pain respectively, p=0.573, Fig 1A], red blood cell compartment [6.1 (4.1–19.5), and 6.3 (4.2–12.3) nmols/g of Hb, high and low pain respectively, p=0.758, Fig 1B], and plasma [0.43 (0.24–4.88), and 0.36 (0.21–1.19) nmols/ml, p=0.442, Fig 1C)]. We then combined high and low pain burden subjects as the SCD group. Compared to controls, SCD participants had significantly higher nitrite levels in whole blood [1.4 (1.2–1.8) and 6.1 (3.1–20.6) nmols/g of Hb for controls and SCD participants, respectively, p<0.001, Fig 1D], red blood cell [2.8 (0.3–3.8) and 6.2 (4.1–15.9) nmols/g of Hb from controls and SCD

participants, respectively, $p < 0.001$, Fig 1E] and in plasma [0.2 (0.13–0.29) and 0.42 (0.24–1.48) nmols/ml for controls and SCD participants, respectively, $p = 0.014$, Fig 1F].

In controls, there was a positive correlation between plasma and whole blood nitrite levels ($r = 0.742$, $p = 0.022$, data not shown). Among SCD participants, there were positive correlations between plasma and whole blood nitrite levels ($r = 0.78$, $p < 0.00001$, supplemental Fig 2A), plasma and red blood cell nitrite levels ($r = 0.84$, $p < 0.0000001$, supplemental Fig 2B) and between whole blood and red blood cell nitrite levels ($r = 0.67$, $p < 0.001$, supplemental Fig 2C). There were no differences in nitrite levels among male and female SCD subjects in whole blood [5.4 (4.4–24.1), and 6.8 (2.0–16.7) nmols/g of Hb, males and females respectively, $p = 0.568$], red blood cells [6.5 (4.1–18.8), and 6.1 (4.1–12.3) nmols/g of Hb, males and females respectively, $p = 0.604$] and plasma [0.46 (0.31–1.76), and 0.25 (0.16–1.15) nmols/ml, males and females respectively, $p = 0.062$].

Eight subjects on the SCD low-pain and 10 on the high-pain group were treated with hydroxycarbamide. As expected, hydroxycarbamide-treated subjects had higher hemoglobin F (HbF) percentage compared to those untreated ($p = 0.011$, Fig 2A). SCD subjects with high- and low-pain burden had similar HbF percentage ($p = 0.531$, Fig 2B). Noticeably, compared to hydroxycarbamide-untreated, hydroxycarbamide-treated subjects had similar nitrite levels in all blood compartments [whole blood ($p = 0.222$), red blood cells ($p = 0.243$), and plasma ($p = 0.182$), Fig 2C–E].

To investigate nitrite availability and signaling in SCD further, we assayed cGMP levels in blood compartments. Subjects with SCD had significantly higher cGMP levels (Fig 3) in red blood cells [18.9 (12.4–25.3) and 11.4 (9.4–12.3) pmols/g Hb, SCD and control subjects respectively, $p = 0.025$] and plasma [1.61 (1.4–2.13) and 0.99 (0.71–1.19) pmols/ml, SCD and control subjects respectively, $p = 0.007$]. Overall, median red blood cell and plasma cGMP levels in SCD subjects were ≈ 1.6 fold higher than in normal volunteers.

In vitro, SCD blood nitrite metabolism/consumption is altered

We then evaluated nitrite metabolism/consumption in blood compartments of SCD subjects and controls to identify possible factors contributing to elevated nitrite levels. After addition of nitrite (10 μ M) to whole blood, there were significant decreases in nitrite levels over 120 minutes, which varied according to genotype (genotype by time interactions, $p = 0.004$). Specifically, after addition of nitrite, over time, there were less decreases from initial nitrite levels in whole blood from SCD subjects compared to controls at 15 ($p = 0.042$), 30 ($p = 0.001$), 60 ($p < 0.001$), and 120 min ($p = 0.006$) (Fig 4A), thus suggesting, that SCD subjects metabolize/consume added nitrite slower than controls.

We then examined nitrite metabolism in red blood cell and plasma compartments separately. When plasma was removed and red blood cells from controls and SCD subjects were diluted to the same hemoglobin levels (2–3 g/dL), after addition of nitrite (10 μ M), there were decreases in nitrite levels overtime ($p < 0.001$), which were similar comparing controls and SCD subjects (genotype by time interaction, $p = 0.298$, Fig 4B). When nitrite was added to plasma from controls or SCD subjects, there were no significant changes in nitrite levels overtime ($p = 0.337$, Fig 4C).

Sickle cell mice have elevated nitrite and cGMP levels

Akin to human SCD experiments, we first measured absolute nitrite content in whole blood from SCD mice and controls (supplemental Fig 3A). Compared to controls, homozygous Townes mice showed a trend towards higher whole blood nitrite content (supplemental Fig 3A) and had significantly lower hemoglobin concentrations (supplemental Fig 3B). In keeping with all experiments and given that the blood compartment that contains the most nitrite are red blood cells [35; 36], and that homozygous SCD mice are profoundly anemic (supplemental Fig 3B), in all experiments we normalized the sample nitrite content to hemoglobin concentration.

We then examined nitrite levels, signaling, and metabolism further in mouse models of SCD (Fig 5). Nitrite levels in young Townes mice (5–7 weeks old) were similar among males, females, homozygous, heterozygous, and controls (data not shown). Similar to findings in humans, in older (15–20-week) Townes mice whole blood nitrite levels were significantly higher in homozygotes compared to heterozygotes ($p=0.006$) or controls ($p<0.001$, Fig 5A). In BERKs, because male mice were unavailable, nitrite levels in blood compartments were investigated only in age-matched BERK females (20–25-week old). There was a significant main effect of genotype for whole blood nitrite levels ($p=0.008$, Fig 5B) and BERK homozygous mice had higher whole blood nitrite levels compared to hemizygous ($p=0.021$) and respective BERK control mice ($p=0.005$).

We then examined cGMP levels in SCD mice. Compared to controls, Townes homozygotes had significantly higher cGMP levels in whole blood ($p=0.002$, Fig 5C), red blood cell ($p=0.001$, Fig 5D), and plasma ($p=0.003$, Fig 5E). Overall, whole blood, red blood cell and plasma cGMP levels were 9–13 fold higher in Townes homozygous mice as compared to control animals (Figs 5C–E). We also examined tissue levels of cGMP and found that tissue cGMP levels in heart, liver and lung were similar across Townes mice genotypes (data not shown). Conversely, kidney cGMP levels were significantly higher (≈ 9 fold) in Townes homozygotes compared to control animals ($p=0.038$, Fig 5F).

As nitrite supplementation has been proposed as a therapeutic intervention in SCD, we investigated its effects on cGMP levels in Townes mice. Compared with vehicle, long-term nitrite supplementation in drinking water yielded significantly higher red blood cell and plasma cGMP levels in controls and homozygotes (effect of treatment, $p<0.001$ in red blood cell and plasma, Figs 5G–H). In red blood cells, nitrite treatment increased cGMP levels ≈ 2 –3 fold in Townes controls and homozygotes (Fig 5G). Similarly, nitrite-treated mice had significantly higher plasma cGMP levels (11-fold and 8-fold in the control and homozygous mice respectively, Fig 5H) compared to respective genotype vehicle-treated animals. Contrary to findings in blood compartments, nitrite treatment yielded no significant changes in renal cGMP levels in either control or homozygous mice (treatment main effect $p=0.242$, data not shown).

In vitro, SCD blood nitrite metabolism/consumption is altered

Using Townes homozygous mouse blood, we examined the effect of temperature on stability of nitrite levels overtime (supplemental Fig 4). Over 120 min after addition of nitrite

(10 μ M), its levels significantly varied according to incubation temperature as there was a significant time by temperature interaction ($p<0.001$). Specifically, when incubated at 37°C, whole blood nitrite levels significantly decreased overtime ($p<0.001$, (supplemental Fig 4A), whereas when incubated at 4°C, whole blood nitrite levels remained stable (for up to 2hrs). Identical results were obtained using blood from Townes control animals (data not shown).

We then examined *in vitro* nitrite metabolism/consumption in Townes mice after the addition of nitrite (10 μ M) to whole blood obtained from controls or homozygous animals (supplemental Fig 4B, all experiments at 37°C). After addition of nitrite (10 μ M) to whole blood, there were significant decreases in nitrite levels over 120 min, which varied according to genotype (genotype by time interaction, $p=0.02$). Specifically, after addition of nitrite, over time there were significantly higher nitrite levels in whole blood from homozygotes compared to control mice at 5 ($p=0.015$), 10 ($p=0.010$), 15 ($p<0.001$), and 30 min ($p<0.001$) (supplemental Fig 4B), thus suggesting that homozygote blood metabolized/consumed added nitrite slower than blood from control mice. However, when red blood cells were diluted to similar hemoglobin concentrations (2–3 g/dL), overtime, nitrite levels in both controls and homozygotes decreased similarly ($p=0.367$), thus suggesting that when blood samples had similar hemoglobin concentration, nitrite was metabolized at similar rate over time (supplemental Fig 4C).

We then examined *in vitro* the possible role of xanthine oxidoreductase (XOR), a purported nitrite reductase, in nitrite handling by whole blood from homozygous Townes mice. The addition of the XOR substrates xanthine (10 μ M), NAD (5mM) or a combination of both drugs did not affect nitrite recovery 30 min after adding nitrite to whole blood from Townes homozygous (supplemental Fig 4D) or controls (data not shown). Similarly, addition of the XOR inhibitor allopurinol (10 μ M) or the NOS inhibitor L-NAME (100 μ M) to red blood cells did not modify nitrite metabolism/consumption in human or mouse erythrocytes (data not shown), suggesting that *in vitro* these enzymes were not involved in nitrite metabolism in this blood compartment.

Acute inhibition of nitric oxide synthase and xanthine oxidoreductase alter nitrite levels in SCD mice

We then examined the potential role of NOS and XOR on the increased nitrite levels in SCD mice using acute administration of L-NIL, an NOS2 inhibitor, L-NAME, a non-selective NOS inhibitor, and allopurinol, a XOR inhibitor (Fig 6). The effects of L-NIL, L-NAME, and allopurinol varied according to genotype and blood compartment. In control mice, compared with vehicle, L-NIL, L-NAME, and allopurinol treatments were not associated with significant changes in nitrite levels in any blood compartment (all $p > 0.109$, Fig 6A–C). However, compared with vehicle, in whole blood, L-NIL-, L-NAME, and allopurinol treatments were associated with decreases in nitrite levels in homozygous but not in control Townes and these effect patterns between genotypes were significantly different (treatment by genotype interaction, $p=0.018$, Fig 6D). In RBC's, compared with vehicle, L-NAME and allopurinol treatments, but not L-NIL, were associated with decreases in nitrite levels in homozygous but not in control Townes and these effect patterns between genotypes were significantly different (treatment by genotype interaction, $p<0.001$, Fig 6E). In plasma from

homozygous, compared with vehicle, L-NIL and allopurinol treatments, but not L-NAME, were associated with decreases in nitrite levels ($p=0.006$ and $p=0.026$ for L-NIL and allopurinol respectively, Fig 6F).

Chronic nitrite supplementation increases nitrite levels, alters hematologic parameters, but does not alter nociception phenotype in SCD mice

Given that therapies that alter NO availability have yielded conflicting results in SCD-associated pain [12; 48], we investigated the effects of nitrite supplementation on nociception phenotype and hematologic parameters in different cohorts of Townes and BERKs mice. Among BERKs, nitrite-treatment at doses of 1g/L, but not 0.05g/L, increased nitrite levels in controls and homozygotes ($p<0.0001$, for overall treatment effect, supplemental Fig 5). Interestingly, the effect of nitrite supplementation varied according to genotype as there was a treatment by genotype interaction ($p=0.001$). Nitrite supplementation at 1g/L was associated with greater increases in nitrite levels in homozygous BERK mice than in control (C57BL/6J) animals (supplemental Fig 5). In Townes, we only examined nitrite supplementation at the dose of 1g/L, which yielded significant increases in plasma nitrite levels in controls, heterozygotes, and homozygotes compared to vehicle ($p<0.0001$, for overall treatment effect, supplemental Fig 5).

Regarding the effect of nitrite supplementation on the nociception phenotype, in BERK mice, compared to vehicle, a four-week nitrite administration in drinking water at doses of 0.05g/L or 1g/L yielded no significant changes from baseline in hot plate latency, cold plate sensitivity, or current threshold in response to 2000, 250, or 5Hz stimulation (supplemental Fig 6). Similarly, in Townes mice, compared to vehicle, nitrite supplementation at 1g/L for four weeks yielded no significant changes from baseline in hot plate latency, cold plate sensitivity, or current threshold in response to 2000, 250, or 5Hz stimulation (supplemental Fig 7).

We also examined the effect of nitrite supplementation in hematologic parameters in Townes mice. In keeping with previous reports [31; 37; 38], homozygous Townes had anemia, leukocytosis, and thrombocytosis (Fig 7A–C). Compared with vehicle, nitrite treatment (1g/L) had no effect on red blood cell or white blood cell counts, regardless of genotype (Fig 7A–B). In contrast, the effect of nitrite on platelet count varied according to genotype as there were treatment by genotype interactions ($p<0.0001$). Specifically, compared to respective vehicle-treated mice, nitrite-treated controls had similar platelet counts ($p=0.342$), whereas nitrite-treated heterozygotes ($p=0.0014$) and homozygotes ($p<0.0001$) had significantly lower platelet counts (Fig 7C). Further, in all genotypes combined, nitrite-treated, compared with vehicle-treated animals, had lower hemoglobin ($p<0.0001$, for overall treatment effect, Fig 7D) and hematocrit ($p=0.0195$, for overall treatment effect, Fig 7E). Regarding mean corpuscular volume (MCV), among vehicle-treated animals, homozygous mice had significantly higher MCV compared to controls and heterozygotes ($p<0.0001$, Fig 7F). Additionally, there was an overall effect of nitrite treatment on MCV as nitrite-treated mice had overall lower MCV compared to vehicle-treated animals ($p=0.003$, for overall treatment effect in all genotypes combined, Fig 7F). Further, the effect of treatment on mean corpuscular hemoglobin concentration varied according to genotype.

Specifically, compared to respective vehicle-treated mice, controls ($p<0.0001$) and heterozygotes ($p=0.009$) had significantly lower, whereas nitrite-treated homozygotes had similar mean corpuscular hemoglobin concentration ($p=0.554$), and these nitrite effect patterns were significantly different ($p=0.0058$, for treatment-by-genotype interactions Fig 7G).

Acute nitrite supplementation and NO scavenging alter bleeding time in controls and homozygous mice

While we did not observe significant changes in pain phenotype with nitrite supplementation in mice, we examined other possible physiologic consequences of nitrite in SCD mice. As mouse bleeding time has been shown to be altered by nitrite supplementation and increased nitrite levels [49], we measured bleeding time to evaluate natural hemostasis in Townes mice (Fig 8). We found that during basal conditions, Townes homozygous had longer bleeding times compared to controls ($p<0.001$, Fig 8). As expected, acute injection of nitrite, compared with vehicle, yielded significant prolongation of bleeding time in control mice ($p=0.012$, Fig 8). As homozygous mice have elevated nitrite levels and nitrite can serve as a NO reservoir, we tested the hypotheses that injection of c-PTIO, a NO scavenger would decrease bleeding time. We found that an injection of c-PTIO, compared with vehicle, yielded significant decreases of bleeding time in homozygous Townes ($p=0.003$, Fig 8).

Nitrate metabolism in human and mice SCD

We also analyzed the capacity of whole blood, red blood cells, or plasma to reduce nitrate into nitrite in SCD subjects and Townes mice. After addition of nitrate ($100\mu\text{M}$) to whole blood or red blood cells from Townes homozygous or control mice nitrite levels were unchanged even after 2hrs incubation at 37°C (data not shown). Addition of xanthine ($10\mu\text{M}$), NAD (5mM) or a combination of both drugs also did not convert nitrate into nitrite in whole blood from Townes mice (data not shown). Similarly, human red blood cells or plasma from controls ($n=8$) or SCD subjects ($n=9$) did not reduce nitrate into nitrite (data not shown), suggesting that no blood compartment examined displayed nitrate reductase activity under the conditions investigated here.

DISCUSSION

We found that during baseline conditions, SCD subjects, compared to healthy controls, have higher nitrite levels in blood compartments. These elevated nitrite levels were observed in SCD subjects with high and low pain burden, who were treated or not treated with hydroxycarbamide, and were associated with higher cGMP levels. Similarly, in two humanized mouse models of SCD, blood nitrite and cGMP levels were elevated compared to respective control animals. We also found that acute inhibition of NOSs and XOR were associated with decreased nitrite levels in blood compartments of SCD, but not control mice, thus suggesting that NOS and XOR contribute to these elevated nitrite levels in SCD. Further, in SCD mice, nitrite supplementation, while associated with further increases in nitrite and cGMP levels, worsened anemia, decreased platelet count, and yielded no changes in nocifensive behavior. Additionally, when we injected a NO scavenger in homozygous SCD mice, there were decreases in bleeding time, a test of hemostasis known to be altered

by elevated nitrite levels. Combined, these data suggest that in SCD, nitrite and NO availabilities are actually increased, rather than decreased as previously hypothesized, and that therapies, which increase nitrite availability do not change SCD-associated pain phenotype.

Why might SCD subjects have higher blood nitrite levels compared to control subjects? We examined two possibilities, the first, that nitrite consumption would be decreased and the second that nitrite production would be increased in SCD mice. One possibility that might lead to decreased consumption is that HbS in SCD subjects metabolizes/consumes nitrite at a slower rate than HbA in controls. We tested this hypothesis *in vitro* by adding nitrite to whole blood of SCD and control subjects and measuring its consumption. At 37°C and 21% oxygen concentration, whole blood from SCD subjects consumed nitrite at a slower rate compared to controls, a finding also observed in SCD mice. However, when nitrite was added to control or sickle red blood cells samples containing similar hemoglobin concentrations, nitrite consumption was similar and independent of genotype. These findings suggest that anemia, rather than differences in nitrite metabolism by HbA and HbS, was the reason for slower nitrite metabolism observed in SCD subjects and mice. Importantly, these findings also highlight and support the notion that when measuring nitrite in whole blood and in red cells, one needs to take into account the sample's concentration of hemoglobin or hematocrit. This approach has been previously proposed by others, who while examining nitrite levels in blood compartments, showed that the blood compartment, which contains the most nitrite are erythrocytes [35; 36].

We then examined the hypothesis that nitrite production, possibly by NOSs activation and NO production, is increased in SCD mice. SCD is known to be associated with an inflammatory state; therefore, we tested the role of inducible NOS *in vivo* and found that an injection of L-NIL, an inducible NOS inhibitor, decreased nitrite levels in whole blood and plasma, but not in RBC, in homozygotes, but not in control Townes. In homozygous Townes, but not in controls, L-NAME, a non-selective NOS inhibitor was associated with decreases in nitrite levels in all blood compartments. Interestingly, L-NAME, but not L-NIL, lowered nitrite levels in RBCs of SCD mice. We posit that these findings are in concert with the reports by others demonstrating that RBCs express an active and functional endothelial-type NOS, which is inhibited by non-selective NOS-inhibitors [50]. Taken together the findings that NOS inhibitors decrease nitrite levels in SCD mice, suggest that in SCD, NOS activation contributes to increased nitrite production, possibly by increasing NO, which can be converted into nitrite.

Experimental evidence indicates that there is an interplay between XOR, NOS, and the nitrate-nitrite-NO pathway [16; 17; 18; 51; 52]. Researchers have shown that XOR activity is elevated in endothelial NOS-deficient mice [52] and that XOR is capable of reducing nitrate into nitrite and nitrite into NO during physiological or more acidic conditions[16; 17; 18; 51; 52]. Others have also shown that in SCD, episodes of hepatic ischemia/reperfusion could lead to the release of XOR into the circulation and in fact, SCD subjects and mice have elevated XOR activity in plasma [53]. *In vitro*, at 21% oxygen concentration, we found that XOR substrates (xanthine and/or NAD) did not accelerate nitrite metabolism and that an XOR inhibitor (allopurinol) failed to block nitrite metabolism in human or mouse red blood

cells, thus suggesting that XOR does not play a role in nitrite consumption in SCD in those conditions. *In vivo*, however, we found that administration of allopurinol, a XOR inhibitor, significantly decreased nitrite levels in SCD mice but not in controls. Taken together, the findings of others showing increased XOR activity in SCD mice and humans and our findings that XOR inhibition decreases nitrite levels in blood compartments of SCD mice suggest that, by mechanisms incompletely understood, XOR plays an important role in nitrite production in SCD.

While nitrite levels in SCD have been less frequently examined, our findings are consistent with levels reported by others [18; 36; 54; 55; 56] but contrary to those by Sullivan et al, reporting high plasma nitrite levels in control subjects and a non-statistically significant lower level in SCD subjects. Conversely, our findings are congruent with those by Elias et al showing an eight-fold increase in SCD blood nitrite levels compared to controls[57]. Here, we used methods for blood collection and nitrite assay, which were shown to be specific for nitrite over several other similar molecules, to be reproducible, and free of nitrite contamination [32; 33]. However, there are several approaches to measure nitrite in biologic matrices; therefore, we posit that differences in blood collection and processing methods, which may include the use of nitrite-contaminated blood collection tubes, could partially explain those reported discrepant results.

In our SCD cohort, we found that nitrite levels were similar in subjects treated with and those not treated with hydroxycarbamide. These findings are contrary to those of others who showed that, compared to healthy volunteers, nitrite levels were higher only in SCD subjects taking hydroxycarbamide[58]. Researchers have shown that *in vitro*, hydroxycarbamide increases intracellular cGMP in erythroid progenitor cells and enhances NO and cGMP production by stimulating eNOS activity in endothelial cells [59; 60]. Others also showed *in vitro* that while hydroxycarbamide increases NO production by stimulating eNOS, it had no effect on nitrite production [61]. Still others have shown that the increased nitrite production by heme-stimulated endothelial cells can be reduced by hydroxycarbamide treatment [61]. Therefore, the relationship between hydroxycarbamide treatment and nitrite/NO production is incompletely understood. Nevertheless, we found that SCD subjects had elevated nitrite levels in blood compartments independently of hydroxycarbamide intake, findings that were replicated in humanized SCD mouse models not taking hydroxycarbamide. Taken together, these data suggest that in SCD, blood nitrite levels are elevated regardless of hydroxycarbamide intake.

There is growing interest in using soluble guanylate cyclase (sGC) activators/stimulators in SCD and clinical trials of these agents are ongoing [62]. Researchers have shown that in BERK mice, acetylcholine-induced and NO-donor induced relaxation of the pulmonary artery is impaired and is improved after long-term treatment with sGC activators, but not sildenafil, a phosphodiesterase inhibitor [62]. These findings were interpreted to suggest that sGC is oxidized in SCD mice, which in turn leads to blunted responses to NO [62]. Here, we found that coupled with elevated nitrite levels, both SCD subjects and homozygous Townes mice had elevated blood levels of cGMP. These findings are in concert with reports by others showing increased cGMP levels in SCD subjects [63]. Further, in homozygous Townes mice, cGMP was also elevated in kidneys, but not in heart, lung, or liver, suggesting that

cGMP elevation is a localized, not a widespread phenomenon. Additionally, when SCD animals were treated with long-term nitrite supplementation, there were additional elevations both in nitrite and cGMP levels in red blood cells and plasma. While researchers have shown that plasma cGMP levels can be derived from particulate guanylate cyclase (activated by natriuretic peptides) and/or sGC (stimulated by nitrate-nitrite-NO pathway) [64], the determination of the specific source of cGMP levels was beyond the scope of this work. Nevertheless, our findings of increased nitrite and cGMP at baseline, both in humans and mice with SCD, and of additional elevation in nitrite and cGMP levels after nitrite treatment do not support the hypotheses of decreased NO bioavailability or of impaired sGC activity in SCD.

In an effort to ascertain whether perturbations in nitrite levels would have physiological consequences, we examined the effect of nitrite supplementation and of NO scavenging on bleeding time, a marker of natural hemostasis shown to be affected by nitrite levels [49]. We found that homozygous Townes mice, which have increased nitrite levels, also have increased bleeding time compared to controls. Additionally, in keeping with previous reports, we found that an acute injection of nitrite increased bleeding time in control mice. We then examined the effect of scavenging NO in Townes mice and found that an injection of c-PTIO, a NO scavenger, yielded decreases in bleeding time in homozygotes. It is interesting that we and others have shown that SCD mice have increased platelet aggregation [13; 65], which might appear incongruent with the findings of increased bleeding time. However, bleeding time is a marker of natural hemostasis *in vivo* and as such, it reflects the combination of many factors, in addition to platelet aggregation, that contribute to bleeding. Taken together, these findings suggest that perturbation of nitrite levels and scavenging of NO in animals with elevated nitrite levels yield perturbation in bleeding time.

Regarding NO availability and SCD-related pain, smaller clinical trials suggested that inhaled NO decreased pain severity and reduced opioid use during pain crisis in children [66] and adults [67]. However, larger prospective double blind randomized trials using strategies that increase NO availability failed to confirm those initial findings [12; 48]. Interestingly, we showed that SCD subjects with high and low pain burden had similarly elevated nitrite and cGMP levels, thus failing to support the notion of impaired NO availability in the disease. Others and we have also shown that SCD mice have sensory fiber hyperalgesia and decreased grip strength, which has been interpreted as to reflect muscle hyperalgesia [31; 42; 68]. Recently we showed that long-term nitrite supplementation improves grip strength in SCD mice, a finding that could reflect improvement in muscle hyperalgesia [22]. Notably, this improvement in grip strength was actually associated with increases in specific force of fast-, but not slow-twitch muscle, suggesting the improvement in grip force could simply reflect improvement in muscle performance. Regarding measures of nociception and nocifensive behaviors, we showed that long-term nitrite supplementation yielded additional increases in nitrite and cGMP levels and no significant change on nocifensive response to noxious stimuli in SCD or control mice. Therefore, taken together, our findings suggest that nitrite supplementation does not improve sensory fiber hyperalgesia in SCD mice and do not support the use of strategies that increase NO availability (for example nitrite or nitrate supplementation, NO-donors, PDE inhibitors, sGC stimulators or activators) to treat sickle cell pain.

Given the technical challenges in measuring nitrite levels in biological matrices, there are a few important points to consider when examining and interpreting our findings. First, we measured nitrite and cGMP levels during steady state, thus, one cannot assume stability of these levels as we did not obtain longitudinal samples. Further, given that red blood cells is the blood compartment containing the most nitrite and SCD subjects and mouse models have significant anemia, we normalized whole blood and red blood cell nitrite measurements to respective hemoglobin levels in order to allow for a direct comparison with values obtained from control samples as proposed by others [35; 36]. We used blood sample collection and processing techniques, which have been shown to be free of nitrite contamination, to be selective for nitrite over many similar chemical species, and that have been validated in human and mouse blood samples in the presence of the nitrite stabilization solution [32; 33]. Additionally, when we performed red blood cell nitrite metabolism assays, samples from all genotypes were diluted to the same hemoglobin concentrations before the assays were carried out. That way, the results of increased nitrite levels were not caused by a normalization effect to the lower hemoglobin content in SCD samples. Further, we argue, that elevation of nitrite levels in SCD blood compartments were unrelated to the fact that subjects did not fast prior to blood collection. In fact, others have shown that blood nitrite (but not nitrate) levels are independent of diet [69]. Nevertheless, our findings indicate that in SCD, both in humans and mouse models, nitrite levels are increased, rather than decreased, in blood compartments, and strategies to increase nitrite levels should perhaps be reconsidered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The Intramural Research Program of the National Institutes of Health, National Institutes of Health Clinical Center and the Children's Research Institute supported this work. The authors are grateful to our patients and their families, to Michael Guerrero, MD, Barbara Speller-Brown, PhD, and Kevin Jackson, BA for help during the study and to Paulette Price for superb technical support. The authors also have the deepest gratitude to Nicholas Kenyon (In memoriam) for maintenance of the SCD mouse colonies and outstanding technical support during the study.

References

- [1]. Morris CR, Gladwin MT, Kato GJ, Nitric oxide and arginine dysregulation: a novel pathway to pulmonary hypertension in hemolytic disorders, *Curr Mol Med* 8 (2008) 620–32. [PubMed: 18991648]
- [2]. Akinsheye I, Klings ES, Sick cell anemia and vascular dysfunction: the nitric oxide connection, *J Cell Physiol* 224 (2010) 620–5. [PubMed: 20578237]
- [3]. Kato GJ, Steinberg MH, Gladwin MT, Intravascular hemolysis and the pathophysiology of sickle cell disease, *J Clin Invest* 127 (2017) 750–760. [PubMed: 28248201]
- [4]. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO 3rd, Schechter AN, Gladwin MT, Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease, *Nat Med* 8 (2002) 1383–9. [PubMed: 12426562]
- [5]. Minneci PC, Deans KJ, Zhi H, Yuen PS, Star RA, Banks SM, Schechter AN, Natanson C, Gladwin MT, Solomon SB, Hemolysis-associated endothelial dysfunction mediated by accelerated NO inactivation by compartmentalized oxyhemoglobin, *J Clin Invest* 115 (2005) 3409–17. [PubMed: 16294219]

- [6]. Gladwin MT, Schechter AN, Ognibene FP, Coles WA, Reiter CD, Schenke WH, Csako G, Wacławski MA, Panza JA, Cannon RO 3rd, Divergent nitric oxide bioavailability in men and women with sickle cell disease, *Circulation* 107 (2003) 271–8. [PubMed: 12538427]
- [7]. Reiter CD, Gladwin MT, An emerging role for nitric oxide in sickle cell disease vascular homeostasis and therapy, *Curr Opin Hematol* 10 (2003) 99–107. [PubMed: 12579034]
- [8]. Hsu LL, Champion HC, Campbell-Lee SA, Bivalacqua TJ, Mancini EA, Diwan BA, Schimel DM, Cochard AE, Wang X, Schechter AN, Noguchi CT, Gladwin MT, Hemolysis in sickle cell mice causes pulmonary hypertension due to global impairment in nitric oxide bioavailability, *Blood* 109 (2007) 3088–98. [PubMed: 17158223]
- [9]. Li L, Chen W, Rezvan A, Jo H, Harrison DG, Tetrahydrobiopterin deficiency and nitric oxide synthase uncoupling contribute to atherosclerosis induced by disturbed flow, *Arterioscler Thromb Vasc Biol* 31 (2011) 1547–54. [PubMed: 21512164]
- [10]. Bunn HF, Nathan DG, Dover GJ, Hebbel RP, Platt OS, Rosse WF, Ware RE, Pulmonary hypertension and nitric oxide depletion in sickle cell disease, *Blood* 116 (2010) 687–92. [PubMed: 20395414]
- [11]. Hebbel RP, Reconstructing sickle cell disease: a data-based analysis of the “hyperhemolysis paradigm” for pulmonary hypertension from the perspective of evidence-based medicine, *Am J Hematol* 86 (2011) 123–54. [PubMed: 21264896]
- [12]. Machado RF, Barst RJ, Yovetich NA, Hassell KL, Kato GJ, Gordeuk VR, Gibbs JS, Little JA, Schraufnagel DE, Krishnamurti L, Girgis RE, Morris CR, Rosenzweig EB, Badesch DB, Lanzkron S, Onyekwere O, Castro OL, Sachdev V, Wacławski MA, Woolson R, Goldsmith JC, Gladwin MT, Hospitalization for pain in patients with sickle cell disease treated with sildenafil for elevated TRV and low exercise capacity, *Blood* 118 (2011) 855–64. [PubMed: 21527519]
- [13]. Wajih N, Basu S, Jailwala A, Kim HW, Ostrowski D, Perlegas A, Bolden CA, Buechler NL, Gladwin MT, Caudell DL, Rahbar E, Alexander-Miller MA, Vachharajani V, Kim-Shapiro DB, Potential therapeutic action of nitrite in sickle cell disease, *Redox Biol* 12 (2017) 1026–1039. [PubMed: 28511346]
- [14]. Kim-Shapiro DB, Gladwin MT, Nitric oxide pathology and therapeutics in sickle cell disease, *Clin Hemorheol Microcirc* 68 (2018) 223–237. [PubMed: 29614634]
- [15]. Kim-Shapiro DB, Gladwin MT, Mechanisms of nitrite bioactivation, *Nitric Oxide* 38 (2014) 58–68. [PubMed: 24315961]
- [16]. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A, Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage, *Proc Natl Acad Sci U S A* 101 (2004) 13683–8. [PubMed: 15347817]
- [17]. Mikula I, Durocher S, Martasek P, Mutus B, Slama-Schwok A, Isoform-specific differences in the nitrite reductase activity of nitric oxide synthases under hypoxia, *Biochem J* 418 (2009) 673–82. [PubMed: 19046140]
- [18]. Ghosh SM, Kapil V, Fuentes-Calvo I, Bubb KJ, Pearl V, Milsom AB, Khambata R, Maleki-Toyserkani S, Yousuf M, Benjamin N, Webb AJ, Caulfield MJ, Hobbs AJ, Ahluwalia A, Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential, *Hypertension* 61 (2013) 1091–102. [PubMed: 23589565]
- [19]. Fens MH, Larkin SK, Oronsky B, Scicinski J, Morris CR, Kuypers FA, The capacity of red blood cells to reduce nitrite determines nitric oxide generation under hypoxic conditions, *PLoS One* 9 (2014) e101626. [PubMed: 25007272]
- [20]. Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ, Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver, *J Clin Invest* 115 (2005) 1232–40. [PubMed: 15841216]
- [21]. Mack AK, McGowan Li VR, Tremonti CK, Ackah D, Barnett C, Machado RF, Gladwin MT, Kato GJ, Sodium nitrite promotes regional blood flow in patients with sickle cell disease: a phase I/II study, *Br J Haematol* 142 (2008) 971–8. [PubMed: 18671702]
- [22]. Wang L, Almeida LEF, Kamimura S, van der Meulen JH, Nagaraju K, Quezado M, Wakim P, Quezado ZMN, The role of nitrite in muscle function, susceptibility to contraction injury, and fatigability in sickle cell mice, *Nitric Oxide* 80 (2018) 70–81. [PubMed: 30114530]

- [23]. Dampier C, Palermo TM, Darbari DS, Hassell K, Smith W, Zempsky W, AAPT Diagnostic Criteria for Chronic Sickle Cell Disease Pain, *J Pain* (2017).
- [24]. Ballas SK, Kesen MR, Goldberg MF, Luty GA, Dampier C, Osunkwo I, Wang WC, Hoppe C, Hagar W, Darbari DS, Malik P, Beyond the definitions of the phenotypic complications of sickle cell disease: an update on management, *ScientificWorldJournal* 2012 (2012) 949535. [PubMed: 22924029]
- [25]. Yawn BP, Buchanan GR, Afenyi-Annan AN, Ballas SK, Hassell KL, James AH, Jordan L, Lanzkron SM, Lottenberg R, Savage WJ, Tanabe PJ, Ware RE, Murad MH, Goldsmith JC, Ortiz E, Fulwood R, Horton A, John-Sowah J, Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members, *JAMA* 312 (2014) 1033–48. [PubMed: 25203083]
- [26]. Hebbel RP, Ischemia-reperfusion injury in sickle cell anemia: relationship to acute chest syndrome, endothelial dysfunction, arterial vasculopathy, and inflammatory pain, *Hematol Oncol Clin North Am* 28 (2014) 181–98. [PubMed: 24589261]
- [27]. Paszty C, Brion CM, Mancini E, Witkowska HE, Stevens ME, Mohandas N, Rubin EM, Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease, *Science* 278 (1997) 876–8. [PubMed: 9346488]
- [28]. Wu LC, Sun CW, Ryan TM, Pawlik KM, Ren J, Townes TM, Correction of sickle cell disease by homologous recombination in embryonic stem cells, *Blood* 108 (2006) 1183–8. [PubMed: 16638928]
- [29]. Darbari DS, Onyekwere O, Nouraie M, Minniti CP, Luchtman-Jones L, Rana S, Sable C, Ensing G, Dham N, Campbell A, Arteta M, Gladwin MT, Castro O, Taylor J.G.t., Kato GJ, Gordeuk V, Markers of severe vaso-occlusive painful episode frequency in children and adolescents with sickle cell anemia, *J Pediatr* 160 (2012) 286–90. [PubMed: 21890147]
- [30]. Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R, Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin, *Science* 318 (2007) 1920–3. [PubMed: 18063756]
- [31]. Wang L, Almeida LE, de Souza Batista CM, Khaibullina A, Xu N, Albani S, Guth KA, Seo JS, Quezado M, Quezado ZM, Cognitive and behavior deficits in sickle cell mice are associated with profound neuropathologic changes in hippocampus and cerebellum, *Neurobiol Dis* 85 (2016) 60–72. [PubMed: 26462816]
- [32]. Almeida LEF, Kamimura S, Nettleton MY, de Souza Batista CM, Walek E, Khaibullina A, Wang L, Quezado ZMN, Blood collection vials and clinically used intravenous fluids contain significant amounts of nitrite, *Free Radic Biol Med* 108 (2017) 533–541. [PubMed: 28416347]
- [33]. Almeida LE, Kamimura S, Kenyon N, Khaibullina A, Wang L, de Souza Batista CM, Quezado ZM, Validation of a method to directly and specifically measure nitrite in biological matrices, *Nitric Oxide* 45 (2015) 54–64. [PubMed: 25445633]
- [34]. Piknova B, Schechter AN, Measurement of nitrite in blood samples using the ferricyanide-based hemoglobin oxidation assay, *Methods Mol Biol* 704 (2011) 39–56. [PubMed: 21161628]
- [35]. Rogers SC, Khalatbari A, Gapper PW, Frenneaux MP, James PE, Detection of human red blood cell-bound nitric oxide, *J Biol Chem* 280 (2005) 26720–8. [PubMed: 15879596]
- [36]. Dejam A, Hunter CJ, Pelletier MM, Hsu LL, Machado RF, Shiva S, Power GG, Kelm M, Gladwin MT, Schechter AN, Erythrocytes are the major intravascular storage sites of nitrite in human blood, *Blood* 106 (2005) 734–9. [PubMed: 15774613]
- [37]. Khaibullina A, Almeida LE, Wang L, Kamimura S, Wong EC, Nouraie M, Maric I, Albani S, Finkel J, Quezado ZM, Rapamycin increases fetal hemoglobin and ameliorates the nociception phenotype in sickle cell mice, *Blood Cells Mol Dis* 55 (2015) 363–72. [PubMed: 26460261]
- [38]. Kenyon N, Wang L, Spornick N, Khaibullina A, Almeida LE, Cheng Y, Wang J, Gupta V, Finkel JC, Quezado ZM, Sickle cell disease in mice is associated with sensitization of sensory nerve fibers, *Exp Biol Med* (Maywood) 240 (2015) 87–98. [PubMed: 25070860]
- [39]. Finkel JC, Besch VG, Hergen A, Kakareka J, Pohida T, Melzer JM, Koziol D, Wesley R, Quezado ZM, Effects of aging on current vocalization threshold in mice measured by a novel nociception assay, *Anesthesiology* 105 (2006) 360–9. [PubMed: 16871071]

- [40]. Finkel J, Guptill V, Khaibullina A, Spornick N, Vasconcelos O, Liewehr DJ, Steinberg SM, Quezado ZM, The three isoforms of nitric oxide synthase distinctively affect mouse nociceptive behavior, *Nitric Oxide* 26 (2012) 81–8. [PubMed: 22202903]
- [41]. Le Bars D, Gozariu M, Cadden SW, Animal models of nociception, *Pharmacol Rev* 53 (2001) 597–652. [PubMed: 11734620]
- [42]. Calhoun G, Wang L, Almeida LE, Kenyon N, Afsar N, Nouraie M, Finkel JC, Quezado ZM, Dexmedetomidine ameliorates nociceptive behavior in humanized sickle cell mice, *Eur J Pharmacol* 754 (2015) 125–33. [PubMed: 25724786]
- [43]. Choi Y, Yoon YW, Na HS, Kim SH, Chung JM, Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain, *Pain* 59 (1994) 369–76. [PubMed: 7708411]
- [44]. Koga K, Furue H, Rashid MH, Takaki A, Katafuchi T, Yoshimura M, Selective activation of primary afferent fibers evaluated by sine-wave electrical stimulation, *Mol Pain* 1 (2005) 13. [PubMed: 15813963]
- [45]. Spornick N, Guptill V, Koziol D, Wesley R, Finkel J, Quezado ZM, Mouse current vocalization threshold measured with a neurospecific nociception assay: the effect of sex, morphine, and isoflurane, *J Neurosci Methods* 201 (2011) 390–8. [PubMed: 21864576]
- [46]. Streiner DL, Best (but oft-forgotten) practices: the multiple problems of multiplicity-whether and how to correct for many statistical tests, *Am J Clin Nutr* 102 (2015) 721–8. [PubMed: 26245806]
- [47]. Rothman KJ, Six persistent research misconceptions, *J Gen Intern Med* 29 (2014) 1060–4. [PubMed: 24452418]
- [48]. Gladwin MT, Kato GJ, Weiner D, Onyekwere OC, Dampier C, Hsu L, Hagar RW, Howard T, Nuss R, Okam MM, Tremonti CK, Berman B, Villella A, Krishnamurti L, Lanzkron S, Castro O, Gordeuk VR, Coles WA, Peters-Lawrence M, Nichols J, Hall MK, Hildesheim M, Blackwelder WC, Baldassarre J, Casella JF, Nitric oxide for inhalation in the acute treatment of sickle cell pain crisis: a randomized controlled trial, *JAMA* 305 (2011) 893–902. [PubMed: 21364138]
- [49]. Park JW, Piknova B, Huang PL, Noguchi CT, Schechter AN, Effect of blood nitrite and nitrate levels on murine platelet function, *PLoS One* 8 (2013) e55699. [PubMed: 23383344]
- [50]. Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozuyaman B, Schnurch HG, Godecke A, Weber AA, Robenek M, Robenek H, Bloch W, Rosen P, Kelm M, Red blood cells express a functional endothelial nitric oxide synthase, *Blood* 107 (2006) 2943–51. [PubMed: 16368881]
- [51]. Kelley EE, Dispelling dogma and misconceptions regarding the most pharmacologically targetable source of reactive species in inflammatory disease, xanthine oxidoreductase, *Arch Toxicol* 89 (2015) 1193–207. [PubMed: 25995007]
- [52]. Peleli M, Zollbrecht C, Montenegro MF, Hezel M, Zhong J, Persson EG, Holmdahl R, Weitzberg E, Lundberg JO, Carlstrom M, Enhanced XOR activity in eNOS-deficient mice: Effects on the nitrate-nitrite-NO pathway and ROS homeostasis, *Free Radic Biol Med* 99 (2016) 472–484. [PubMed: 27609225]
- [53]. Aslan M, Ryan TM, Adler B, Townes TM, Parks DA, Thompson JA, Tousson A, Gladwin MT, Patel RP, Tarpey MM, Batinic-Haberle I, White CR, Freeman BA, Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease, *Proc Natl Acad Sci U S A* 98 (2001) 15215–20. [PubMed: 11752464]
- [54]. Gladwin MT, Shelhamer JH, Schechter AN, Pease-Fye ME, Wacławiw MA, Panza JA, Ognibene FP, Cannon RO 3rd, Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans, *Proc Natl Acad Sci U S A* 97 (2000) 11482–7. [PubMed: 11027349]
- [55]. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, Kelm M, Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action, *Proc Natl Acad Sci U S A* 98 (2001) 12814–9. [PubMed: 11606734]
- [56]. Rassaf T, Bryan NS, Maloney RE, Specian V, Kelm M, Kalyanaraman B, Rodriguez J, Feelisch M, NO adducts in mammalian red blood cells: too much or too little?, *Nat Med* 9 (2003) 481–2; author reply 482–3. [PubMed: 12724738]

- [57]. Elias DB, Rocha LB, Cavalcante MB, Pedrosa AM, Justino IC, Goncalves RP, Correlation of low levels of nitrite and high levels of fetal hemoglobin in patients with sickle cell disease at baseline, *Rev Bras Hematol Hemoter* 34 (2012) 265–9. [PubMed: 23049438]
- [58]. Nader E, Grau M, Fort R, Collins B, Cannas G, Gauthier A, Walpurgis K, Martin C, Bloch W, Poutrel S, Hot A, Renoux C, Thevis M, Joly P, Romana M, Guillot N, Connes P, Hydroxyurea therapy modulates sickle cell anemia red blood cell physiology: Impact on RBC deformability, oxidative stress, nitrite levels and nitric oxide synthase signalling pathway, *Nitric Oxide* 81 (2018) 28–35. [PubMed: 30342855]
- [59]. Cokic VP, Beleslin-Cokic BB, Tomic M, Stojilkovic SS, Noguchi CT, Schechter AN, Hydroxyurea induces the eNOS-cGMP pathway in endothelial cells, *Blood* 108 (2006) 184–91. [PubMed: 16527893]
- [60]. Cokic VP, Andric SA, Stojilkovic SS, Noguchi CT, Schechter AN, Hydroxyurea nitrosylates and activates soluble guanylyl cyclase in human erythroid cells, *Blood* 111 (2008) 1117–23. [PubMed: 17993617]
- [61]. da Guarda CC, Santiago RP, Pitanga TN, Santana SS, Zanette DL, Borges VM, Goncalves MS, Heme changes HIF- α , eNOS and nitrite production in HUVECs after simvastatin, HU, and ascorbic acid therapies, *Microvascular Research* 106 (2016) 128–136. [PubMed: 27089822]
- [62]. Potoka KP, Wood KC, Baust JJ, Bueno M, Hahn SA, Vanderpool RR, Bachman T, Mallampalli GM, Osei-Hwedie DO, Schrott V, Sun B, Bullock GC, Becker-Pelster EM, Wittwer M, Stampfuss J, Mathar I, Stasch JP, Truebel H, Sandner P, Mora AL, Straub AC, Gladwin MT, Nitric Oxide-Independent Soluble Guanylate Cyclase Activation Improves Vascular Function and Cardiac Remodeling in Sickle Cell Disease, *Am J Respir Cell Mol Biol* 58 (2018) 636–647. [PubMed: 29268036]
- [63]. Conran N, Oresco-Santos C, Acosta HC, Fattori A, Saad ST, Costa FF, Increased soluble guanylate cyclase activity in the red blood cells of sickle cell patients, *Br J Haematol* 124 (2004) 547–54. [PubMed: 14984506]
- [64]. Tsutamoto T, Kinoshita M, Ohbayashi Y, Wada A, Maeda Y, Adachi T, Plasma arteriovenous cGMP difference as a useful indicator of nitrate tolerance in patients with heart failure, *90* (1994) 823–829.
- [65]. Vogel S, Arora T, Wang X, Mendelsohn L, Nichols J, Allen D, Shet AS, Combs CA, Quezado ZMN, Thein SL, The platelet NLRP3 inflammasome is upregulated in sickle cell disease via HMGB1/TLR4 and Bruton tyrosine kinase, *Blood Adv* 2 (2018) 2672–2680. [PubMed: 30333099]
- [66]. Weiner DL, Hibberd PL, Betit P, Cooper AB, Botelho CA, Brugnara C, Preliminary assessment of inhaled nitric oxide for acute vaso-occlusive crisis in pediatric patients with sickle cell disease, *Jama* 289 (2003) 1136–42. [PubMed: 12622584]
- [67]. Head CA, Swerdlow P, McDade WA, Joshi RM, Ikuta T, Cooper ML, Eckman JR, Beneficial effects of nitric oxide breathing in adult patients with sickle cell crisis, *85* (2010) 800–802.
- [68]. Kohli DR, Li Y, Khasabov SG, Gupta P, Kehl LJ, Ericson ME, Nguyen J, Gupta V, Hebbel RP, Simone DA, Gupta K, Pain-related behaviors and neurochemical alterations in mice expressing sickle hemoglobin: modulation by cannabinoids, *Blood* 116 (2010) 456–65. [PubMed: 20304807]
- [69]. Lundberg JO, Weitzberg E, Gladwin MT, The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics, *Nat Rev Drug Discov* 7 (2008).

HIGHLIGHTS

- Sickle cell disease (SCD) is thought to be associated with nitric oxide (NO) deficit.
- SCD subjects and SCD mouse models have elevated blood nitrite and cGMP levels.
- Nitrite supplementation increased nitrite levels but does not change pain phenotype in mice
- SCD mice have increased bleeding time, which are decreased with NO scavengers
- These findings indicate that clinical trials seeking to increase nitrite in SCD should be reconsidered.

Figure 1. Nitrite levels are elevated in blood compartments of sickle cell disease (SCD) subjects independently of pain burden

Dot density plot and median nitrite levels in whole blood (A and D), red blood cells (B and E), and plasma (C and F) in controls and SCD subjects. The control group includes nine subjects (five African-Americans and four Caucasians) who had similar nitrite levels in all blood compartments ($p=1.00$ for whole blood and red blood cell and $p=0.071$ for plasma) and are shown combined. Compared to controls, SCD participants with low- ($n=13$) and high-pain ($n=13$) burden had significantly higher nitrite levels in whole blood and red blood cell (A and B). In plasma (C), SCD participants with high-, but not those with low-pain

burden, had higher nitrite levels compared to controls. Because SCD participants with high- and low-pain burden had similar nitrite levels in whole blood, red blood cell, and plasma ($p=1.00$, Fig 1A-C)], data was combined as the SCD group. Compared to controls ($n=9$), SCD participants ($n=26$) had significantly higher nitrite levels in whole blood (D, $p<0.001$), red blood cell (E, $p<0.001$), and plasma (F, $p=0.014$). The horizontal bar in this and successive graphs, when applicable, depicts the median. Statistical analysis was conducted with a non-parametric test, Kruskal-Wallis test, (one-way ANOVA on ranks).

Figure 2. Hydroxycarbamide (HU) intake has no significant impact on nitrite levels in blood compartments

Dot density plot and median hemoglobin F (HbF) percentages (A and B) and nitrite levels in whole blood (C), red blood cell (D), and plasma (E) in sickle cell disease (SCD) subjects taking or not taking HU. As expected, SCD subjects on HU had higher HbF percentage compared to those who were not on the drug (A, $p=0.011$). B. Percentages of HbF in SCD subjects with low and high-pain burden were similar ($p=0.531$). Noticeably, in all blood compartments [whole blood (C, $p=0.222$), red blood cell (D, $p=0.243$), and plasma (E, $p=0.182$)] nitrite levels in subjects taking HU were similar to those in subjects not taking HU (C, D, and E).

Figure 3. Participants with sickle cell disease (SCD) have higher cyclic guanosine monophosphate (cGMP) in blood compartments compared to controls

Dot density plot and median cGMP levels in controls and SCD participants in red blood cell (RBC, A) and plasma (B). Subjects with SCD had significantly higher red blood cell (A, $p=0.025$) and plasma (B, $p=0.007$) cGMP levels compared to controls. Overall, the median red blood cell and plasma cGMP levels in SCD subjects were ≈ 1.6 fold higher than that in normal volunteers.

Figure 4. Nitrite metabolism in control and sickle cell disease (SCD) subjects

Results are shown as least square means $\pm$ standard error of the mean. A. After addition of nitrite (10 μ M) to whole blood, there were significant decreases in nitrite levels over 120 min, which varied according to genotype as there were genotype by time interactions ($p=0.004$). After addition of nitrite, there were less decreases from initial nitrite levels in whole blood from SCD subjects ($n=10$) compared to controls ($n=5$) at 15 ($p=0.042$), 30 ($p=0.001$), 60 ($p<0.001$), and 120 min ($p=0.006$). B. When plasma was removed and red blood cells (RBC) from controls ($n=8$) and SCD subjects ($n=9$) were diluted to the same hemoglobin levels (2–

3 g/dL), after addition of nitrite (10 μ M), there were decreases in nitrite levels overtime ($p<0.001$), which were independent of genotype as there were no genotype by time interaction ($p=0.298$). C. When nitrite was added to plasma from controls ($n=8$) or SCD subjects ($n=9$), there were no changes in nitrite levels overtime ($p=0.337$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. Nitrite and cyclic guanosine monophosphate (cGMP) levels in blood compartments from humanized sickle cell disease (SCD) mice

Dot density plot and median nitrite levels (A and B) and cGMP levels in blood compartments and tissue (C to H). A. In Townes mice whole blood nitrite levels were significantly higher in homozygotes compared with heterozygotes ($p=0.006$) and control Townes ($p<0.001$). B. Similarly, BERK homozygous mice had higher whole blood nitrite levels compared to hemizygous ($p=0.021$) and respective control (C57BL/6J) mice ($p=0.005$). Regarding downstream effects of NO, compared to controls, homozygous Townes, had significantly higher cGMP levels in whole blood ($p=0.002$, C), red blood cell

($p=0.001$, D), and plasma ($p=0.003$, E). Overall, median whole blood, red blood cell and plasma cGMP levels were 9–13 fold higher in Townes homozygous mice as compared to control animals. F. Kidney levels of cGMP were significantly higher (≈ 9 fold) in Townes homozygotes compared to control animals ($p=0.038$). In a study of the effects of long term nitrite supplementation, nitrite treatment was associated with significant increases in cGMP levels in red blood cell (G) and plasma (H) cGMP levels (both $p<0.001$, for overall effect of treatment). P values reflect-post hoc comparisons with respective control groups.

Figure 6. Acute inhibition of nitric oxide synthase and xanthine oxidoreductase alter nitrite levels in SCD mice

Dot density plot and median nitrite levels in blood compartments of controls (A, B, and C) and Townes homozygous mice (D, E, and F) after acute administration of L-N⁶-(1-iminoethyl)lysine (L-NIL), an NOS2 inhibitor, N^ω-Nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor, or allopurinol, a xanthine oxidoreductase inhibitor. P values reflect ad hoc comparison between respective treatment and vehicle for given genotypes. The effect of L-NIL, L-NAME, and allopurinol varied according to genotype and blood compartment. In control mice, L-NIL, L-NAME, and allopurinol treatments were not

associated with significant changes in nitrite levels in any blood compartment compared with vehicle (all $p > 0.109$, A-C). D. Compared with vehicle, in whole blood, overall L-NIL-, L-NAME, and allopurinol treatments were associated with decreases in nitrite levels in homozygous but not in control Townes in a significantly different effect pattern (treatment by genotype interaction in whole blood, $p=0.018$). E. In red blood cells, compared with vehicle, treatment with L-NAME and allopurinol, but not L-NIL, were associated with decreases in nitrite levels in homozygotes but not in control Townes in a significantly different effect pattern (treatment by genotype interaction in RBC, $p<0.001$). F. In plasma from homozygotes, compared with vehicle, treatment with L-NIL and allopurinol, but not L-NAME, were associated with decreases in nitrite levels.

Figure 7. Effect of nitrite supplementation on hematologic parameters in Townes mice

Results are shown as least-square means $\pm$ 95% confidence interval. Hetero represent heterozygotes and homo, homozygotes. In keeping with previous reports [31; 37; 38], homozygous Townes had anemia (A), leukocytosis (B), and thrombocytosis (C). Compared with vehicle, nitrite treatment had no effect on red blood cell (A) or white blood cell (B) counts, regardless of genotype. C. In contrast, the effect of nitrite on platelet count varied according to genotype as there were treatment by genotype interactions ($p < 0.0001$, for interaction). Specifically, compared to respective vehicle-treated mice, nitrite-treated controls had similar platelet counts ($p = 0.342$), whereas nitrite-treated heterozygotes ($p = 0.0014$) and homozygotes ($p < 0.0001$) had significantly lower platelet counts and these nitrite effect patterns were significantly different. In all genotypes combined, nitrite-treated animals, compared with vehicle-treated, had lower hemoglobin ($p < 0.0001$, for overall treatment effect, D) and hematocrit ($p = 0.0195$, for overall treatment effect, E). F. Regarding mean corpuscular volume (MCV), among vehicle-treated animals, homozygous mice had significantly higher MCV compared to controls and heterozygotes ($p < 0.0001$). Additionally, there was an overall effect of nitrite treatment on MCV as nitrite-treated mice had overall lower MCV compared to vehicle-treated animals ($p = 0.003$, for overall treatment effect in all genotypes combined). G. The effect of treatment on mean corpuscular hemoglobin concentration varied according to genotype. Specifically, compared to respective vehicle-treated mice, controls ($p < 0.0001$) and heterozygotes ($p = 0.009$) had significantly lower, whereas nitrite-treated homozygotes had similar mean corpuscular hemoglobin concentration ($p = 0.554$), and these nitrite effect patterns were significantly different ($p = 0.0058$, for treatment by genotype interactions). Symbols above nitrite-treated bar reflect

post-hoc comparisons with respective vehicle-treated group within a given genotype. * reflects $p < 0.05$, † reflects $p = 0.01$, ‡ reflects $p = 0.001$, and § reflects $p = 0.0001$. Hetero represents heterozygote and homo homozygote.

Figure 8. Acute nitrite supplementation and nitric oxide (NO) scavenging alter bleeding time in controls and homozygous mice

Dot density plot and median bleeding times. P values reflect comparisons between indicated groups. Hetero represent heterozygotes, homo, homozygotes, and c-PTIO, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium. Bleeding times were examined to evaluate physiologic effects of nitrite/NO on natural hemostasis in Townes mice. During basal conditions, Townes homozygous had longer bleeding times compared to controls ($p<0.001$). As expected, acute injection of nitrite, compared with vehicle, yielded significant prolongation of bleeding time in control mice ($p=0.012$, Fig 8). As homozygous mice have elevated nitrite levels and nitrite can serve as a NO reservoir, we then tested the hypotheses that injection of an NO scavenger would decrease bleeding time. After an injection of c-PTIO, homozygous Townes had significantly lower bleeding time compared with vehicle-injected homozygotes ($p=0.003$).