Title: IRON REDUCTION BY PHLEBOTOMY FOR THE TREATMENT OF DIABETES AND NONALCOHOLIC FATTY LIVER DISEASE

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Study Title: IRON REDUCTION BY PHLEBOTOMY FOR THE TREATMENT OF DIABETES AND NONALCOHOLIC FATTY LIVER DISEASE

Principal Investigator, Co-investigator(s): PI: Donald A. McClain MD, PhD; Co-I's: Chinenyewah Usoh MD; John Buse MD, PhD (UNC); Laura Young MD (UNC), Klara Klein MD, PhD (UNC)

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Background, Rationale and Context

1.B. Overview. Iron is a risk factor for diabetes and one of its principal complications, NASH. Extensive studies in rodent models and small clinical trials in humans have demonstrated significant and even curative benefit from iron reduction by blood donation. This proposal is to perform a larger randomized, placebo-controlled trial that will test the hypothesis that this simple, safe, inexpensive, and even socially beneficial approach is generalizable to a larger population of individuals with prediabetes and early T2DM. In addition to the large trial for efficacy, we propose in subcohorts to investigate mechanism (changes in insulin sensitivity vs. secretory capacity) and to monitor indices of NASH more intensively. In the application, I will first review the data linking iron with diabetes and NASH, present our Preliminary Data supporting efficacy of iron reduction for both conditions, and describe the proposed low-risk clinical trial to test our hypothesis. If iron reduction succeeds in improving glycemic control and affecting NASH progression, the study would have great impact: (1) Although this trial will test the "iron hypothesis" only in those in the upper half of normal iron, and even though that is by definition half of all people with diabetes, the data reviewed below in **1.C** indicate that iron confers excess risk across its entire "normal" range, so most people with diabetes could benefit from iron reduction; (2) The animal models suggest that the beneficial effect of iron will be long-lived, but even if it were to be eventually overcome, for example by continued weight gain, delaying disease onset by only a few years would still have immense benefits in terms of healthcare costs and the delay in the burden of complications those individuals would otherwise suffer; (3) Finally, iron is also associated with other chronic diseases such as Alzheimer's (see below), and the insights gained in this study may be applied to those conditions as well.

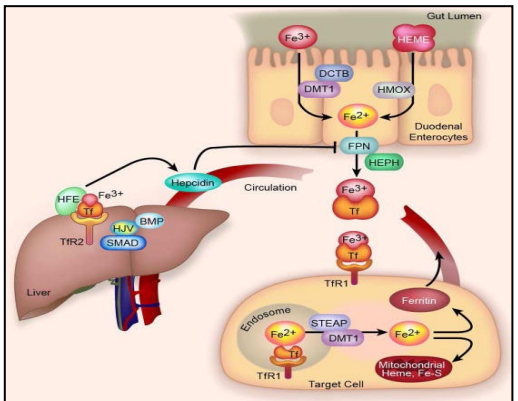

Fig. 1: Iron regulation. Iron enters the circulation from the gut via the iron exporter ferroportin (FPN). Transferrin (Tf)-bound iron enters cells after endocytosis of the Tf Receptor. In the liver, iron stimulates production of hepcidin that feeds back to downregulate FPN.

1.C. Iron and the risk for diabetes. Iron is a required cofactor for fuel oxidation and energy production. The cell's need for iron in the face of its potential danger as an oxidant has given rise to a complex system, coordinated across tissues, to tightly regulate its levels, distribution, and bioavailability (**Fig. 1**). In yeast, the switch from fermentative (glycolytic) metabolism to oxidative phosphorylation is dependent on the presence of iron in the environment. Conversely iron, a potentially dangerous oxidant, is not imported into the cell unless there is a need for oxidative phosphorylation. Thus, there has arisen over evolution an important link between iron homeostasis and metabolism, and we have shown many of those connections are conserved in humans.

Although iron deficiency is one of the most common nutritional deficiencies in the world, humans can also experience pathologic iron overload in conditions such as hereditary hemochromatosis (HH), where the dangers of excess tissue iron were first appreciated. HH, for example, is associated with diabetes, nonalcoholic steatohepatitis (NASH), and an increased risk of Alzheimer's disease (1). The crucial fact that iron plays a causal role in HH has been demonstrated by improved insulin secretion (2), and protection from progression of liver disease (3) after phlebotomy to normalize body iron stores. Iron overload resulting from frequent blood transfusions is also associated with diabetes risk, and that risk has decreased since the initiation of aggressive iron chelation therapy in patients with beta thalassemia (4).

Beyond pathologic iron overload, the question has arisen if there is also a relationship between T2DM and tissue iron levels in the broad "normal" range. Epidemiologic evidence demonstrates a clear association between diabetes risk and serum ferritin, a generally reliable biomarker for tissue iron stores. In the NHANES cohort the odds ratios for diabetes in those with elevated serum ferritin levels are 4.9 for men and 3.6 for women (**Fig. 2**, Ford studies) (5). *Importantly, progressively increasing risk is seen through the entire range of normal ferritin (6)*. Because ferritin is also a marker of systemic inflammation, these relationships were initially attributed to the inflammation associated with diabetes. Studies have shown, however, *the diabetes risk associated with high iron is not accounted for by HH or inflammation but rather by increased dietary intake* (7). Recent studies of gestational diabetes have shown that the increased risk is associated in particular with intake of heme from red meat, which is more efficiently absorbed than non-heme iron (8). These studies suggest that eating as little as 1.5 pounds of red meat per week can raise iron to the levels that are associated with T2DM.

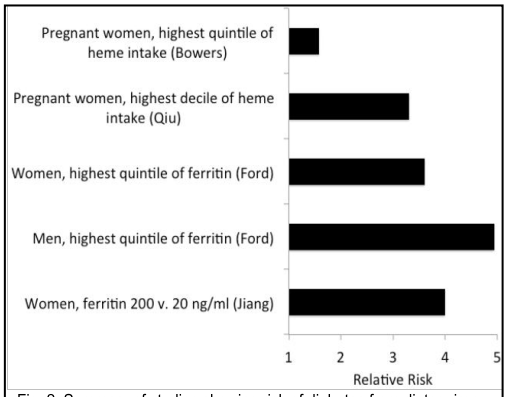

Fig. 2. Summary of studies showing risk of diabetes from dietary iron and/or serum ferritin levels, taken from the studies referenced in the text (Bowers [11], Qiu [10], Ford [5], and Jiang [6]).
Plotted are the relative risks from four studies (Bowers et al., 2011) (Qiu et al., 2011) (Ford and Cogs

Similar relationships among iron and T2DM risk, insulin resistance, or both are seen in Europeans and African-Americans (6, 9), gestational DM (8, 10, 11) and prediabetes (12). The relative risk of diabetes as a function of iron/ferritin in key studies is summarized in **Fig. 2**. High ferritin also doubles the risk for metabolic syndrome (MetS), the constellation of impaired glucose tolerance, obesity, hyperlipidemia, and hypertension, after accounting for other risk factors (13). The detrimental effects of high-normal iron on tissue function take on extra importance because of iron's association with a number of other diseases including NASH (14), Parkinson's and Alzheimer's diseases (15), colon/breast cancer (16), and cardiovascular disease (17).

Demonstrated mechanisms for the beneficial effects of lowering iron in "garden variety" T2DM and MetS include improvements in insulin secretion (2) and insulin sensitivity (18, 19), the principal determining factors of T2DM. Increased insulin sensitivity is mediated at least in part by adiponectin, which is directly regulated by iron (20). Proof of a causal role of iron in T2DM, and more importantly its reversibility, have been shown in rodent diabetes models (21) and by relatively small studies in humans that demonstrated improvements in fasting glucose, hemoglobin A_{1C} (HgbA1C), and insulin sensitivity after modest phlebotomy to reduce iron from the upper to the lower ranges of normal (20, 22, 23).

Low iron/ferritin is also a marker for so-called "healthy obesity," namely obesity that does not proceed (or perhaps has not yet proceeded) to MetS and diabetes. Comparing individuals with BMI >40, some with no evidence of MetS other than waist girth, and others that had all elements of MetS (hypertension, glucose intolerance or diabetes, high serum triglycerides [Tg], and low HDL), we found serum ferritin to be nearly twice as high in the MetS group (20). Possible mechanistic contributors are the regulation of both adiponectin and leptin by iron cited above (20, 24). In rodents, it is also possible to maintain healthy obesity by decreasing bioavailable iron in adipocytes: Mice on the Ob/Ob background with adipocyte-targeted over expression of an iron-sulfur cluster binding protein, MitoNEET, *weigh up to 140 g but do not develop diabetes or NAFLD* (25).

1.D. Iron and the progression of nonalcoholic fatty liver disease (NAFLD). NAFLD, a condition that has been termed the hepatic manifestation of MetS and T2DM, is present in a majority of people with T2DM and is becoming the most common reason for liver transplantation. Many pathways contribute to NAFLD, but just as all obesity does not proceed to diabetes, not all NAFLD proceeds to NASH, and excess tissue iron is shared as a risk factor for both progressions (see above and (14, 26)). A link between iron and the progression of NAFLD to NASH has also been noted in genome wide association studies wherein increased risk of NASH is seen individuals with mutations in the genes that cause thalassemia and HH, both of which result in iron overload (27). NAFLD and/or NASH are also associated with polymorphisms in genes connected to pathways that we have shown to be iron-regulated, including leptin, adiponectin, and PPARγ coactivator 1 α (PGC1α) (28). In these cases, the effect of the polymorphisms that confer diabetes risk is the same as that of high iron, namely decreased pathway activity (Preliminary Data and (28)). This congruence of genetic underpinnings of NAFLD/NASH with the effects of iron validates many of the linkage that we propose to test.

The issue of the causality of the relationship between elevated serum ferritin and increased risk for progression to NASH is as germane to this disease as to diabetes. NASH is by definition an inflammatory disorder that could increase ferritin by that mechanism and confound the relation of iron to the disease. However, large studies have demonstrated *in biopsy-proven NAFLD/NASH that ferritin is more closely related to liver iron*

content than to markers of inflammation (29).

Most importantly, proof of causality rests on interventional studies that demonstrate improvement or delayed progression of disease with iron reduction. Reducing iron by phlebotomy or chelation in humans has been shown to improve NAFLD/NASH (29-31). Other studies are conflicting, but they have employed a wide range of thresholds and targets for tissue iron and differing inclusion criteria, and suffer from underpowering and differing time windows of analysis after iron reduction. A recent meta-analysis concluded there was no benefit to NASH of iron reduction by phlebotomy (32). At odds with that conclusion, that very same analysis did reveal that phlebotomy results in a significant reduction in the canonical marker of NASH, the serum transaminase ALT. Furthermore, the two most prominent "negative" studies in that analysis were one in which the pre-treatment population started with *normal* transaminases (hence, not showing improvement was a foregone conclusion) and the other followed subjects with NASH for only 6 months, a time that had been previously shown in a positive study to be insufficient to demonstrate improvement (33). Thus, based at least in part on a seriously flawed analysis, phlebotomy (or blood donation), which is a safe, simple, acceptable and promising therapy, has not been widely adopted. Even if it were, thresholds for initiating phlebotomy, optimal targets for iron levels, the best rates to achieve those targets, and the longevity of the effects are not known. Furthermore, we have an incomplete understanding of all the pathways impacted by iron modulation. Thus, our laboratory has continued to study the basic science of iron and metabolism even while also pursuing funding for clinical trials.

1.E. Preliminary Data**:** *Phlebotomy for prediabetes and T2DM)*. To demonstrate the benefits of reducing iron on glucose homeostasis, we recently completed a pilot study in which subjects (N=11, 9M and 2F, aged 57.2±1.2 yr, BMI 30.6±2 kg/m^2) with prediabetes (N=3) or early T2DM (N=8) were treated with phlebotomy in order to reduce serum ferritin concentrations. No subjects had evidence of systemic inflammation as assessed by medical history or elevated C-reactive protein. Individuals treated with phlebotomy achieved a significantly lower serum ferritin level with biweekly to monthly 1 Unit donations (average 3.4±0.5 Units, or ~1.7 L) of blood, providing further evidence that the elevations in ferritin were related to iron and not inflammation. Women started with lower ferritin (92±2 ng/mL) compared to men (219±32 ng/mL, p<0.04) and thus achieved less absolute lowering of their ferritin (56±7 ng/mL) than did the men (150±33 ng/mL). Average weights increased slightly but not significantly in both groups over the course of the study (1.8 kg in treated, 3.1 kg in controls, p=NS). Blood hemoglobin concentrations tended to decrease in the treated group (p=0.10); only one subject declined to below the normal range (by 0.1 g/dL, less than 1% below normal) and recovered to normal by the end of the study. There were no serious adverse events and no fainting.

Improved glucose tolerance after phlebotomy. Phlebotomy treatment resulted in significant lowering of area under the glucose curve (AUCg) during a 120 min oral glucose tolerance test (average improvement 1340±570 [mg/dL]*min, p<0.05 by paired t test). Non-treated controls increased their AUCg value by an average of 1110±670 (mg/dL)*min, significantly different from the treated group (**Fig. 3**, p<0.04). The decrement in the AUCg after phlebotomy tended to be correlated with the starting AUCg value (r=0.667, p=0.13) and the starting ferritin value (r=0.641, p=0.063). AUCg values also trended to decrease more in those with prediabetes than diabetes (-10.3±2.2% compared to -2.9±2.3%, p=0.06), suggesting that early intervention, perhaps before critical loss of beta cell mass, will prove to have the greater beneficial effect.

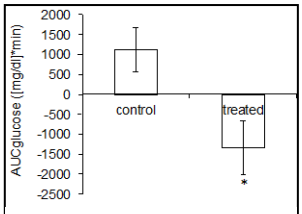

Fig. 3. Individuals (N=12) with diabetes or prediabetes and serum ferritin in the upper half

Disposition index determined by frequently sampled intravenous glucose tolerance test (FSIGTT) is improved by phlebotomy. A subset of the subjects (1 control, 8 treated) underwent FSIGTT before and after iron reduction. The disposition index (DI), which is the product of the values for insulin sensitivity (S_I) and acute insulin response to glucose (AIRg), serves as a better predictor of diabetes risk than either S_i or AIRg alone (34). Among the treated subjects, DI Improved in seven of eight (P<0.04 for the entire group, by paired t test). The single control subject undergoing FSIGTT experienced a 72% worsening in DI over the study period. Among all of the subjects, the increase in DI (log transformed) was significantly correlated to the change in ferritin during the study (**Fig. 4**, p<0.04). The reasons for the improvement in DI varied. Every treated subject improved in terms either of insulin secretion (N=6 of 9, average increase in those 6 of 23.7%, p=NS), insulin sensitivity (N=6 of 9, average increase in those 6 of 51.9%, p=NS), or both (N=3 of 9).

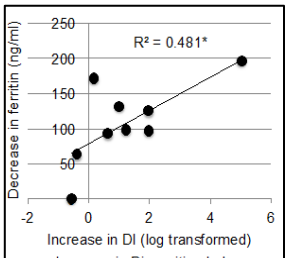

Fig. Increase in Disposition Index as a function of decrease in ferritin after phlebotomy. *p<0.05

In this small study sample, there was a hint that iron reduction to the very lower limit of normal was not optimally beneficial, and a recent epidemiologic study has suggested a possible optimal ferritin level in the range

of ~40-60 for men and ~30-50 for women, values that are in the 2nd lowest decile of normal.

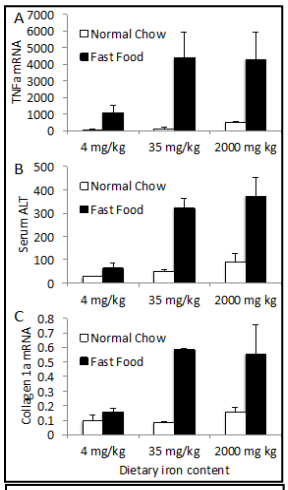

Fig 5. (A) Liver TNFα mRNA, (B) serum ALT, and (C) liver collagen 1α mRNA in mice fed the

Improvement in indices of NASH with reduced dietary iron intake. We have demonstrated in a mouse model that a low iron diet protects from NASH (**Fig. 5**). Mice were fed a "fast food" (FF) diet (~30% fat and fructose-supplemented water) that had previously been shown to result in obesity, insulin resistance, and hepatic inflammation. We varied the iron content from low (4 mg iron/kg chow, but not resulting in iron-deficient insofar as animals maintain normal blood Hgb) to a level seen in some normal chow (35 mg/kg) to a high level (2000 mg/kg, resulting in tissue iron seen in the high end of normal humans). We measured mRNA for TNF α, an inflammatory cytokine thought to play a role in NASH pathogenesis (**Fig. 5A**), the canonical serum marker of NASH, ALT (**Fig. 5B**), and mRNA for the fibrotic protein collagen 1α (**Fig. 5C**). In mice fed the FF diet, all three markers increased, but only in the mice on the higher iron diets. The intermediate iron diet also resulted in a delay in the rise of ALT measured at earlier times (not shown).

We also have demonstrated in humans that reduction of iron in individuals with NAFLD/NASH is associated with improvement hepatic inflammation (serum transaminases, **Table 1,** unpublished) and other markers of metabolic syndrome including HgbA1C and lipids (not shown).

	Pre-phlebotomy	Post-phlebotomy
Serum ferritin	398±319 (ng/mL)	47±52*
ALT	64±46 (IU/L)	36±19*
AST	41±26 (IU/L)	28±12*

Table 1: Individuals (N=40) with elevated ferritin and a diagnosis of NAFLD or NASH received phlebotomy to lower ferritin to 50 ng/mL. *p<0.005.

OBJECTIVES. Based on the rationale and preliminary data presented, *we hypothesize that tissue iron levels in the higher range of normal confer excess risk of T2DM and another common complication of diabetes, NASH. We also hypothesize that this risk is reversible, and markers of both diabetes and NASH will improve and/or progress less in individuals who undergo iron reduction by phlebotomy (blood donation), sufficient to decrease the biomarker of tissue iron, serum ferritin, from the upper half of normal to the two lowest deciles of normal.* The study is an extension of that which resulted in our pilot data, and is aimed at testing our hypothesis in a larger cohort that will provide data on appropriate target populations and threshold and target levels of iron for treatment. Because of the ease, safety, and acceptability of the treatment that would also carry with it an independent societal benefit to blood banks, we believe that this proposed trial could have a major impact on diabetes, NASH, and ultimately other conditions associated with excess nutrition and aging.

METHODS AND MEASURES

DESIGN: We propose a randomized, placebo-controlled trial to determine if reducing tissue iron stores by phlebotomy (blood donation) will improve measures of glycemia in Type 2 Diabetes (T2DM) or prediabetes. Adult subjects (N=240 total) aged 40-75, half with prediabetes and half with early T2DM (controlled by diet or up to 2 nonhypoglycemic agents) with ferritin values in the upper half of normal will be randomized to receive real or sham phlebotomy (blood donation), performed in the same manner as at Red Cross donation centers. Glycemia will be assessed by HgbA1C (primary endpoint), fasting glucose, and continuous glucose monitoring (CGM) before and after treatment. Glycemic outcomes will be assessed 6 and 12 months after achieving the target ferritin. The primary endpoint for which this study aim is powered is a change in HgbA1C at 6 months. As secondary outcomes, we will determine if that improvement is stable over a year, and if there are changes associated with iron reduction in serum alanine transaminase (index of NASH), insulin sensitivity by homeostasis model assessment (HOMA-IR), weight, lipids, blood pressure, changes in the number of diabetes medications for participants with diabetes, and changes in glycemic status (normal, prediabetes or diabetes) for participants with prediabetes. We will also correlate the degree of change in glycemia with the starting ferritin levels and the overall change in ferritin to determine targets and thresholds for iron reduction.

There will be two optional sub studies that will be conducted only at Wake Forest Baptist Medical Center. One will look more closely at liver complications of diabetes and the other will explore whether improvements in glucose levels are due to more insulin production or improved insulin action.

Iron may also play a role in the progression of fatty liver to scarring and cirrhosis. Since 75% of people with diabetes have some degree of fatty liver, we would also like to study how your liver reacts to the lowering of iron.

This will require some additional test, namely an MRI and a liver Ultrasound (Fibroscan). There will be approximately 40 people recruited at Wake Forest Baptist Medical Center for the liver sub study.

The Glucose Tolerance Mechanism substudy will look at the mechanism that your body uses to regulate blood sugar levels by insulin, this will require the frequently sample intravenous glucose tolerance test (FSIVGTT). There will be approximately 48 people recruited at Wake Forest Baptist Medical Center.

SETTING: The trial will be performed in two academic health care centers, Wake Forest School of Medicine and Baptist Health Center, and the University of North Carolina at Chapel Hill. Study procedures and intervention will take place in those two institutions' Clinical Research Units supported by their NIH Clinical and Translational Science Awards. At UNC, non-phlebotomy visits may occur at the Eastowne Clinical Research Unit for ease of parking and access.

Subjects selection criteria

Subjects will have type 2 diabetes mellitus, or prediabetes, defined by HgbA1C criteria.

- **Inclusion Criteria.** Ages 40-75; At least 3 months since diagnosis of prediabetes or diabetes; HgbA1C value within three months or at screening of 5.7-6.4% for those with prediabetes, undiagnosed on no medication HgbA1C 6.5-6.9 and 7-8.5% for those with diabetes (the upper limit of the latter to reduce the likelihood of major changes in glycemic intervention during the trial period, and the lower limit to allow some room for improvement); Serum ferritin levels within 1 year or at the time of screening in the upper half of the normal range (>50 ng/mL for women and >100 ng/mL for men). C-reactive protein levels up to 11.0. Subjects will be on stable lifestyle therapy and 0-2 antihyperglycemic agents that are non-hypoglycemic (i.e. no sulfonylureas, glinides or insulin) to limit the chances of hypoglycemia if, as hypothesized, participants become more insulin sensitive with iron reduction. Aim 2 – serum ALT> 1.5 times the upper limit of normal, or; liver stiffness of > 12.5 kPa by Fibroscan transient elastography.

- **Exclusion Criteria.** Documented current anemia, hemoglobin levels within 0.5 g/dL of the lower limit of normal (<12.5 g/dL for women and 13.5 g/dL for men), or recent blood loss; bleeding diatheses (coagulation abnormalities or treatment with anticoagulants); serious chronic infections or chronic inflammatory conditions that could elevate ferritin as an "acute phase reactant;" C-reactive protein greater than 10.0, the upper limit of normal, to further validate the lack of significant chronic inflammation; active cancer diagnosis (excluding skin cell cancers other than melanoma); renal insufficiency (eGFR<60 ml/min); history of orthostatic hypotension; heavy alcohol use (NIH criteria, for men greater than 4 drinks on any day or 14/week, for women >3 drinks on any day or > 7/ week); pregnancy or premenopausal women of childbearing age, unless unable to become pregnant because of oral contraceptive use, IUD, birth control implants, surgical loss of ovaries or uterus. Aim 2 – individuals meeting the additional inclusion criteria for aim 2 will be tested for anti-HAV IgM, HBs Ag, anti-HBc. IgM, anti HCV IgM and IgG. Subjects who prove positive for any of these viral serologies, except for HCV IgG will be excluded. The latter will be tested for HCV RNA by PRC, and if negative they will be eligible for enrollment

- **Sample Size.** In our pilot study of the effects of phlebotomy, we did not measure HgbA1C, but did perform glucose tolerance testing where the difference in area under the curve from pre- to post-treatment was 1130±1970 mg-min/dl. Assuming this average change would be maintained through the day, and converting this change to the relative change in HgbA1C, the change would be expected to be a decrease of ~0.6% (in A1C units, not a change of 0.6% magnitude). With the variance observed, this means a sample size of 32 patients in each group will provide 80% power to detect a difference between the beginning and final HgbA1C in either treatment group at $p<0.05$ when pre- and post-phlebotomy results are compared within subjects. This agrees with the published results from another group that did measure HgbA1C, wherein they achieved a significant change ($p<0.001$) with an N of 31, but a shorter observation period and much less reduction in ferritin. A larger sample size (60/group, 240 total in Aim 1, half diabetes and half prediabetes, with

120 each being randomized to control and phlebotomy) will be recruited to facilitate possible subgroup analysis (e.g. stratification by age, treatment, race, sex) and to allow an up to 20% dropout rate, which is significantly higher than the experience of previous similar studies at Wake Forest University, wherein the dropout rates were in the 12-15% range. (In our pilot study, there were no dropouts.) Thus, we will be theoretically powered to analyze each target ferritin group separately, although the initial analysis will pool the two treatment groups.

- Multisite study. This is a multisite study, coordinated by WFUHS, and equal numbers will be recruited at each site, 120 at WFUHS and 120 at UNC Chapel Hill. The Liver substudy will recruit 40 people and the Glucose Tolerance Mechanism substudy will recruit 48 people. The sub studies will be conducted only at Wake Forest Baptist Medical Center.

Interventions and Interactions

The essential features of interventions and interactions used to generate data for the study should be described.

- ***Intervention***: Periodic (every 8 weeks) phlebotomy of 500 mL (one "Unit") sufficient to reduce serum ferritin from the upper half of normal to the lowest 20% of normal (Males 20-40, Females 20-40 ng/ml) (35). In our pilot study, subjects reached similar ferritin levels with donation of 3.4±0.5 Units. In that case subjects donated one Unit per month and there were no adverse events; that interval has been increased in this study to 8 weeks to conform to current Red Cross practices.
- ***Sham Phlebotomy***: As any other study, interventions are subject to placebo effects, and the same is true for phlebotomy. We are thus including a sham phlebotomy as described in the procedures, below.
- ***Study Protocol.*** The inclusion and exclusion criteria are listed in **3.A**. We are including arms to address prediabetes and diabetes separately because there is the possibility that the former will respond more robustly to therapy because they will not have had the disease long enough to develop a significant degree of insulin deficiency, a feature of T2DM that may be difficult to reverse in middle-aged or older adults. Our small pilot trial showed improvement in both groups, but 2/3 of those with prediabetes reverted to normal glucose tolerance, a surprisingly good result. For the reason of reversibility cited above, we are not including anyone on insulin; furthermore, anyone on insulin or other agents with the potential to cause hypoglycemia are also excluded because if their insulin sensitivity improves with iron reduction, as the Preliminary Data suggest, they could be more prone to hypoglycemia.

  Serum ferritin will be used as the primary assessment of tissue iron stores. It is an excellent and reliable marker of tissue iron, even in those with significant comorbidities including advanced NASH (26). There is an inflammatory component to obesity and T2D, and hence there is a small increase in ferritin with obesity noted in some but not all studies (e.g. Fig. 3 in (36)). However, the magnitude of the average difference with obesity (~20 ng/ml) is tiny, 7% compared to the normal range in the population (20-~300 ng/mL). The paper showing the largest difference with obesity is only true when the compared to BMI <25, whereas there is *no change at all* going from the group with BMI of 25-30 (ave. ferritin 144), to 30-35, 35-40, and >40 (ave. ferritin 146)! (37). NHANES data show that people with diabetes and high-ferritin are heavier consumers of red meat, and ferritin is not accounted for by markers of inflammation (7), confirmed in a more recent study (38). We showed no relation of CRP to ferritin in obese T2D (20). That ferritin is overwhelmingly reporting iron status rather than inflammation is also backed by biopsy-based studies in NASH (29). *In every subject* in our pilot, ferritin fell uniformly and promptly with phlebotomy, which would not happen if ferritin were mainly reporting inflammation. To the degree that obesity *per se* contributes to ferritin, it is overwhelmed by the contribution of iron. We will further exclude the possibility that it may be falsely reporting inflammatory stress by excluding individuals with elevation of the inflammatory marker C-reactive protein. A second measure of tissue iron, serum "soluble transferrin receptor," will be obtained as confirmation of the assessment of ferritin, but not used for inclusion/exclusion because it is less well-validated.

- ***Study Procedures*: Basic Study.** These are summarized in the accompanying table.

	Screening (Not fasting)	Visit 1 (not fasting)	Visit 2 (fasting)	Visit 3 (not fasting)	Visit 4 (Not fasting)	Visit 5 (not fasting)	Visit 6 (not Fasting)	EOS 1 (fasting)	EOS 2 (fasting)
Consent	X								
Inclusion/Exclusion	X								
Medical History	X								
Review Medications	X		X	X	X	X	X	X	
Vital Signs (following BP protocol)	X		X	X	X	X	X	X	X
Physical Exam	X							X	X
Adverse Events	X		X	X	X	X	X	X	X
CGM Placement		X						X (returned by mail)	X (returned by mail)
Return CGM and Download Data			X						
Hemoglobin-WFU CRU	X		X	X	X	X	X	X	X
Ferritin, serum	X (if not available)		X	X	X	X	X	X	X
HgbA1c	X (if not available)							X	X
CRP	X							X	X
Insulin (fasting)			X					X	X
Lipid panel (fasting)			X					X	X
CMP (14)			X					X	X
CBC platelets no Diff	X							X	X
*Fibroscan			*X					*X	*X
Blood for Research Storage (to be sent to Wake Forest)	X		X	X	X	X	X	X	X
Phlebotomy Procedure (including all vital sign measurements prior and post procedure)			X	X	X	X	X		
Phlebotomy De-identified Discarded samples sent to the Olin Physical Lab – WFBH site only			X	X	X	X	X		

* –Fibroscan at WFBH site only

- **a. Screening Visit (Not fasting)*:*** Consent, assessment of inclusion/exclusion criteria, serum ferritin (if not available within last year), CBC, Platelets, no differential, C-Reactive Protein (CRP), Hemoglobin, and HgbA1C (if not available through the electronic health record in the last 3 months). Subjects will be counseled to reduce weight if they are overweight or obese, using the protocol of the Diabetes Prevention Program (39). Subjects who complete the screening visit will be compensated $100 for their time.
    - a.1 Rescreening: Due to the COVID-19 pandemic delay in research visits, any subject that was previously screened but was not able to initiate the study can be rescreened; if eligible all subsequent visit will proceed in accordance with the protocol.
- **b. Visit 1 (not fasting) (~30 min):** Placement of continuous blood glucose monitor (CGM). This is a small waterproof "pod" with a subcutaneous probe that provides a continuous record of glucose levels (every 5 min) for a period of 6-10 days. We will use the Abbott Libre that is FDA approved for monitoring glucose in patients with diabetes. This will provide more information than a single fasting glucose, an important consideration because of the variability of fasting glucose and the additional information provided regarding postprandial and nighttime levels. An added advantage is that after the initial placement and removal, when the subjects have gained familiarity with it, the device can be returned by mail back after the monitoring period. Subjects who complete Visit 1 will be compensated $50 for their time.
- **c. Visit 2 (7-14 days) (fasting) (~1 hr):** Report to the Clinical Research Unit (CRU) at University of North Carolina at Chapel Hill (UNC) or Wake Forest (WF) School of Medicine. Review Adverse Events (AE's). Measure outcome variables (weight, blood pressure; blood for, insulin, lipid panel, CMP (14), Ferritin) and the Ultrasound Fibroscan (WFBH site only). Subjects who complete Visit 2 will be compensated $50 for their time.

Randomization (1:1) to control (sham phlebotomy) or phlebotomy groups with final target ferritin levels of 20-40 ng/ml for males and 20-40 for females. Phlebotomy will not be performed if hemoglobin is lower than 12.5 g/dL for women and 13.0 g/dL for men; in that case, subjects will undergo repeat hemoglobin measurement every 2 months. If the hemoglobin value increases above the threshold for phlebotomy at Visits 3 or 4, phlebotomy will be performed; otherwise, participants will have an "end of study" visit 1 at six months. If blood pressure declines by > 20 mm Hg systolic, or pulse increases by > 30 bpm upon standing, phlebotomy will not proceed.

Phlebotomy: Both groups will have a sleep mask-style blindfold placed or a barrier (e.g. a towel) will be placed between all subjects and the phlebotomy bag in order to prevent the subject from knowing if blood was drawn, and have a phlebotomy needle placed, usually in an antecubital vein. The treatment group will have 1 Unit (~500 mL) of blood removed, while in the placebo group the needle will remain clamped. After the usual 8-10 minutes required for removal of the unit, both groups will have the needle removed, be given a zero-calorie rehydration drink (12 oz) and remain seated for at least 5 min or until comfortable to stand. A snack will be offered. Blood pressure will be monitored after the procedure and if there is hypotension by the above criteria (see *Randomization)*, the subject will be asked to remain another 5-10 min and encouraged to finish the drink or have a second. The phlebotomies or sham phlebotomies will be performed by research nurses in the CRU, who have the training and support to respond to a medical emergency. For considerations related to phlebotomy safety and the use of the placebo group, please refer Visit 3.*WFBH participants only*: The discarded phlebotomy blood will be de-identified and will be picked up by a representative from the Dr. Kim-Shapiro Lab, the sample will be stored in the Olin Physical Lab on the Reynolda campus, room 216. The blood will be used for in vitro experiments aiming to prevent thrombosis in extracorporeal circulation devices. An Institutional Review Board (IRB) must also approve any future research study using your blood sample.

Review of laboratory findings: Ferritin results will not be routinely available prior to phlebotomy, but will be reviewed 1-3 days after the visit. Once the serum ferritin results reach the target range (20-40 ng/ml), the subject will be moved to the End of Study sequence.

- **d. Visit 3 (8 weeks after Visit 2) (not fasting) (~1 hr.):** Review AE's. Draw blood for ferritin and perform on site determination of hemoglobin. If hemoglobin is lower than 12.5 g/dL for women and 13.0 g/dL for men, phlebotomy will not be performed and subject will be scheduled 8 weeks out for Visit 4. If the serum ferritin results from this visit have reached the target range (20-40 ng/ml), the subject will be moved to the End of Study sequence. If not, the subject will continue to Visit 4. Subjects who complete Visit 3 will be compensated $50 for their time.
- **e. Visit 4 (not fasting):** Review AE's. Draw blood for ferritin and perform on site determination of hemoglobin. If hemoglobin is lower than 12.5 g/dL for women and 13.0 g/dL for men, phlebotomy will not be performed and subject will be scheduled 8 weeks out for Visit 5. At this visit, after the sham phlebotomy, all of the Placebo Group will be scheduled to the "end of study" sequence. Three phlebotomies were the average needed for the treatment group in the pilot study to achieve goal ferritin. If serum ferritin results of the treatment group subjects from this visit have reached the target range (20-40 ng/ml), the subject will be moved to the End of Study sequence. If not, the subject will continue to Visit 5. Subjects who complete Visit 4 will be compensated $50 for their time.
- **f. Visits 5-6, if needed (not fasting):** Hemoglobin and Ferritin will be drawn. If hemoglobin is lower than 12.5 g/dL for women and 13.0 g/dL for men, phlebotomy will not be performed and subject will be scheduled 6 months out to start the End of Study sequence. If the serum ferritin results from Visit 5 have reached the target range (20-40 ng/ml), the subject will be moved to the End of Study sequence. If not, the subject will continue to Visit 6. After Visit 6, all subjects, regardless of serum ferritin results, will move to the End of Study sequence. Subjects who complete these visits will be compensated $50 for their time per visit.
- **g. End of Study Visit 1 (6 months ± 2 weeks after last phlebotomy visit) (fasting) (~1 hr.):** Patient should come in fasting. Review AE's. Perform outcome measures (blood pressure, weight, CMP (14), CBC platelets no diff., insulin, HgbA1C, CRP, lipid panel, ferritin. Fibroscan (WFBH site only), place CGM, which the subject will mail back in a prepaid mailer. Subjects who complete this visit will be compensated $100 for their time.

- **h. End of Study Visit 2 (6 months ± 2 weeks after E.O.S. 2) (fasting) (~1 hr.)**: Patient should come in fasting. Review AE's. Perform outcome measures (CGM placement, blood pressure, weight, CMP (14), CBC platelets no diff., insulin, lipid panel, HgbA1C, ferritin, CRP, Fibroscan (WFBH site only)). Place CGM, which the subject will mail back in a prepaid mailer. Subjects who complete this visit will be compensated $100 for their time.

- Study procedure: Liver Substudy

- The Screening Visit and Visit 1 will be the same as the Basic Study

- In addition to the other studies planned Visit 2 of the Basic Study, those in the Liver Substudy will have: Magnetic Resonance Imaging (MRI), FIBROSCAN done at Visit 2.

- Both Control and Treatment Groups will have an Ultrasound Fibroscan, the Fibroscan is a noninvasive imaging study that evaluates the degree of liver stiffness, which is a measure of the scaring or fibrosis that can occur with diabetes or fatty liver. It does this by determining the speed of sound waves through the liver utilizing a sonogram. The Magnetic Resonance Imaging (MRI) is a test that uses powerful magnets, and a computer to make detailed pictures inside your body. Neither test uses radiation.

- Following Visit 2, the RANDOMIZATION, PHLEBOTOMY, and END OF STUDY (E.O.S.) visits will occur just as in the Basic Study, the End of Study 1 will repeat the Fibroscan and End of Study 2 will repeat the Fibroscan and MRI.

- Liver Substudy Calendar: includes all main requirements with the addition of the Fibroscan and MRI

Procedure	Screening Visit (Not Fasting)	Month 0		Months 2-8 (as needed)			Month 11-12	Month 17-18
		Visit 1 (Not fasting)	Visit 2 (Fasting)	Visit 3 (Not Fasting)	Visit 4 (Not Fasting)	Visit 5, 6, ect. (Not fasting)	E.O.S. 1 (Fasting)	E.O.S. 2 (Fasting)
*Fibroscan			X				*X	*X
*MRI			**X					**X

* – Fibroscan (additional test for Liver substudy)
** - MRI

- Study procedures: Glucose Tolerance Mechanism substudy

- The Screening Visit and Visit 1 will be the same as the Basic Study.

- In addition to the other studies planned at Visit 2 those in the Glucose Tolerance Mechanism Substudy will have a Frequent Sample Intravenous Glucose Tolerance Test (FSIVGTT) at Visit 2.

- Both Control and Treatment Groups will have a glucose test that uses an IV it is called a Frequent Sample Intravenous Glucose Tolerance Test (FSIVGTT). There will be a plastic catheter (IV) placed in a vein in your arm and blood samples will be taken. A second IV will be placed in your other arm and dextrose (sugar water) will be given through that IV. For this test, you will be given 300mg/kg (or an average total of 20 grams) of glucose IV over one minute with bloods for insulin and glucose determination collected at one minute intervals for 6 minutes, then at two minute intervals until 18 minutes. At 20 minutes, you will be given 0.04 Unites/kg or regular insulin, or an average of 2-3 Units total. Samples of blood will continue to be taken until a total of 180 minutes have elapsed. This amount of insulin is much less, than your own body will be making in response to the glucose we gave you.

- During this visit, the total amount of blood taken from you will be approximately ¾ cup (10 tablespoons). When your tests are completed, you will be given a snack and meal voucher.

- Following Visit 2, the RANDOMIZATION, Phlebotomy, and END OF STUDY (E.O.S.) visits will occur just as in the Basic Study, with the addition of the FSIVGTT at E.O.S 1 and E.O.S. 2.

- Glucose Tolerance Mechanism Substudy Calendar: includes all Main requirements with the addition of the FSIVTT test

Procedure	Screening Visit (Not Fasting)	Month 0		Months 2-8 (as needed)			Month 11- 12	Month 17- 18
		Visit 1 (Not Fasting)	Visit 2 (Fasting)	Visit 3 (Not Fasting)	Visit 4 (Not Fasting)	Visit 5, 6, ect (Not Fasting).	E.O.S. 1 (Fasting)	E.O.S. 2 (Fasting)
FSIVGTT			**X				**X	**X
*Fibroscan			*X				$*_X$	$*_X$

** – Frequent Sample Intravenous Glucose Tolerance Test (FSIVGTT)
* – Fibroscan

Outcome Measure(s)

The primary endpoint will be an improvement in glycemia as assessed by HgbA1C at the first end of study visit, 6 months after phlebotomy. Acutely, HgbA1C will be lowered by blood donation, because that test measures the lifetime exposure or a red blood cell (RBC) to glucose; phlebotomy will result in new RBC synthesis and hence younger RBC, but that effect should disappear soon after the normal RBC lifespan of ~3.5 to 4 months, well before the first post-treatment assessment at 6 months.

A secondary endpoint will be HgbA1C at 12 months to probe longevity of the effect. Based on animal data, we expect that value to be further improved relative to 6 months, but we have no human data on which to support that hypothesis. We do not anticipate that the effect will decline over that time based on reaccumulation of iron. This is a slow process: In individuals with hemochromatosis, for example, iron overload and its adverse effects may not appear until the 5th or 6th decades. In fact, we even anticipate continued improvement as the high levels of tissue iron redistribute and re-equilibrate as processes triggered by decreasing iron proceed, such as autophagy of ferritin complexes, and unloading of macrophages in the face of low hepcidin (Fig. 1).

Fasting glucose will also be assessed at 6 and 12 months after phlebotomy, with secondary endpoints being an improvement at 6 and 12 months. CGM will also be performed using the Abbott Freestyle Libre Pro system for 7-14 days to enhance validation of HgbA1C measures; it allows determination of multiple fasting levels, daily average glucose and various indices of glucose excursions, e.g. postprandial maxima and areas under the curve.

Other secondary endpoints include improvement in insulin sensitivity (decreased HOMA-IR calculated from fasting glucose and insulin), discontinuation of oral antihyperglycemic agents because of improved HgbA1C, change in ALT, changes in weight or blood pressure, and conversion from prediabetes (HgbA1C criteria) to either diabetes or normal glucose tolerance. The secondary endpoints were chosen because they are inexpensive and frequently assessed as part of usual clinical care, and because of the literature suggesting that they are also improved by phlebotomy. Outcomes will also be analyzed using initial, final, and change in ferritin as a continuous variable to interrogate possible optimal thresholds and targets for therapy.

Liver Substudy Outcome Measures

The primary endpoint is a decrease in ALT 12 months after completion of phlebotomy**.** For this endpoint, we will include as a separate analysis individuals in Aim 1 with elevated ALT attributed to NASH (by exclusion of viral hepatitis, etc.) but who elected not to participate in Aim 2.

Secondary endpoints will include improvements (or decreased deterioration) compared to controls in: (1) Model of End-stage Liver Disease (MELD) scores (64) as determined from the history and serum testing; (2) Fibroscan and MRI indices of hepatic fibrosis; (3) ALT and imaging indices will analyzed as a function of hepatic iron, change in hepatic iron, hepatic stiffness, and hepatic fibrosis, and using initial, final, and change in ferritin as a continuous variables, to interrogate possible optimal thresholds and targets for therapy.

Glucose Mechanisms Substudy Outcome Measures

Our primary endpoint will be an improvement in the FSIGTT DI, which is the best predictor or diabetes of the many indices arising from MinMod analysis of FSIGTT data (41). Secondary analyses will be the indices that directly quantify the two hallmarks and principal pathogenic factors in T2DM, namely insulin secretory capacity (AIRg) and insulin sensitivity (Si). As in Aim 2, and as in the pilot study on which this Aim is modeled, we will pool the diabetes and prediabetes groups for the primary analysis. This study is powered for that group analysis based on our preliminary data, although we will subanalyze by subgroups and with ferritin, AIRg, and Si as continuous variables to determine if there is evidence for an optimal ferritin target.

Analytical Plan

Statistical Plan and Data Analysis.

The primary analysis will be an analysis of covariance to compare the mean change in the primary endpoint for each aim (HgbA1C at 6 months after phlebotomy for Aim 1, serum ALT at 12 months after phlebotomy for Aim 2, and DI at 6 months after phlebotomy for Aim 3) between the iron depletion and control groups in the full cohort of patients after controlling for the baseline levels of those measurements. The baseline HgbA1C is included as a covariate to increase study power by accounting for regression to the mean between the two time points.

Secondary Analyses. The effects of iron depletion on secondary continuous outcomes will be evaluated using analysis of covariance with adjustment for the baseline level of the factor being analyzed as well as the baseline HgbA1C level. For example, we will determine the degree of response (e.g. change in average glucose by CGM) as a function of starting ferritin, ending ferritin, and change in ferritin for determination of dose-response relationships. Treatment effects on dichotomous outcomes (e.g., conversion of pre-diabetes to diabetes) will be evaluating by exact logistic regression, controlling again for the baseline level of the analyzed factor. Ordinal logistic regression will be applied to compare categorical variables with more than 2 ordered categories. In expanded models, interaction terms between the treatment assignment and baseline HgbA1C level will be added to evaluate if the treatment effect differs across the HgbA1C range, and, in particular, between the diabetic and pre-diabetic patients in the study.

***S**tatistical Power*. In our pilot study of the effects of phlebotomy, we did not measure HgbA1C, but did perform glucose tolerance testing where the difference in area under the curve from pre- to post-treatment was 1130±1970 mg-min/dl. Assuming this average change would be maintained through the day, and converting this change to the relative change in HgbA1C, the change would be expected to be a decrease of ~0.6. With the variance observed, this means a sample size of 32 patients in each group will provide 80% power to detect a difference between the beginning and final HgbA1C in either treatment group at $p<0.05$ when pre- and post-phlebotomy results are compared within subjects. This agrees with the published results from another group using HgbA1C, wherein they achieved a significant change ($p<0.001$) with an N of 31, but a shorter observation period and much less reduction in ferritin (28). A larger sample size (60/group, diabetes and prediabetes, control and phlebotomy) will be recruited to facilitate possible subgroup analysis (e.g. stratification by age, treatment, race, sex) and to allow for dropout. (Previous studies requiring FSIGTT at WF, had dropout rates were in the 12-15% range; in our pilot study, there were none.) Thus, we will be theoretically powered to analyze each target ferritin group separately, although the initial analysis will pool the two treatment groups.

The N for the change in transaminase levels by the same criteria (Aim 2) is 13, and in DI (Aim 3) is 16. Because the natural history is for both glycemia and NAFLD/NASH to deteriorate over time, the comparison of the control group with the treated group should be even more robust.

Human Subjects Protection

***. Study management and monitoring*.** The Data Coordinating Center (DCC) will be housed at the Wake Forest University Department of Public Health Sciences. This group has served as the coordinating center for several large and sentinel NIH- and CDC-funded clinical trials in the areas of metabolism, metabolic syndrome, and diabetes, including SPRINT, ACCORD, SEARCH, LIFE, and LookAHEAD.

A. The occurrence of any significant clinical events (e.g. cardiovascular, renal or cerebrovascular) will be monitored throughout the trial. These events will be tracked by patient interview during study visits. After learning of an event, details will be recorded and transmitted to the DCC, IRB and DSMB.

B. Quality control primarily concerns three areas: (i) personnel training, (ii) data collection and (iii) sample collection. The DCC will implement the methods for quality control, including double data entry and audit of a sample of forms. The DCC will enforce standards to assure that personnel are adequately trained.

C. Our DCC will leverage the expertise and infrastructure of the Wake Forest Department of Public Health

Sciences to provide a web-based portal for the study. The portal will support overall data management.

D. The DCC will prepare monthly reports based on the last month's and cumulative data for screening, recruitment and randomization numbers, and follow-up measurements. The numbers will be categorized by demographics. These data will be reviewed in monthly meetings with the DCC, investigators, and coordinators.

Data Monitoring Committee

A Data and Safety Monitoring Board (DSMB) will be appointed consisting of experts in diabetes, biostatistics, and hematology. The DSMB will evaluate scientific issues of the trial and monitor data, especially those relevant to outcomes and safety. The DSMB will have access to all available trial data and may request specific information from the DCC. The DSMB will review all reports of serious adverse events. In addition, interim results of the trial will be reviewed at 6 and 9 months in order to assure safety and minimize the impact of possible adverse effects. Files will be secured per HIPAA and local regulations. Files will be secured and only accessible to study staff, regulatory agencies and the IRB. Participants will be identified only by codes. Adverse Events (AEs) will be evaluated at every study visit by specific questioning, and as appropriate, by physical examination. The nature, time of onset, course, corrective action (including medication dose changes or discontinuation) and outcome of the AE will be documented in the AE form. Each AE will be classified as: (i) expected or unexpected; (ii) serious (SAE) or not: (iii) related or unrelated to the trial. We will file reports in a most timely manner to the local IRBs and to the DCC. All AEs will be followed until resolution.

Subject Recruitment Methods

Recruitment. A systematic approach to recruitment will be followed. The plan is to recruit a total of 240 subjects. Recruitment will proceed over the first two years to allow completion of the study in four. The Data Coordinating Center will assist in recruitment by providing recruitment schedule goals. The recruitment process will be monitored at the monthly meetings, carefully and continuously so any shortfalls, including shortfalls in proportion of female or minority patients are detected and promptly corrected. Both WF and UNC also have Research Participant Recruitment Units as part of their NIH-funded Clinical and Translational Science Awards sites. These offer resources such as recruitment best practices consultation, assistance with developing advertising and marketing, and websites that describe ongoing research studies for potential research subjects.

Recruitment will be facilitated by the federated electronic warehouse of patient data. Eligibility criteria for the study were entered and the numbers of eligible patients determined, which supports feasibility. All were patients seen within six months in Forsyth County within the Wake Forest Baptist Medical system, and were limited to those who have had HgbA1C values recorded.

- Females 50-75 (to enrich postmenopausal), HgbA1C 5.6-6.4%, no DM code in chart (i.e. prediabetic) **832**
- Females 50-75 yo (to enrich for postmenopausal), HgbA1C 6.5-8.0%, no insulin use recorded (i.e. DM reasonably well-controlled on oral meds) **837**
- Males aged 30-75, HgbA1C 5.6-6.4%, no DM code in chart (i.e. prediabetic) **720**
- Males aged 30-75, HgbA1C 6.5-8.0%, no insulin use recorded (i.e. DM reasonably well-controlled on oral meds) **961**

Of these potential subjects, by definition 1/2 will have ferritins in the upper half of normal, and furthermore people with diabetes have higher ferritins than the general population, hence there are >3350 potential subjects whom we could screen of whom over 1675 will fit eligibility criteria. With IRB approval, letters will be sent to the potential WF subjects through their primary care physicians, with stamped and preaddressed letters to the potential subjects. At UNC, recruitment will be performed through mass email to a research registry and a combination of MyChart messaging through the patient portal, and letters and telephone calls from the study team.

We will also recruit from our own clinic sites. Wake Forest University School of Medicine has an outstanding history of performing clinical trials. This expertise has recently been recognized with the award to Wake Forest of an NIH Clinical and Translational Science Award (CTSA) that helps fund infrastructure for clinical investigations; Dr. McClain is Director of the Clinical and Translational Science Institute funded by the CTSA. Dr. McClain, as Director of the Center on Diabetes, will have excellent access to recruit subjects for this study. The University Diabetes Center sees ~300 patients per week. The substudy recruitment will only be at Wake Forest Baptist Medical Center.

The Co-Investigator at UNC, Dr. John Buse (Director, Division of Endocrinology, UNC Chapel Hill), is one of the leading diabetes clinical trialists in the world, for example Director of the recent LEADER trial of liraglutide. Numbers of available participants is higher at UNC than WF, where there is a large diabetes research registry, consisting of over 70,000 people with diabetes and 20,000 people with prediabetes. At both sites, we will focus initial enrollment efforts on patients who have prior ferritin values available to minimize screen failures. UNC and Wake Forest have full IRB reciprocity.

Advertising: IRB-approved trial recruitment brochures will be developed and translated into Spanish to allow non-English speaking patients to enroll in each trial. We have Spanish-speaking individuals in the Research Subject's Advocates offices who will assist in this and in consenting Spanish speaking subjects.

***Retention and adherence*:** Our retention strategy is directly tied to our recruitment methodology, as demonstrated by other randomized clinical trials at Wake Forest and UNC. It is easier to maintain regular contact and adherence than to bring back a subject who has missed data collection activities. We will use established adherence enhancement strategies and develop monitoring tools to track the progress for each subject, to schedule visits and to measure compliance. It is important for the research personnel to keep in mind that volunteers give their time and information to the study in exchange for relatively little obvious known benefits. Excellent customer service is essential to repay their commitment.

Informed Consent

Signed informed consent will be obtained from each subject, by the investigators or study coordinators. Consents will be obtained prior to screening in the Clinical Research Units of WFUHS or UNC-CH.

Confidentiality and Privacy

Confidentiality will be protected by collecting only information needed to assess study outcomes, minimizing to the fullest extent possible the collection of any information that could directly identify subjects, and maintaining all study information in a secure manner. To help ensure subject privacy and confidentiality, only a unique study identifier will appear on the data collection form. Any collected patient identifying information corresponding to the unique study identifier will be maintained on a linkage file, store separately from the data. The linkage file will be kept secure, with access limited to designated study personnel. Following data collection subject identifying information will be destroyed *(state the anticipated time the data will be destroyed, e.g. three years after closure of the study, and the method of destruction),* consistent with data validation and study design, producing an anonymous analytical data set. Data access will be limited to study staff. Data and records will be kept locked and secured, with any computer data password protected. No reference to any individual participant will appear in reports, presentations, or publications that may arise from the study.

Data and Safety Monitoring

The principal investigator will be responsible for the overall monitoring of the data and safety of study participants. The principal investigator will be assisted by other members of the study staff.

Reporting of Unanticipated Problems, Adverse Events or Deviations

Any unanticipated problems, serious and unexpected adverse events, deviations or protocol changes will be promptly reported by the principal investigator or designated member of the research team to the IRB and sponsor or appropriate government agency if appropriate.

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Appendix

1. Consent form