CLINICAL RESEARCH PROTOCOL

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

DATE: December 18, 2025

PROTOCOL NUMBER: 14-DK-0060

Protocol Title: Famine from Feast: Linking Vitamin C, Red Blood Cell Fragility, and Diabetes

Short Title: Inpatient Diabetes Study

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Precis:

Diabetes type two is a debilitating disease that leads to chronic morbidity such as accelerated microvascular disease. Accelerated microvascular disease may produce blindness, end stage renal disease, myocardial infarction, stroke, and limb ischemia. Strategies to prevent or delay microvascular disease have the potential to improve the lives of millions and prevent catastrophic illness. The major focus of prevention of microvascular disease in diabetes has been on the endothelium and its role in protection of blood vessels. An unexpected means to prevent microvascular disease in diabetes may be coupled to the function of vitamin C in red blood cells (RBCs) of diabetic subjects. Based on new and emerging data, vitamin C concentrations in RBCs may be inversely related to glucose concentrations found in diabetes. Based on animal data, we hypothesize that RBCs with low vitamin C levels may have decreased deformability, leading to slower flow in capillaries and microvascular hypoxia, the hallmark of diabetic microangiopathy. Low vitamin C concentrations in RBCs of diabetic subjects may be able to be increased, by using vitamin C supplements. Findings in animals may not accurately reflect effects in humans because of species differences in mechanisms of vitamin C entry into RBCs. Therefore, clinical research is essential to characterize vitamin C physiology in RBCs of diabetic subjects. In this protocol we will investigate physiology of vitamin C in RBCs of diabetic subjects as a function of glycemia, without vitamin C supplementation (stage 1) and with vitamin C supplementation (stage 2). We will screen type II diabetic subjects on insulin and/or oral hypoglycemic medication(s) and select those with hemoglobin A1C concentrations of $\leq 12\%$. To investigate how response to the nutritional interventions in individuals with diabetes varies from normal, nondiabetic controls will also be recruited and studied. Selected subjects will be hospitalized twice, each time for approximately one week. The primary objective of the first hospitalization (stage 1) will be to evaluate the effect of hyperglycemia on vitamin C RBC physiology regardless of baseline vitamin C concentrations (without any vitamin C supplementation). The second hospitalization (stage 2) investigates the effect (if any) of vitamin C supplementation to changes in RBC physiology during periods of normal (euglycemic) and elevated (hyperglycemic) glucose concentrations. As inpatients, subjects will have two venous sampling periods each of approximately 24 hours.

On admission, subjects may be fitted with continuous glucose monitors (CGMs), subjects will be transitioned to an individualized inpatient diabetes regimen determined by investigators, based on pre-admission diabetes regimen and glycemic control. For participants with diabetes, the inpatient diabetes regimen will be titrated to achieve euglycemia (fasting and pre-meal glucoses <140mg/dl) prior to the first sampling period (euglycemic sampling). The first sampling period will be performed under conditions of euglycemic control for 24 hours. The second sampling period will be performed under controlled hyperglycemia induced by decreasing doses of the diabetes regimen and providing a high carbohydrate load diet (70-75% carbohydrate). Correction-scale insulin will be provided for glucoses >350-400mg/dl. Hyperglycemia will not exceed 9 hours and will be reversed by reinstituting insulin. For nondiabetic controls, an oral glucose tolerance test (75 grams dextrose) will be administered on admission. Controls will receive the same metabolic diets and undergo the sampling schedule as the cohort with diabetes. During the two sampling periods, samples will be withdrawn via venous catheter for RBC deformability, vitamin C concentrations and other related research studies. Following completion of stage 1, subjects considered for participation in stage 2 will be provided a prescription for vitamin C 500mg twice daily. Given that vitamin C and vitamin E are related antioxidants, and that both vitamins appear to be associated with RBC rigidity, diabetic subjects

may also be given a prescription for 400 international units (IU) of vitamin E (RRR alpha tocopherol) daily. Subjects will continue vitamin C and E supplementation for a minimum of 8 weeks depending on RBC vitamin C concentrations. To evaluate any effect of vitamin E supplementation, plasma and RBC vitamin E levels may be measured concurrently with vitamin C levels during various phases of stages 1 and 2. All subjects will be seen as outpatients at biweekly or monthly intervals with regular measurement of plasma and/or RBC vitamin C concentrations. Target RBC vitamin C concentration >30uM is required prior to stage 2 inpatient sampling studies. Vitamins C and E supplementation will be discontinued upon inpatient admission for stage 2. Risk of both vitamin supplements are minimal as both supplementation doses are safe. Outcomes are to measure RBC rigidity and vitamin concentrations before and after supplementation. After a minimum of 8 weeks (depending on RBC vitamin C levels), subjects will be hospitalized again, and sampling repeated as described. In this manner, each subject serves as his/her own control, and deformability of red blood cells can be determined in relation to glycemia and to vitamin C concentrations in RBCs and plasma. Subjects will be required to consume standardized meals during inpatient stays. All meals will be prepared by the NIH Clinical Center Metabolic Kitchen. To avoid obscuring plasma vitamin C changes that may result from hyperglycemia, dietary vitamin C content will be approximately 30-35 mg per meal. Additionally, to avoid confounding vitamin E measurements, diets will provide approximately 6 mg alpha tocopherol per day. Standardized meals at the 2nd inpatient admission will be provided to match what was consumed by the subject at their 1st inpatient admission.

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1. Introduction:

Diabetes type 2 is an epidemic public health issue with grave consequences if left untreated. Untreated diabetes results in accelerated microvascular disease and chronic debilitating morbidities and mortality. Diabetic microvascular angiopathy is the leading cause of blindness, end stage renal disease and amputations worldwide, as well as myocardial infarction, stroke and peripheral arterial disease.

Strategies to prevent or delay microvascular disease could improve the lives of millions and prevent catastrophic illness. To date, the focus of prevention of microvascular disease in diabetes has been on the endothelium and its role in protection of blood vessels. Vascular smooth muscle abnormalities, platelet dysfunction, abnormal coagulation and impaired vascular repair are other major pathologies proposed to lead to diabetic vasculopathy (Beckman 2002, Cubbon 2013). However, the pathogenesis of microangiopathy in diabetes remains largely unknown.

It is not often appreciated that diabetes affects RBC structure and function. Surprisingly, a large body of evidence indicates that diabetes leads to a progressive decline in RBC deformability (Peterson 1976, McMillan 1978, Kamada 1991, Virtue 2004, Diamantopoulos 2004, Brown 2005, Shin 2007, Kung 2009, Keymel 2011, Buys 2013). RBC deformability measures the ability of the cell to change shape upon entering into capillaries. RBC deformability is vital to RBC function and plays a major role in microvascular flow. Impaired deformability adversely affects capillary perfusion (Simchon 1987, Parthasarathi 1998).

Impaired deformability of diabetic RBCs leading to blood flow disturbance was linked to greater morbidity of diabetic patients with coronary artery disease (Keymel 2011). Others reported impaired RBC function and deformability to be a consequence of hyperglycemia and suggested its use as a measure of disease severity (Shin 2006 & 2007). Consistent with decreased deformability, increased RBC fragility and decreased RBC survival in uncontrolled diabetes contributed to organ failure (Kung 2009). RBC lifespan was shortened in diabetic patients and improved with diabetes control (Peterson 1976). Anemia is twice as common in patients with diabetes compared to control subjects, although anemia in diabetes has multiple causes. Anemia in diabetes was associated with increased cardiovascular morbidity and mortality, retinopathy and foot ulcers (Thomas 2005). Additional reports showed a direct correlation between decreased RBC deformability and severity of diabetic microangiopathy, especially with retinopathy and nephropathy (Parthasarathi 1998).

There have been few hypotheses that focus on RBCs and their ability to deliver oxygen as contributory to diabetic vascular disease. Despite existing deformability data, there has been little focus on the importance of decreased deformability of RBCs in diabetes, or how defects in deformability could contribute to diabetes vascular complications, other than to describe possible associations. Similarly, improving RBC deformability in diabetes has not been an area of focus. This is due, in part, to the current lack of understanding of the underlying pathophysiology leading to RBC stiffness in diabetes. A sluggish microcirculation as a result of stiffer RBCs compromises tissue oxygenation. Thus, strategies that improve RBC deformability could modify microvascular hypoxia, with significant clinical implications.

RBC deformability has been difficult to measure reliably. One simple test is based on osmotic fragility (Parpart 1947), although the test is time consuming and cumbersome and does not provide full information. Many prior studies of RBC deformability used sedimentation or other indirect measurement techniques, because of simplicity and low cost. Unfortunately, the results of these tests may be subjective and not reproducible. The best technique to quantitate deformability, ektacytometry, has been used infrequently because of high cost of the instrument,

need for a highly skilled operator, and difficulty of the instrumentation itself. Recent advances in ektacytometery have improved operation and make the technique more feasible, if cost can be surmounted (Baskurt 2009, Musielak 2009). Osmotic fragility measurements are incorporated in current ektacytometer measurements. Previous research has shown that RBC deformability and hemolysis, as measured by ektacytometry and osmotic fragility testing are interrelated. Impaired RBC deformability caused a reduction in cell survival (Clark 1983). Using a modern ektacytometer, other measurements related to RBC deformability can be obtained, including elongation properties, membrane stability and rouleaux formation.

There are several possibilities that suggest links between RBCs, vitamin C, and diabetes. Multiple studies reported lower vitamin C levels in diabetic subjects, especially those with microvascular complications such as retinopathy and nephropathy (Som 1981, Ali 1989, Sinclair 1991, Will 1996, Lindsay 1998, Cunningham 1998, Chen 2005). Many of these reports, unfortunately, relied on vitamin C assays that had substantial artifact, making interpretation of findings difficult (Will 1996, Padayatty 2003). In an early hypothesis, it was proposed that diabetic vascular disease might result from a vitamin C deficient state (Mann 1975). However, the hypothesis lacked specific mechanism links between vitamin C and diabetes and had minimal supporting evidence. In one study, low vitamin C levels in patients with diabetes were associated with presence of microvascular disease, but data were again confounded by assay artifact (Ali 1989).

Another possible connection between vitamin C and diabetes has a direct clinical basis. Consistent with a hypothesized role for a RBC deformability defect in diabetes, anemia and hemolysis are clinical manifestations of vitamin C deficiency (Hart 1964, Cox 1968). Unfortunately, direct deformability measurements are not available in patients with low vitamin C levels. Published clinical data, from case reports, are confounded because it was likely that patients had multiple vitamin deficiencies. In epidemiologic studies, vitamin C deficiency was associated with increased risk of stroke (He 2010). Case reports and epidemiologic data are consistent with a postulated link between RBCs vitamin C and diabetes, but neither data set is definitive. Causality is not established by observational data or associations alone. Other limitations of the findings are that moderate to severe deficiency was needed before effects were observed.

A mechanism link between RBCs, vitamin C and diabetes is possible based on transport pathways, particularly the structural similarity between oxidized vitamin C (dehydroascorbic acid, DHA) and glucose. As background, vitamin is transported by two sodium-dependent transporters, encoded by genes which express transporter proteins SLC23A1 and SLC23A2. Data from mice in which the genes for either SLC23A1 or SLC23A2 were knocked out showed that vitamin C entered nearly all tissues as ascorbic acid, via sodium-dependent transporters (Levine 2011). In contrast, RBCs only seemed to transport oxidized ascorbic acid (dehydroascorbic acid, DHA) via facilitated glucose transporters (GLUTs) (Hughes 1968, Vera 1993, Rumsey 1997, Liang 2001). Indeed, RBCs lack sodium-dependent vitamin C transporters SLC23A1 and SLC23A2 (May 2007). Once DHA enters cells, it is reduced immediately to ascorbate, such that intracellular DHA is not detectable by current methods. Previous studies described DHA and ascorbic acid transport mechanisms in RBCs (Montel-Hagen 2008, May 2001 & 2011). However, major limitations of these studies are that assays for RBC ascorbate and DHA have been poor, until the past year (Li 2012). For this reason, no data are available using physiologic concentrations of ascorbic acid and DHA to measure transport into RBCs. Without a sound assay for vitamin C in RBCs, false conclusions can be reached (Montel-Hagen 2008,

Carruthers 2009).

A new assay has become available to measure vitamin C in RBCs (Li 2012). This assay can be used to probe intracellular RBC vitamin C concentrations in healthy patients and those with diabetes (See Fig. 1; discussed in preliminary data).

There are at least 3 independent reasons as to why vitamin C concentrations in RBCs may be lower in diabetic patients compared to healthy subjects: glucose may compete with DHA for entry (Fig 1, left branch): ascorbate may be oxidized within red cells after DHA entry and internal RBC reduction, due to accelerated sorbitol formation (Fig 1, left branch); and plasma ascorbate concentrations may be low in diabetic subjects, so that less DHA forms and enters RBCs (Fig 1, right branch). With respect to competition between DHA and glucose, DHA in RBCs is transported by GLUTs. GLUTs are sodium-independent facilitated glucose transporters. This mechanism is distinct from ascorbate transport by sodium dependent transporters SLC23A1 and SLC23A2 (Wilson 2005). Using expressed transporter systems, data showed that GLUTs transport DHA with affinities higher than that of glucose itself, and DHA transport by GLUTs was inhibited by glucose (Vera 1993, Rumsey 1997, Corpe 2013). Whether competition between DHA and glucose occurs in human RBCs has never been addressed with direct measurements and proper controls (Montel-Hagen 2008, Carruthers 2009, Li 2012). A second mechanism to explain lower vitamin C RBC concentrations in diabetic subjects is a coupled to accelerated oxidation of ascorbic acid, after DHA transport and internal reduction to ascorbate. Oxidation within the RBC could occur via the sorbitol pathway, or by action of other oxidants generated by hyperglycemia. The last possibility to account for low vitamin C levels in RBCs of diabetic subjects is decreased substrate availability, possibly via a renal leak. If less ascorbate is available in plasma, less is available to oxidize to DHA. Ascorbate in plasma is filtered at the renal glomerular capillary tuft and reabsorbed via the sodium-dependent transporter SLC23A1 located on proximal tubular cells. Normally, above a certain threshold of serum vitamin C, tubular transporters are saturated and vitamin C is lost in the urine. However, patients with diabetes may have increased renal clearance of vitamin C (Iino 2005, Chen 2006), perhaps due to increased glomerular filtration rate (GFR) seen in early diabetes or to tubule disease that occurs as part of diabetic nephropathy. One group reported that patients with chronic kidney disease have low levels of plasma vitamin C, and that these had lower vitamin C levels at any given GFR compared with non-diabetic patients with chronic kidney disease (Takahashi 2010). In another report, low vitamin C levels were found in diabetic subjects at very early stages of microalbuminuria, before overt nephropathy (Hirsch 1998). Unfortunately, in studies describing how the kidney handled vitamin C in diabetes, only indirect measurement techniques were used. Further, there were no simultaneous measurements of urine and plasma vitamin C determinations, which are necessary to assess renal clearance.

These multiple mechanisms make it plausible that hyperglycemia from diabetes could lead to local RBC vitamin C deficiency, thus predisposing RBCs to less deformability. Less deformable RBCs will travel through capillaries slower, thereby impairing tissue oxygenation and initiating microvascular damage (Fig. 1). Our hypothesis (Fig 1) is novel and represents a unique perspective compared to the current paradigm: a shift of emphasis from endothelium to the RBCs as the culprit in diabetic microvascular disease. In ongoing experiments (described below, see supporting evidence), low vitamin C concentrations were found in human RBCs from healthy blood donors, as a function of plasma concentrations (Fig. 2A). These data show that low vitamin C levels in RBCs are possible and do occur. Similarly, low RBC vitamin C concentrations in mice result in more fragile RBCs, as measured by osmotic fragility which is

correlated with deformability as discussed above. Vitamin C concentrations in RBCs from mice are inversely related to blood glucose in vivo (Fig. 5C, see supporting evidence below).
The proposed study will enable us to investigate whether localized RBC vitamin C deficiency is present in human with diabetes. We will also test in humans, in a dynamic fashion, whether there is an inverse relationship between hyperglycemia and RBC vitamin C content. Concurrently, we will use the unique ektacytometer available at our lab to measure RBC deformability before and after vitamin C supplementation. Renal clearance with respect to vitamin C will also be quantitated. Together, the data obtained will provide a new understanding of vitamin C physiology in subjects with diabetes, regarding both RBC function and renal function. Outcomes of our study, if the hypothesis holds true, have enormous potential for future prevention of diabetic microvascular disease. If RBC deformability improves with vitamin C, then supplementation could be a preventative measure, to decrease microvascular complications. Vitamin C supplementation is simple, with minimal cost and risk. Thus, this line of clinical investigation has great potential for public health benefit, including relieving and reducing suffering in addition to reducing health care costs. The proposed study will provide new physiologic dynamic data about vitamin C in subjects with diabetes. Furthermore, as mentioned before, RBCs with low vitamin C concentrations result in the phenotype of increased fragility and hemolysis in mice, but this has been observed in human RBCs. Human RBC handles oxidized vitamin C differently compared to mice, therefore we do not know if our hypothesis holds true in humans, especially in subjects with diabetes. Whether the findings from mice apply to humans is one essential reason for conducting this study. The data obtained may serve as an essential foundation for future intervention studies.

2. Objectives:

2.1. General aim:

In subjects with type 2 diabetes and healthy volunteers, characterization of RBC physiologic properties in relation to: vitamin C concentrations in RBCs and plasma; concurrent glucose concentrations in plasma; and vitamin C urine clearance.

2.2.Specific objectives:

2.2.1. Primary goals:

a) Whether RBCs have low vitamin C concentrations in patients with poorly controlled diabetes, as measured by Hba1C.
b) Whether ascorbate in RBCs of diabetic subjects is inversely related to acute glycemic control, over hours.
c) Whether acute glycemic control affects urinary leakage of ascorbate (vitamin C).
d) Whether acute changes in glycemia and/or red blood cell ascorbate (vitamin C) modify RBC deformability.

2.2.2. Secondary goals:

a) Whether RBC vitamin C concentrations can be increased by vitamin C supplementation over several weeks in diabetic subjects.
b) Whether RBC deformability is affected by vitamin C supplementation.

We plan the following procedures per protocol:

1. Measurement of vitamin C concentrations in RBC and plasma in participants with type 2 diabetes and nondiabetic controls.

2. RBC deformability, osmotic fragility, rouleaux formation, and aggregation will be measured to determine whether these measures are associated with hyperglycemia and RBC ascorbate concentrations.

A. We will screen outpatient type 2 diabetic subjects with HgA1C of $\leq$ 12% and nondiabetic controls.

B. Selected subjects will be hospitalized at the Clinical Center. Upon admission, subjects will be transitioned to an individualized inpatient diabetes regimen determined by investigators, based on pre-admission glycemic control and diabetes regimen. Oral hypoglycemic medication(s) may be withheld on admission, and patients may be transitioned to insulin-based therapies. Once adequately controlled on insulin regimens, euglycemic sampling will be performed via indwelling venous catheter. Glucose and ascorbate will be measured hourly for a maximum of 9 hours, then every 3-4 hours afterwards. After completion of euglycemic sampling (24 hours), insulin regimen will be withheld to produce controlled hyperglycemia < 400 mg/dl for a maximum of 9 hours. Subjects will be provided a high carbohydrate diet (70-75% carbohydrate) on the day of hyperglycemia. Sampling scheme described above will be repeated for hyperglycemic sampling.

C. We will measure RBC deformability in these subjects under conditions of hyperglycemia and euglycemia. Ascorbate will be measured in plasma and RBCs. The measurements will allow us to determine whether hyperglycemia is inversely associated with RBC vitamin C concentrations.

D. After discharge, subjects considered for participation in stage 2 will be supplemented with oral vitamin C 500 mg twice daily for a minimum of 8 weeks. Once RBC vitamin C concentrations are > 30uM, subjects may be eligible for stage 2 inpatient study.

E. Given that vitamin C and vitamin E are related antioxidants, and that both vitamins appear to be associated with RBC rigidity, stage 2 participants will also be supplemented with 400 IU (international units) of vitamin E (RRR alpha tocopherol).

F. Supplemented subjects will be re-hospitalized and the measurements in sections B and C repeated.

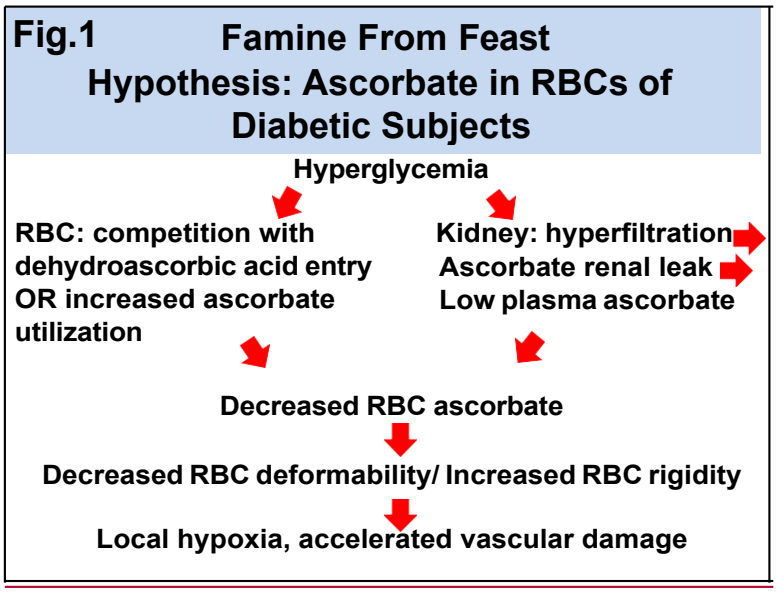
3. Quantitation of vitamin C urine concentrations in subjects with type 2 diabetes and nondiabetic controls as a function of plasma and RBC vitamin C, plasma glucose, insulin concentrations, creatinine clearance, and presence or absence of proteinuria. We will determine renal ascorbate threshold and ascorbate clearance in in-patient subjects with diabetes by timed measurements.

4. Determination of whether there is an inverse relationship between RBC ascorbate and RBC sorbitol. Samples will be obtained and stored for future sorbitol analyses.

5. In addition, blood samples will be obtained for measurement of RBC vitamin E levels.

3. Hypothesis:

We propose that in diabetes (“feast”) there is localized deficiency of vitamin C (“famine”) in RBCs, resulting in decreased RBC deformability. If true, aberrant RBC deformability in diabetes may be reversed with vitamin C supplementation. It follows that if vitamin C supplementation can restore RBC deformability, it also has the potential to prevent or diminish diabetic vascular complications by improving oxygen delivery by RBCs and thereby reversing microvascular hypoxia (Fig.1).



4. Supporting evidence:

The hypothesis in Fig 1 is supported by preliminary and published data (Li 2012). We examined whether low RBC ascorbate, as well as plasma ascorbate, is found in humans and mice. The results show that plasma vitamin C levels of some random healthy subjects were very low, and that human RBC ascorbate was a function of plasma vitamin C concentration (Fig. 2A) (Li 2012). The difference between RBCs and other cell types is seen clearly when neutrophil vitamin C concentrations (Levine 1996 & 2001) as a function of plasma ascorbate are compared to erythrocyte vitamin C concentrations as a function of plasma ascorbate in healthy subjects (Fig. 2B). Normal mice and those unable to make vitamin C ($gulo^{-/-}$) also show a linear relationship between RBC and plasma ascorbate (Fig 2C).

Note: in these and all figures, symbols represent mean +/- S.D.

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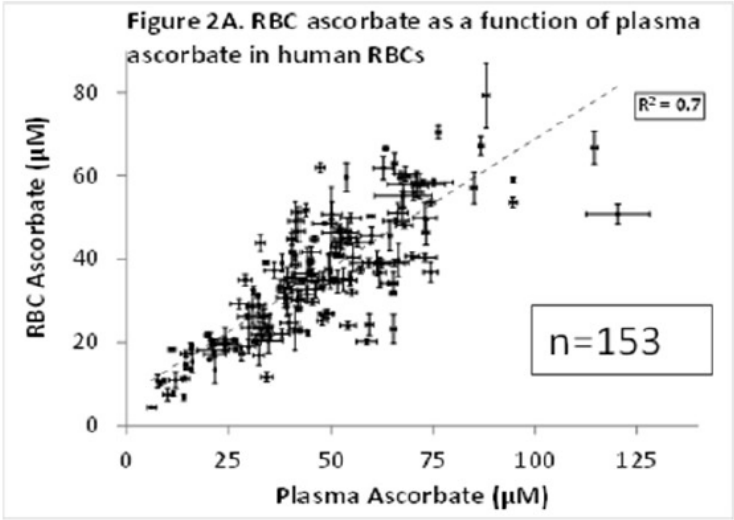


Figure 2A.
RBC ascorbate as a function of plasma ascorbate in human RBCs. The results show that plasma vitamin C levels of some random healthy subjects were very low, and that human RBC ascorbate was a function of plasma vitamin C concentration.

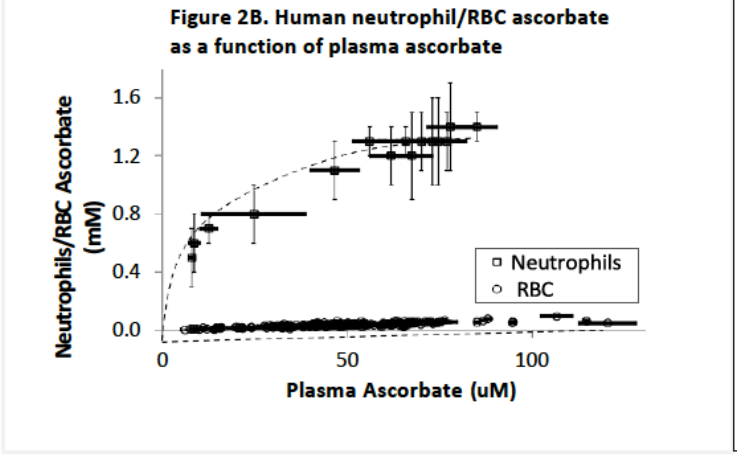


Figure 2B.
The difference between RBCs and other cell types is seen clearly when neutrophil vitamin C concentrations as a function of plasma ascorbate are compared to erythrocyte vitamin C concentrations as a function of plasma ascorbate in healthy subjects

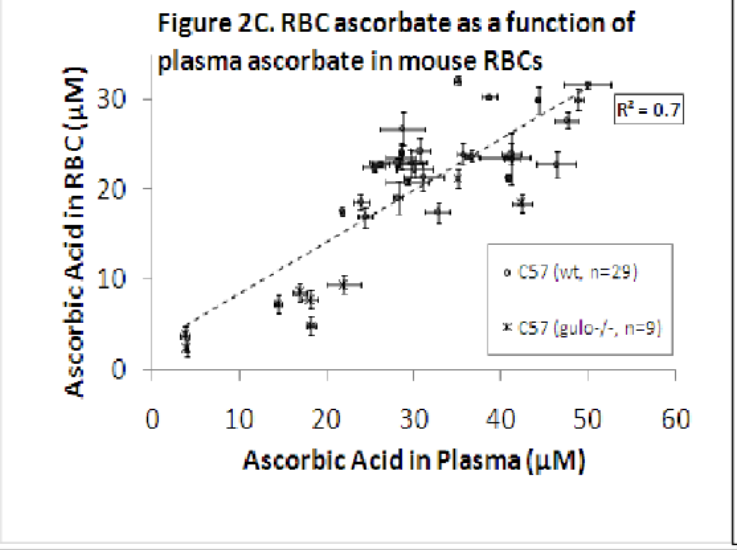


Figure 2C.
Plasma and RBC vitamin C measurements were performed using wildtype mice (C57 strain) and gulo -/- mice. Gulo-/- mice (mice that need vitamin C for survival) were deprived of vitamin C in their drinking water for 1-3 weeks before blood was sampled.

We next tested whether there was a relationship between RBC vitamin C concentrations and the osmotic fragility of mouse RBCs, using hypotonic saline to produce osmotic fragility as an index of deformability (Clark 1983). The amount of hypotonicity at which 50% of RBCs hemolyzed was shifted rightwards with low ascorbate concentrations (Fig. 3A), indicating increased fragility (Clark 1983). The amount of hypotonicity causing 50% lysis was expressed as a function of RBC ascorbate. As ascorbate declined, less hypotonicity was needed to cause lysis, again indicating increased fragility (Fig. 3B).

Figure 3A. Hemolysis, induced by hypotonicity, as a function of low and high ascorbate within RBCs from gulo $^{-/-}$ and WT mice

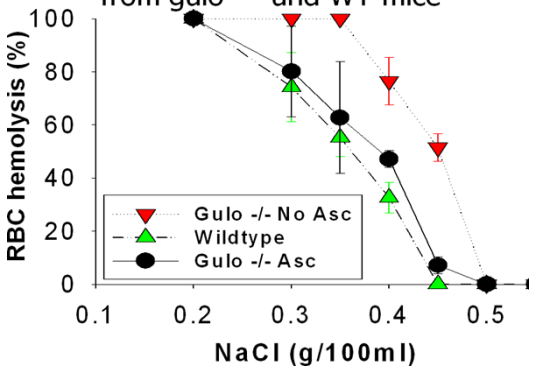


Figure 3B. 50% hemolysis, induced by hypotonicity, as a function of ascorbate in RBCs from gulo $^{-/-}$ mice given varying ascorbate doses

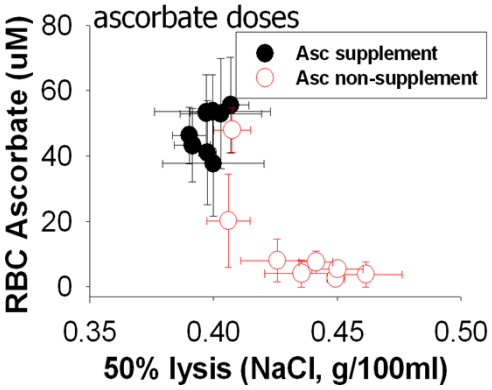


Figure 3B. The amount of NaCl (sodium chloride) necessary to induce 50% hemolysis in RBCs (Parpart 1947, see 3A) is displayed as function of RBC ascorbate concentration in samples from gulo-/- mice supplemented with vitamin C or deprived of it from 1-4 weeks, N=5 mice per point.

Figure 3A. RBC Osmotic fragility was determined in wildtype mice (C57, plasma level 76 uM); gulo-/- mice (plasma level 4 uM); and gulo-/- mice given vitamin C in drinking water (plasma level 64 uM). Osmotic fragility was determined at different NaCl concentrations (x-axis) by spectophotometric analyses to detect RBC hemolysis (y-axis) (Parpart 1947), N=5

To test how glucose and RBC ascorbate intersect, we investigated the substrate used by erythrocytes to achieve their internal ascorbate concentrations. As shown in figure 4A (human RBCs) and figure 4B (mouse RBCs), only DHA is transported into RBCs, not ascorbate. We tested transport across a range of ascorbate and DHA concentrations, and the findings confirmed those displayed. Ascorbate at concentrations up to 50 uM was not transported into RBCs at all. We specifically displayed data for 2 uM DHA because it is a physiologically relevant concentration. Data describing glucose transporters expressed in *Xenopus laevis* oocytes indicate that DHA and glucose are both transported by glucose transporters, and that the affinity of DHA for these transporters is much higher (>100 fold) than that of glucose (Vera 1993, Rumsey 1997,

Corpe 2013). As DHA entry into RBCs may also occur via glucose transporters (May 2001, Montel-Hagen 2008, Carruthers 2009), there could be potential interface in RBCs between DHA entry and glucose.

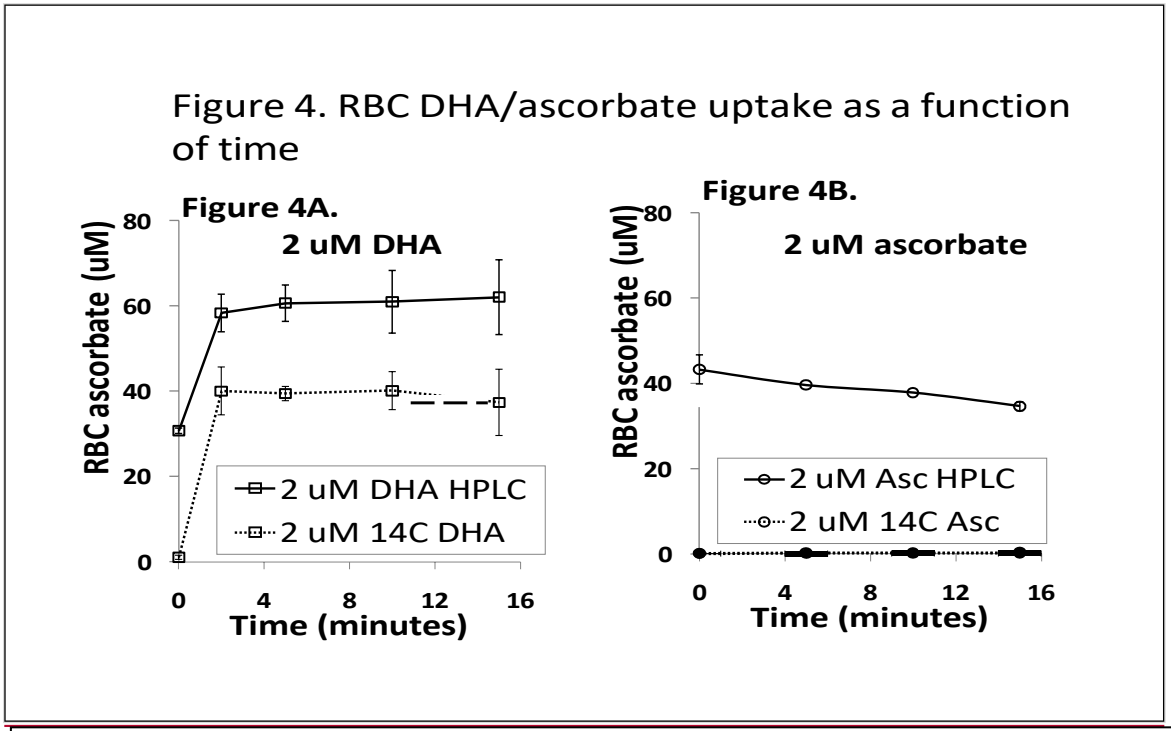
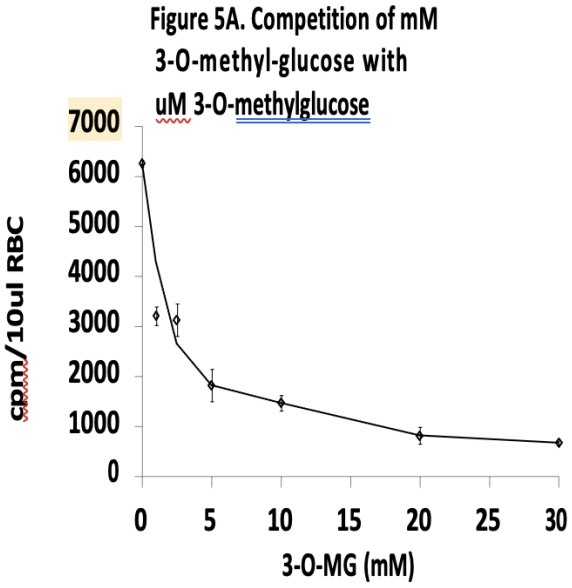
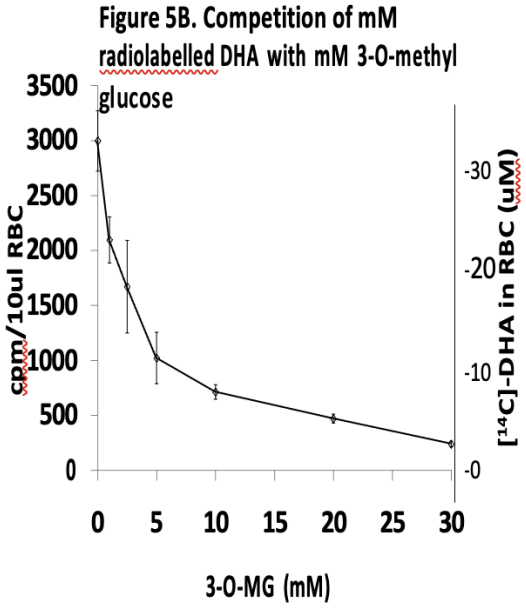


Figure 4. Human RBCs were incubated with 2 uM DHA (L) or 2 uM ascorbate (R) for indicated times, Ascorbate measured by HPLC (solid lines) or scintillation spectrometry (dashed lines). Experiments were performed in presence of 5 mM glucose.

To test transport further, we investigated whether or not glucose could compete with DHA entry into RBCs. We used the glucose analog 3-0-methyl glucose because it is a pure glucose transport substrate and not metabolized after entry. As a control, we showed that millimolar concentrations of 3-0-methyl glucose competed with 5 uM radiolabelled 3-0-methyl glucose for entry into isolated human RBCs (Fig. 5A). Similarly, the same concentration of radiolabelled DHA was competed by millimolar concentrations of 3-0-methyl glucose (Fig. 5B). Similar findings were obtained for mouse RBCs (data not shown). DHA concentration (5 uM) was selected to reflect the lowest potential *in vivo* concentration in plasma that was also compatible with scintillation spectrometry measurement. To explore whether glucose *in vivo* was inversely related to ascorbate in RBCs, we measured RBC ascorbate in leptin deficient (AZIP) mice with (AZIP) and without hyperglycemia and in fed and fasted wild type controls) (Kim 2000, Kennedy 2010). Note that fed AZIP mice have hyperglycemia, which resolves with fasting (Kim 2000). As predicted, an inverse relationship was found (Fig. 5C) between plasma glucose and RBC ascorbate concentrations. Taken together, these experiments show that glucose competes with DHA for entry into RBCs *in vitro* and *in vivo*.





Figures 5A, 5B. Human RBCs were incubated with 5 uM [3H]3-0-methyl glucose (A) or 5 uM [14C]DHA (B) without or with mM unlabelled 3-0-methyl-glucose for 1 minute 4°C. [3H] 3-0-methyl-glucose uptake (A) or [14C] DHA uptake (B) was measured by scintillation spectrometry.

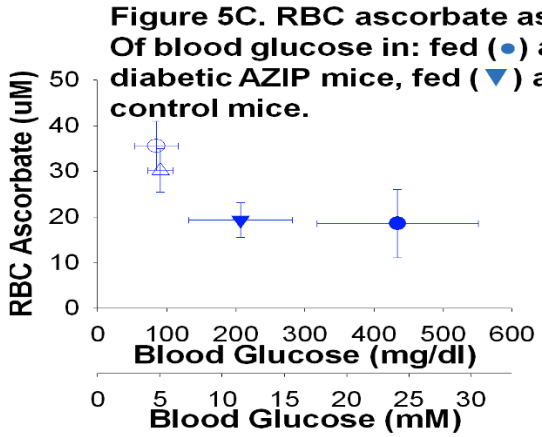


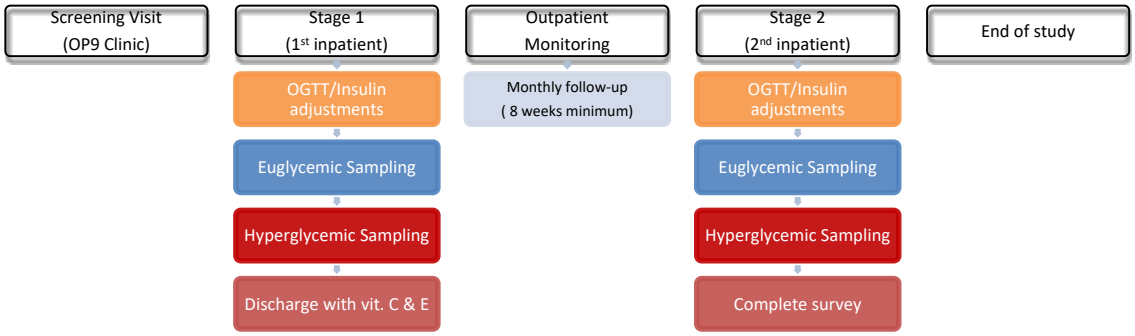
Figure 5C. Blood was withdrawn from AZIP (leptin deficient) mice (Kim 2000, Kennedy 2010) that were fed or fasted 16 hours; RBC ascorbate (y axis) and glucose (x axis). Plasma ascorbate values were unchanged (not shown, note that AZIP mice synthesize ascorbate).

We propose that in diabetes with hyperglycemia, RBC vitamin C content falls due to glucose competition with DHA. This competition may lower RBC vitamin C concentration, which increases RBC fragility. Increased fragility, in turn, is associated with decreased life span and impaired deformability. In addition, we propose that compromised renal reabsorption of vitamin C independently occurs in diabetes. Decreased renal reabsorption of ascorbate could be due to hyperfiltration and/or damage to the vitamin C reabsorptive transporter. In either case, the results would be lower plasma ascorbate than otherwise expected without diabetes. Lower plasma ascorbate in turn decreases the availability of DHA, which is formed from vitamin C in plasma, for entry into RBCs. We further propose that impaired RBC deformability can be improved by raising RBC ascorbate concentrations, using vitamin C supplementation.

5. Study design and methods:

5.1. Overall design:

The study is a prospective physiologic/natural history study. The study population will be up to 100 adult male or female subjects with diabetes type 2 (HgA1c $\leq$ 12%) or without diabetes (nondiabetic controls) between 18 and 65 years old. Each subject will act as his/her own control. Subjects will be recruited de novo. Also, participation will be offered to volunteers who enroll in protocol 04-DK-0021: Urinary vitamin C loss in subjects with or without diabetes.



The study will consist of two parts: stage 1 and stage 2. Following an initial examination, all selected subjects will be studied as inpatients in stage 1. Following completion of stage 1, subjects will be considered for participation in stage 2, which includes vitamin C and E supplementation.

Subjects may be invited to re-enroll and repeat study procedures if there are issues with testing, compliance, or changes to the protocol that would affect aggregated results.

5.2. Schedule of activities:

Procedures	Screening[g]	Baseline[g] Day 0	Inpatient Stage 1 Day 1 (euglycemic	Inpatient Stage 1 Day 2 (hyper-	Follow-up Visits during Outpatient Supplement	Inpatient Stage 2 (euglycemic	Inpatient Stage 2 (hyper-glycemic)
Informed consent[cd]	X						
Demographics[cd]	X						
Medical history[cd]	X						
Physical exam [cd]	X						
Vital signs[cd]	X		X	X	X	X	X
Height & weight[cd]	X						
EKG[cd]	X						
Screening blood assessments[acd]	X						
Urine pregnancy test[bcd]	X		X		X	X	
Review of 24-hour insulin and/or oral hypoglycemic agent required[c]	X		X	X	X	X	X
Eye examination & fundus photograph[c]	X						
Dietician interview[cd]		X					
Oral glucose tolerance test (OGTT)[d]			X				
Continuous glucose monitor (CGM) sampling[cd]			X	X		X	X
Research blood sampling [cde]		X	X	X	X	X	X
Research urine sampling [cdf]			X	X	X	X	X
Vitamin C & Vitamin E supplementation[cd]					X	X	X
Vitamin C & Vitamin E pill counting[cd]					X	X	X
Standardized meals[cd]			X	X		X	X
SF-36 health related quality-of-life survey[c]				X			X
Adverse event review and evaluation[cd]	X	X	X	X	X	X	X
Complete Case Report Forms (CRFs)[cd]	X	X	X	X	X	X	X

a: CBC, electrolytes, blood urea nitrogen, creatinine, mineral profile, lipid profile, hepatic profile, HbA_1C, thyroid function, hepatitis B & C tests, HIV test
b: for women with child-bearing potential only
c: for people with diabetes
d: for people without diabetes
e: plasma and RBC vitamin C assessments; insulin and glucose concentrations
f: urine glucose levels, vitamin C and vitamin E levels, F2-isoprostanes
g: screening and baseline visits will occur on the same day with baseline procedures happening only after provision of informed consent

5.3. Initial evaluation:

All subjects will undergo an initial evaluation that will consist of a complete history and physical examination, EKG, urinalysis, CBC, electrolytes, blood urea nitrogen, creatinine, mineral profile, lipid profile, hepatic profile, HbA_1C, thyroid function tests, screening for hepatitis B and C, HIV test and a urine pregnancy test for women of childbearing age. The protocol will be explained to the subjects and informed consent obtained. Specific laboratory evaluation may include plasma and RBC vitamin C levels. As part of screening in participants with diabetes, subjects 24 hour insulin requirement +/- oral hypoglycemic agents will be evaluated and reviewed. Per investigator discretion subjects with diabetes may be sent for a routine comprehensive dilated eye exam by the ophthalmology group. A fundus photograph may be taken per the ophthalmologist's discretion. Patients will be asked to discontinue vitamin C and/or E supplementation 2 weeks prior to first inpatient study. During the screening visit, patients will be interviewed by a dietician in order to assess their calorie intake and food preferences.

5.4. Inpatient study:

Stage 1:

Selected subjects who meet the study criteria as determined during screening process will be admitted as inpatients to the National Institutes of Health (NIH) Clinical Center metabolic unit. This unit is equipped with a nursing staff specialized in inpatient care of research subjects and serial testing. The duration of inpatient stay is estimated to be one week. During the admission, subjects will have two sampling periods of 24 hours each. Upon admission, subjects will be transitioned to an individualized inpatient diabetes regimen determined by investigators, based on pre-admission glycemic control and diabetes regimen. In order to achieve optimal glycemic monitoring and for safety reasons, subjects may be fitted with a Dexcom continuous glucose monitor (CGM) upon inpatient admission. CGM will be used to supplement, rather than replace, fingerstick glucose measurements. CGM monitoring will include a sensor fitted subcutaneously and a wireless transmitter that allows for remote glucose monitoring by the research team. CGM will also include a receiver that displays glucose trends in real time. One key objective of CGM monitoring will be to take advantage of the customizable alarms to anticipate hypoglycemic or severe hyperglycemic episodes and intervene before they occur. CGMs may be discontinued anytime per researcher's clinical discretion.

Over the first 24-48 hours, for participants with diabetes, insulin doses will be titrated to achieve and maintain euglycemia (premeal and fasting glucose <140 mg/dl). Glucose will be monitored by fingerstick measurements premeals and before bedtime (four times daily). When euglycemic conditions are achieved (approximately day 2-3), an intravenous catheter will be inserted for the first 24 to 48 hours and euglycemic sampling initiated for 24 hours. During this time, for the first 9 hours: blood samples will be drawn every one to two hours for glucose and insulin concentrations (clinical chemistry), plasma and RBC vitamins C and E concentration, and RBC deformability measurements. After 9 hours, blood sampling will be adjusted to every 3-4 hours until completion of euglycemic sampling.

Timed urine for vitamin C measurements (and other labs as clinically determined) will be scheduled periodically for the duration of sampling period. Glucose levels will be monitored before meals and patients maintained on a standardized diet. Due to the possible antioxidant

interactions between vitamin E (located in RBC membrane) and vitamin C (Tanaka 1997), measurements of vitamin E levels will be done. To quantify changes in oxidative stress, we will optionally obtain plasma and urine F2-isoprostanes (F2-IsoPs). Plasma F2-IsoPs may be collected every 1-4 hours for the first 9 hours, then 3-6 hours until completion of sampling period. Similarly, aliquots for urine for F2-IsoPs may be obtained from timed urine collections (every 3-4 hours and/or overnight). Blood will also be collected and stored for future measurements of dehydroascorbic acid, sorbitol and glyoxal-related compounds. Following completion of euglycemic sampling, hyperglycemic sampling will be initiated (approximately day 4). On morning of controlled hyperglycemia (glucose 200-400 mg/dL), basal-bolus insulins will be held, however correction scale insulins will be instituted for glucoses >350-400mg/dL. Glucose (fingerstick) checks every 1-2 hours will be continued per investigator discretion.

Patients will be provided a high carbohydrate diet. Sampling scheme during controlled hyperglycemia will be the same as the euglycemic sampling. To ensure safety, the maximum glucose elevation will not exceed 350-400 mg/dL and the duration of hyperglycemia will not exceed 9 hours. During this time, the subjects will be closely monitored clinically to detect any early evidence of side effects due to the expected hyperglycemia. In case of any clinical or biochemical evidence of hyperosmolarity, dehydration or ketosis the experiments will be stopped and intravenous or subcutaneous insulin therapy (short acting insulin) and/or intravenous fluids administered per investigator discretion and clinical judgment. At the end of 9 hours maximum of hyperglycemia, insulin and/or oral hypoglycemic medication(s) will be re-administered with the goal of achieving patient baseline glycemic control. The type and dosage of the insulin and/or oral hypoglycemic medication(s) will be based on initial assessment and home regimen of the subject. Glucose checks every 1-2 hours will be continued per investigator discretion while insulin and/or oral hypoglycemic medication(s) is re-administered until baseline glycemic control for the subject is achieved**.** To summarize, the sampling period that includes 9 hours of hyperglycemia will have a total duration of 24 hours, with a total of approximately 417 mL of blood drawn for the research laboratory assessments over the inpatient stay. Afterwards, the venous catheter will be removed, and the subject will be managed to achieve euglycemia.After the second sampling, subjects will be asked to complete a brief SF-36 health related quality of life survey and receive pre-discharge counseling on outpatient clinic follow-up.
Subjects will be discharged from the Clinical Center with a prescription for 500 mg twice daily vitamin C and 400 international units (I.U.) RRR alpha-tocopherol (vitamin E).

For the nondiabetic controls, a 2-hr oral glucose tolerance test will be administered on day 1 with 75grams of dextrose to evaluate for impaired glucose tolerance. Participants will receive the metabolic diet and have the inpatient sampling schedule as participants with diabetes. However fingerstick glucose monitoring is optional and glycemic targets are nonapplicable.

Outpatient Follow-up:

Subjects will be asked to return to NIH biweekly or monthly during the time when they are taking vitamin C and E supplements to ensure compliance via measurement of urine and plasma vitamin C levels (14 mL of research blood drawn per outpatient visit). In addition, pill counting will be used as a standard procedure of checking compliance with taking the vitamin C supplements at each visit. During this time, we will recommend a regimen of insulin +/- hypoglycemic agents to the subjects for optimal control of their diabetes. We will follow-up with the subjects in regard to optimal control of diabetes in between the two inpatient visits and may

periodically check HgA1c and glucose.

Stage 2:

Subjects may be considered for stage 2 inpatient study no less than 8 weeks duration from stage 1 study. Once the RBC vitamin C concentrations are optimal (>30 uM), subjects may be re- admitted to Clinical Center metabolic unit and undergo the same protocol as described above in arm 1. Oral vitamin C and E supplementation may be discontinued on admission. The inpatient diet, glucose monitoring and sampling scheme will be the same as described for the first inpatient study.

Inpatient Diet for Stages 1 & 2:

Subjects will be required to consume standardized meals during inpatient stays. All meals will be prepared by the NIH Clinical Center Metabolic Kitchen. Energy from the standardized diet will be provided based on estimated needs using the Mifflin St. Jeor equation with an activity factor of 1.4. To avoid obscuring plasma vitamin C changes that may result from hyperglycemia, dietary vitamin C content will be approximately 30-35 mg per meal. Additionally, to avoid confounding vitamin E measurements, diets will provide approximately 6 mg alpha tocopherol per day. From day of admission to completion of euglycemic sampling, the standardized diet will provide 20% kcal from protein, 30% kcal from fat and 50% of kcal from carbohydrate. On the day of hyperglycemic sampling, the standardized diet will be changed to provide 10% kcal from protein, 20% kcal from fat and 70% kcal from carbohydrate. After the 9-hour "controlled hyperglycemia" sampling, diets will return to the original macronutrient composition (20% protein, 30% fat, 50% carbohydrate). After all sampling is completed, patients will no longer receive standardized meals and can order from room service. While on standardized diets subjects will be asked to consume all provided food and drink within the allotted time. Any remaining uneaten food will be weighed back. Standardized meals at the 2nd inpatient admission will be provided to match what was consumed by the subject at their 1st inpatient admission.

5.5. Dispensing and preparation of oral vitamins C and E supplementation:

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Vitamin C and E supplements will be supplied through the NIH pharmacy, using commercially available products. The dose and frequency of vitamin C was selected because 500 mg orally twice daily is above that amount needed to saturate plasma and tissues in healthy people (Levine 1996 & 2001), and accounts for a possible renal leak in diabetic subjects (Chen 2006). For safety, this dose is below the upper limit dose of 2000 mg daily, and twice daily administration should be acceptable to patients. The dose of vitamin E was chosen given its safety profile, and that this dose when administered with vitamin C is safe (Wang 2014). All steps in procurement of the ascorbate and vitamin E supplements will proceed as directed by the NIH Clinical Center Pharmacy Department.

There will be no IND obtained for the use of any of the commercial agents used in this study. This study meets the criteria for exemption for an IND as this investigation is not intended to support a new indication for use or any other significant change to the labeling; the dietary supplements are already approved and marketed and the investigation is not intended to support a significant change in advertising; and the investigation does not involve a route of administration

or dosage level in use in a patient population or other factor that significantly increases the risks.

5.6. RBC deformability, plasma/RBC vitamin C and other laboratory measurements:

Dr. Levine's lab has the unique advantage of having the LoRRca MaxSys ecktacytometer, one of only two in North America, to measure RBC deformability (Baskurt 2009, Musielak 2009). With this instrument we can characterize RBC deformability, aggregation, osmotic fragility, and rouleaux formation as a function of vitamin C concentrations.

Vitamin C will be measured by high-performance liquid chromatography (HPLC) with coulometric electrochemical detection. This is a unique assay for measurement of RBC ascorbate and allows reliable and precise measurements. This technique, developed in Dr. Levine's lab, is a sensitive and accurate assay able to detect vitamin C in the RBC at concentrations as low as 25 nM. Interference has been a major problem with previous assays. The current assay provides for ascorbate stability and has overcome a major problem with previous RBC ascorbate assays (i.e. the presence of iron which is a catalyst for ascorbate oxidation) (Li 2012).

Clinical biochemical measurements will be performed using standard assays of the NIH Clinical Center Clinical Chemistry Department. Plasma a-tocopherols will be measured by HPLC with cuolometric electrochemical detection by Dr. Levine's lab. Blood samples will be collected and stored for future measurements of dehydroascorbic acid, sorbitol, and glyoxal-related compounds.

Measurements (fee for service) of plasma and timed urine F2-isoprostanes (F2-IsoPs) will be sent for quantification with mass spectrometry, to Dr. Ginger Milne at the Eicosanoid Core Laboratory of Vanderbilt University.

6. Inclusion criteria:

Stage 1

-Male or female 18-65 years old, able to give informed consent.
-Diabetes type 2 HgA1C $\leq$ 12% on insulin and/or oral hypoglycemic agents or nondiabetic without any prior history or diagnosis of diabetes.
-In general good health with no other significant illness.
-Mild concomitant disease such as mild hypothyroidism (TSH <10) is acceptable.
-Blood pressure with or without medication <160/90 mmHg with no known significant target organ damage (end organ damage includes the following: proliferative retinopathy, serum creatinine >1.5 or EGFR < 55 mL/min, symptomatic ischemic heart disease, severe congestive heart failure, advanced peripheral vascular disease.
-Willingness to use effective contraceptive methods such as barrier method for the duration of study (female subjects).

Stage 2

Above criteria with addition of RBC vitamin C concentration >30 uM prior to inpatient studies.

7. Exclusion criteria (Stage 1 and 2):

-Diabetic type 1 subjects will be excluded due to the possibility of ketosis and hemodynamic instability with lack of insulin.

-Any subjective or objective evidence of microangiopathy such as history of claudication, symptomatic peripheral vascular disease, symptomatic coronary artery disease, stroke, retinopathy, nephropathy (serum creatinine >1.5 or EGFR < 55 mL/min).
-Diabetic subjects with retinopathy to avoid accelerated retinopathy with hyperglycemia.
-Concomitant disease such as severe heart failure, severe liver disease (transaminases > 3 times normal), or severe systemic disease of any sort.
-Pregnancy, breastfeeding.
-History of diabetic ketoacidosis or hyperosmolar coma.
-Subjects with clear evidence of non-compliance with protocol/study instructions.
-Subjects who are unwilling or lack capacity to provide informed consent.

8. Inclusion of Vulnerable Participants

8.1. Participation of NIH Staff or family members of study team members

NIH staff and family members of study team members may be enrolled in this study as this population meets the study entry criteria. Neither participation nor refusal to participate as a subject in the research will have an effect, either beneficial or adverse, on the participant's employment or position at NIH.

Every effort will be made to protect participant information, but such information may be available in medical records and may be available to authorized users outside of the study team in both an identifiable an unidentifiable manner.

The *NIH Frequently Asked Questions (FAQs) for Staff Who are Considering Participation in NIH Research* and the Leave Policy for NIH Employees Participating in NIH Medical Research Studies will be made available. Please see section 14.4 for consent of NIH staff.

9. Monitoring subjects:

Prior to enrollment, per protocol, subjects will have a history taken and a physical examination performed, including screening for diabetic complications. We will review available recent outpatient eye examination reports. In case reports are not available or reliable we will refer subjects to NIH ophthalmology service per the investigator discretion based on history and physical examination. Laboratory screening will include all the tests specified above in "Study design and methods- initial evaluation", including screening for HIV and for hepatitis B and C. During the hyperglycemia period a clinical unit nurse will be present at all times. Prior to each study day women of child-bearing age will have pregnancy test. In order to ensure compliance in taking the vitamin C supplements in between the two inpatient studies, urine and plasma vitamin C levels will be measured biweekly or monthly. In addition to this, we will use pill counting as a standard procedure of checking compliance with taking the vitamin C supplements.

10. Data safety and monitoring:

The principal investigator will function as the data and safety monitor and report any adverse events to the Institutional Review Board (IRB).

11. Definition of Adverse Event (AE):

Adverse event means any untoward medical occurrence associated with the use of an intervention in humans, whether or not considered intervention-related (21 CFR 312.32 (a)).

10.1 Definition of Serious Adverse Events (SAE):
A Serious Adverse Event is any Adverse Event that:

- Results in death;
- Is life-threatening (places the subject at immediate risk of death from the event as it occurred);
- Results in inpatient hospitalization or prolongation of existing hospitalization; · Results in a persistent or significant disability/incapacity;
- Results in a congenital anomaly/birth defect; OR
- Based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed above in this definition (examples of such events include allergic bronchospasm requiring intensive treatment in the emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse).

10.2 Classification of an Adverse Event

10.2.1 Severity of Event

For AEs not included in the protocol defined grading system, the following guidelines will be used to describe severity.

- Mild – Events require minimal or no treatment and do not interfere with the participant's daily activities.
- Moderate – Events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- Severe – Events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually potentially life-threatening or incapacitating. Of note, the term "severe" does not necessarily equate to "serious".

10.3 Relationship to Study Procedures

All AEs will have their relationship to study procedures assessed by an appropriately trained clinician based on temporal relationship and his/her clinical judgment. The degree of certainty about causality will be graded using the categories below.

Related – The AE is known to occur with the study procedures, there is a reasonable possibility that the study procedures caused the AE, or there is a temporal relationship between the study procedures and the event. Reasonable possibility means that there is evidence to suggest a causal relationship between the study procedures and the AE.

Not Related – There is not a reasonable possibility that the study procedures caused the

event, there is no temporal relationship between the study procedures and event onset, or an alternate etiology has been established.

10.4 Expectedness

A clinician with appropriate expertise will be responsible for determining whether an AE is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study procedures.

10.5 Adverse Event Recording and Reporting

Only moderate to severe AEs related to research procedures, regardless of expectedness, will be recorded. If a research procedure is conducted simultaneously with a clinical procedure and the study team is not able to distinguish if the AE occurred because of the clinical care or because of the added research procedures, the AE will be assumed to be related to the research and will be recorded. AEs will be recorded only when research procedures are conducted, and only AEs that are noted during these visits will be recorded unless the study team is later notified of an occurrence of an AE that is related to the research procedure. The research team will not follow up with the participant to assess AEs between visits or after the subject has completed participation.

In consultation with the PI, a trained member of the study team will be responsible for conducting an evaluation of all AEs and shall report the results of such evaluation to the NIH Institutional Review Board (IRB) as per Policy 801.

10.6 Unanticipated Problems

10.6.1 Definition of Unanticipated Problems (UP)

Any incident, experience, or outcome that meets all of the following criteria:

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the Institutional Review Board (IRB)-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied; and
- Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places participants or others (which many include research staff, family members or other individuals not directly participating in the research) at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or expected.

10.6.2 Unanticipated Problem Reporting

The investigator will report unanticipated problems (UPs) to the NIH Institutional Review Board (IRB) as per Policy 801.

10.7 Protocol Deviations and Non-Compliance

It is the responsibility of the investigator to use continuous vigilance to identify and report deviations and/or non-compliance to the NIH Institutional Review Board as per Policy 801. All deviations must be addressed in study source documents, reported to NIDDK Program Official. The investigator is responsible for knowing and adhering to the reviewing IRB requirements.

10.7.1 NIH Definition of Protocol Deviation

A protocol deviation is any changed, divergence, or departure from the IRB-approved research protocol.

- Major deviations: Deviations from the IRB approved protocol that have, or may have the potential to, negatively impact the rights, welfare or safety of the subject, or to substantially negatively impact the scientific integrity or validity of the study.

- Minor deviations: Deviations that do not have the potential to negatively impact the rights, safety or welfare of subjects or others, or the scientific integrity or validity of the study.

12. Withdrawal from the study:

The subject may withdraw from the study at any time for any reason. The subject has the right to refuse a particular test. In such cases, a decision will be made on whether the subject may continue the study. This might be grounds for dismissal from the study if the refusal of testing impacts results, study quality or subject safety. Grounds for dismissal from the study will include new development of exclusion criteria, permanent loss of capacity to consent, or development of any complications from hyperglycemia such as dehydration, ketosis, hemodynamic instability or hyperosmolarity.

13. Subject monitoring and follow-up:

All patients will receive follow-up care through their local physician. In the event that subjects do not have a primary care or local physician to aid in long term diabetes control, we will suggest care through the mobile medical Suburban Hospital clinic staffed by NIH endocrine faculty and fellows. Another alternative to obtaining guidance for diabetes care would be to join existing protocols designed for diabetes care at NIH i.e.09-DK-0140. The investigators will offer information on how to contact or enroll in either program.

During the minimum 8-week period in between the two inpatient studies, we will follow-up with patients once or twice a month as an outpatient and an appropriate regimen of insulin +/- hypoglycemic agents will be suggested to patients. By controlling hyperglycemia as an outpatient, we ensure subject safety.

Study procedures will be subject to audits and/or monitoring visits to ensure compliance with the protocol and applicable regulatory requirements consistent with the NIDDK quality

assurance program plan. Audit and/or monitoring visits results will be reported to the Principal Investigator for further reporting as appropriate. Study documents and pertinent hospital or clinical records will be reviewed to verify that the conduct of the study is consistent with the protocol plan.

14. Human subject protections:

14.1. Rationale for subject selection:

Subjects of all gender, race and ethnicity will be considered for inclusion in this study if they meet the study's inclusion and exclusion criteria.

14.2. Recruiting strategies:

Subjects will be recruited from patients enrolled in protocol 04-DK-0021, entitled "Urinary vitamin C loss in subjects with or without diabetes". Subjects will also be actively recruited de novo. All study subjects will be screened and consented under this protocol. We will use the following recruitment strategies: posting flyers in community libraries, on community announcement boards, and in free clinics; and posting recruitment messages and contact information on NIH CC social media sites, on the OPR recruitment website, and on CCTV monitors in Building 10. We also plan to email recruitment notices through listservs and to recruit through ResearchMatch. Patients may be also recruited by word of mouth through research subjects and other physicians.

We plan for 12 subjects with diabetes and 12 nondiabetic controls to complete this pilot study. In order to complete a total of 24 subjects, we have set the accrual ceiling at 100 subjects in order to cover possible subject withdrawals and drop-outs. Based on previous observations (Chen 2006), as many as one third of diabetic subjects have some degree of vitamin C deficiency. However, the prevalence of vitamin C deficiency in diabetes is uncertain, in part because of unreliable assays for vitamin C (Will 1996). To recruit 2 4 subjects, we estimate that we will screen approximately 100 subjects and that enrollment will be accomplished within 12-18 months. We estimate that we will be able to study subjects within 2 years, in a rolling fashion, by enrolling subjects as soon as they are screened and identified as eligible.

14.3. Consent process:

The informed consent document will be provided as a physical or electronic document to the participant or consent designee as applicable for review prior to consenting. A designated study investigator will carefully explain the procedures and tests involved in this study, and the associated risks, discomfort and benefits. In order to minimize potential coercion, as much time as is needed to review the document will be given, including an opportunity to discuss it with friends, family members and/or other advisors, and to ask questions of any designated study investigator. A signed informed consent document will be obtained prior to any research activities taking place.

The initial consent process as well as re-consent, when required, may take place in person or remotely (e.g., via telephone or other NIH approved remote platforms used in compliance with policy, including HRPP Policy 303) per the discretion of the designated study investigator and with the agreement of the participant. Whether in person or remote, the privacy of the subject will be maintained. Consenting investigators (and participant, when in person) will be located in a private area (e.g., clinic consult room). When consent is conducted remotely, the participant will be

informed of the private nature of the discussion and will be encouraged to relocate to a more private setting if needed. If the consent process is occurring remotely, participants and investigators will view individual copies of the approved consent document on screens at their respective locations; the same screen may be used when both the investigator and the participant are co-located.

Note: When required, the witness signature will be obtained similarly as described for the investigator and participant below.

Consent will be documented with required signatures on the physical document (which includes the printout of an electronic document sent to the participant) or on the electronic document. The process for documenting signatures on an electronic document is described below.

When a hand signature on an electronic document is used for the documentation of consent, this study will use the following electronic platform to obtain the required signatures

- iMedConsent platform (which is 21 CFR Part 11 compliant)

Should past participants who either withdrew before completing the study or participated when the study procedures (ie. dietary levels of vitamins C and E) were different choose to enroll again, we will use their data from the most recent participation in this study towards our accrual numbers and towards our primary aims. Previous data with outdated study procedures will not be counted towards the primary objectives of this study but may be used for supplemental measures of intra-subject comparisons of dietary Vitamin C levels on outcomes.

14.4 Considerations for consent of NIH staff, or family members of study team members

Consent for NIH staff will be obtained as detailed above with following additional protections:

Consent from staff members will be obtained by an individual independent of the staff member's team whenever possible. Otherwise, the consent procedure will be independently monitored in order to minimize the risk of undue pressure on the staff member.

15. Analysis of the study:

The outcomes of this study for analysis are to:

-Understand if vitamin C content of RBCs and glycemia affect RBC's physiologic functions of deformability and related ektacytometer parameters.
-Evaluate whether vitamin C supplementation affects RBC vitamin C content.
-Evaluate vitamin C renal clearance as a function of glycemia.

16. Statistical analysis:

Data will be entered in a spreadsheet/database and will undergo appropriate statistical analysis.

Normally distributed continuous data will be summarized using means, standard deviations, and confidence intervals, while skewed continuous data will be summarized using medians and interquartile ranges. Categorical or discrete data will be summarized and reported using counts and percentages. Unless otherwise stated, all statistical analyses will be performed using a two-sided hypothesis test at 5% alpha level.

The primary endpoints are:

1. Change in RBC deformability elongation indices (EI) from stage 1 to stage 2
2. Change in RBC deformability maximum elongation indices (EImax) from stage 1 to stage 2
3. Change in RBC deformability half maximal shear stress ($SS_{0.5}$) from stage 1 to stage 2

The secondary endpoints are:

1. Change in RBC deformability elongation indices before and after hyperglycemic intervention during stage 1
2. Change in RBC deformability maximum elongation indices (EImax) before and after hyperglycemic intervention during stage 1
3. Change in RBC deformability half-maximal shear stress (SS0.5) before and after hyperglycemic intervention during stage 1

The tertiary endpoints are:

1. Change in vitamin C concentration in plasma from stage 1 to stage 2
2. Change in vitamin C concentration in urine from stage 1 to stage 2
3. Change in vitamin C concentration in RBC from stage 1 to stage 2
4. Change in vitamin C concentrations in plasma before and after hyperglycemic intervention during stage 1
5. Change in vitamin C concentrations in RBC before and after hyperglycemic intervention during stage 1
6. Change in vitamin C concentrations in urine before and after hyperglycemic intervention during stage 1
7. Change in vitamin E concentration in plasma from stage 1 to stage 2
8. Change in vitamin E concentration in RBC from stage 1 to stage 2
9. Change in plasma isoprostane concentrations from stage 1 to stage 2
10. Change in plasma isoprostane concentrations before and after hyperglycemic intervention during stage 1
11. Change in urine isoprostane concentrations from stage 1 to stage 2
12. Change in urine isoprostane concentrations before and after hyperglycemic intervention during stage 1
13. Change in SF-36 health related quality of life survey from stage 1 to stage 2

Analyses will focus on both correlations among variables at a particular time and on changes from stage 1 to stage 2 using mixed model regression. Exploratory analyses will compare the cohort with diabetes and the nondiabetic controls. Additional details will be provided in the statistical analysis plan.

We expect, based on the relationship between plasma glucose levels and RBC elongation index in diabetic patients previously described (Keymel 2011), that a patient's change in RBC elongation index would be on average -0.0342 units, with variability of 0.0282. Assuming that variability of hyperglycemia in our subjects is similar and that there is a moderate correlation of 0.2 between a patient's baseline and hyperglycemic elongation index values, 11 patients are needed to have 80% power (SAS Proc Power). To account for potential dropouts and other unanticipated follow-up issues, we propose a study size of N=50 subjects should be sufficient in order to have 11 patients completing phase 2.

We anticipate enrolling several participants who have previously participated in this study with a lower dietary Vitamin C allowance. We may analyze these intra-subject data towards supplemental aims of comparing effects of dietary Vitamin C levels on other test outcomes. Due to the small sample size, these analyses will be exploratory in nature.

17. Benefits:

This is purely a research study with little or no direct benefit to subjects. The information that we gain from this study may provide a foundation for future studies exploring a role of vitamin C in preventing or delaying microvascular complications of diabetes. The study may increase our understanding of how vitamin C supplementation may alter the natural course of diabetes leading to microangiopathy, thus providing a potential therapeutic modality for diabetics in the future. We will also gain new knowledge in vitamin C renal clearance. It is possible that results of some screening tests that we perform on our volunteers may be abnormal. If this is the case, we will notify them of these results and discuss it with them and their physician.

18. Remuneration:

Subjects will be compensated $50 for the screening assessment at the NIH. If the subject completes the first inpatient admission in which he or she stays for an initial evaluation of their insulin requirements with controlled diet and frequent blood sampling to determine glycemic control they will be compensated $500. Subjects who complete the minimum 8 week course of oral vitamin C supplements as an outpatient and the second inpatient admission with frequent blood and urine sampling will be compensated $1500. Subjects will be reimbursed for the screening visit at the time of the visit. Payments for the two inpatient visits and the course of oral vitamin C as an outpatient will only be made upon successful completion of the study. If the subject participates in our protocol but does not successfully complete the study, he or she will be compensated at a rate of $40 for every day of participation while they are an inpatient.

19. Risks and discomforts:

18.1.Hyperglycemia/Hypoglycemia risks:

A potential hazard of this study is the development of significant hyperglycemia in diabetic subjects who are taken off their insulin during the inpatient study day. The risk from relatively short periods (9 hours) of moderate controlled hyperglycemia is minimal. If moderate to severe hyperglycemia remains untreated for 24 hours or longer, type II diabetic subjects could be at risk for hyperosmolarity, dehydration, ketosis or altered mental status. Therefore, hourly monitoring of blood glucose will be performed over the time period of controlled hyperglycemia in order to aid in early detection of potential metabolic derangement. Furthermore, we may make use of the continuous glucose monitoring (CGM) to anticipate hypoglycemic or severe hyperglycemic episodes and intervene before they occur. Based on clinical discretion, additional clinical studies such as electrolytes may be obtained if needed. Subjects will be under close clinical supervision during this time. Maximum glucose value will not exceed 400 mg/dL. In case glucose values are higher than 400 mg/dL, subcutaneous or intravenous insulin and or intravenous fluids will be administered per the investigator's discretion to control hyperglycemia. The study will only be resumed per investigator discretion after a complete clinical and laboratory evaluation.

As with any diabetic patient on insulin therapy, there's always a risk of hypoglycemia from excessive insulin doses, or mis-timed insulin intake relative to food intake. This risk will be

mitigated by frequent and regular testing, especially during sampling periods. Insulin administration will be closely coordinated with meal schedule. For hypoglycemic episodes, management may include glucose tablets for asymptomatic, stable and/or conscious patients. Intravenous or subcutaneous dextrose may be use if patient is unconscious or unable to ingest oral glucose tablets. To avoid confounding effects from dietary vitamin C sources, fruit juices without predetermined vitamin C contents may not be used for hypoglycemic management.

18.2. Blood drawing and line placement for procedures:

There are minimal risks associated with blood draws and line placement for procedures. The patient may experience some discomfort or hematoma at the site of venipuncture and there is a remote risk of local infection. Proper aseptic technique practiced regularly by the research nursing staff will minimize these complications. Rarely (<1%) of patients who have a line placed for procedures develop bacteremia which requires antibiotic therapy. We anticipate drawing approximately 877 mL of research blood for the entire duration of a subject's participation in this study, based on this average blood per visit breakdown:

Visit	Research Blood (mL)	Occurrences per subject	Total (mL)
Screening	15	1	15
Inpatient Stay	417	2	834
Outpatient Visit	14	2	28

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While the total blood drawn from each participant is approximately 877 mL, research blood drawn will not exceed the limit of 550 mL per 8 weeks of research participation, because there is at least 8 weeks between the two inpatient stays.

18.3. Privacy risks:

A patient identification code will be provided so that the identity of the research subjects will only be known to NIH investigators.

18.4. Risks related to clinical relevance:

Standard clinical tests that are performed by the Clinical Center laboratories will be placed in the medical record. Clinical tests not performed at NIH will be reviewed as part of the medical record review. Non-clinical lab improvement amendments (CLIA) certified tests will not be included in the medical record or routinely discussed with the patient. In view of the research nature of this protocol, the non-CLIA certified results cannot be meaningfully interpreted outside the narrow focus of this study.

18.5. Risks associated with ocular examination:

Dilating or anesthetic eye drops may sting. They may cause an allergic reaction or if contaminated may cause infection. Dilating drops can also cause acute glaucoma in eyes especially if predisposed to develop this condition. There is little risk of glaucoma being triggered in this way, but if it does occur, it will be treated promptly. In rare instances the cornea might be scratched during measurement of intraocular pressure or use of contact lens used for examination purpose only. A corneal abrasion of this sort may be painful but heals quickly with no lasting effect. If the ophthalmologist chooses to do a fundus photograph, the light form the camera may cause some transient discomfort and glare and may result in a brief transient decrease in vision.

18.6. Risks associated with continuous glucose monitoring (CGM):

Subjects may wear a Dexcom CGM during their inpatient admission. There is minimal risk associated with the device. Possible side effects include but are not limited to local infection, inflammation, pain or discomfort, bleeding at the insertion site, bruising, itching. A medical provider will be available should any of these problems occur.

20.Research use, storage and disposition of human samples and data:

As with all clinical data, the findings will be kept confidential. Volunteer clinical data will be protected and tracked using standard operating procedures in the medical record department. Any subject data stored in the research office will be kept in locked files or on computers that are

password protected. Blood samples and research data will be assigned a unique code known only to the investigators, which will serve as a link to the individual's identity and to other information collected as part of this research protocol. Research samples will be stored indefinitely in a freezer in the laboratory at NIH in Bethesda, MD. The principal investigator under any and all circumstances will report loss of data or samples to the IRB. Coded samples and data may be shared with other NIH collaborators involved in this study as well as with members of our laboratory to support ongoing study analysis. Samples for vitamin E measurements will be stored and analyzed by Dr. Levine's lab. The code key will be held by the investigators.

In the future, samples and data collected from participants in this study may be used in further research, with approved secondary research protocols by the PI, Dr. Ebenuwa. We may also share samples and data with NIH collaborators, or potentially with outside collaborators with material and/or data sharing agreements, or IRB approval as applicable. No samples or data will be used or shared from subjects who were given an option and said "no" to future research or sharing in the consent form.

21.Privacy and confidentiality:

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor(s). This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor. All research activities will be conducted in as private a setting as possible.

The study monitor, other authorized representatives of the sponsor, representatives of the Institutional Review Board (IRB), and/or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, Institutional policies, or sponsor requirements.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored at the NIH CC. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. The study data entry and study management systems used by clinical sites and by research staff will be secured and password protected. At the end of the study, all study databases will be de-identified and archived in records at NIH CC. To further protect the privacy of study participants, a Certificate of Confidentiality has been issued by the National Institutes of Health (NIH). This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose identifying information on research participation in any civil, criminal, administrative, legislative, or other proceeding, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose

information that would identify research participants, Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to participants.

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