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Prediabetes, Elevated Iron, and All-Cause Mortality: A Cohort Study

Running Title: Prediabetes, Elevated Iron, and All-Cause Mortality

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ABSTRACT

Objectives: Data have indicated low to nonexistent increased mortality risk for individuals with prediabetes, but it is unclear if the risk is increased when the patient has elevated iron markers. Our purpose was to examine the mortality risk among adults with prediabetes in the context of co-existing elevated transferrin saturation (TS).

Setting: Data collected by the third National Health and Nutrition Examination Survey 1988-1994 (NHANES III) in the United States and by the National Center for Health Statistics for the National Death Index from 1988-2006.

Participants: Individuals age 0 and older who participated in the NHANES and provided a blood sample.

Primary Outcome Variable: Mortality was measured as all-cause mortality.

Results: Adjusted analyses show that prediabetes has a small increased mortality risk (HR =1.04; 95% CI, 1.00-1.09). Among persons with prediabetes who had elevated TS, they had an increased mortality risk (HR=1.56; 95% CI, 1.12-2.17) compared with those with normal TS levels and normal glucose. Persons who had prediabetes and elevated iron on two markers (TS/ferritin) had an even higher increased hazard ratio for death (2.28; 95% CI, 1.25-4.14) compared with those who had normal iron markers and normal glucose.

Conclusion: The mortality risk of prediabetes is low. However, among individuals who have co-existing elevated iron markers, the risk rises substantially.

STRENGTHS AND LIMITATIONS OF STUDY

- This study utilizes a nationally representative population-based cohort that allows generalization of results to the population of the United States.
- Mortality was left-censored to control for the potential misclassification of people who were very ill at baseline.
- Biomarkers for elevated iron and prediabetes were available only at baseline.
- We were able to only observe individuals for 12 years, which may be insufficient to adequately see an effect on mortality for prediabetes.

INTRODUCTION

Prediabetes is defined by blood glucose concentrations that are higher than normal, but lower than established thresholds for diabetes.[1] Prediabetes is a high risk state for the development of not only diabetes, but also associated complications. Recent data have shown that in developed countries such as the United States and the United Kingdom more than a third of adults have prediabetes but the vast majority of these do not realize it.[2-4]

Several studies have shown that prediabetes is a mortality risk.[5,6] On the other hand, other studies have found that prediabetes is not a mortality risk.[7–10] Still other studies showed that the relationship was not very robust and was dependent on the measure of prediabetes.[11]

Three different meta-analyses of observational studies have concluded that elevated iron indices like serum ferritin and transferrin saturation (TS) are strongly associated with increased risk for developing diabetes.[12–14] In addition to increased risk of diabetes, elevated TS or elevated ferritin is associated with increased mortality in the general population. [15-20] Further, recent evidence suggests that among patients with diabetes, mortality risk increases in the presence of elevated ferritin or elevated TS.[21,22] We therefore hypothesize that the mortality risk of individuals with prediabetes will be increased in the presence of elevated iron markers.

Considering that only about 10% of the US population with prediabetes realize that they have prediabetes, a better understanding of the potential mortality risk is warranted. Consequently, the purpose of this study was to evaluate the association

between prediabetes, elevated serum TS, elevated TS and elevated ferritin combined, and mortality in a large, nationally representative cohort.

METHODS

We conducted longitudinal analyses of the third National Health and Nutrition Examination Survey, 1988-1994 (NHANES III) linked to mortality data collected through the National Death Index. Mortality data were available through December 31, 2006. The NHANES III survey provides population estimates of the United States and was conducted from October, 1988 through October, 1994. The NHANES III used complex, multi-stage, stratified, clustered samples of civilian, noninstitutionalized population. A total of 30,818 persons were examined in their homes or in mobile examination centers (MEC) which visited 89 communities across the United States. The health examination included collection of blood and urine specimens for the conduct of various laboratory analyses.

The NHANES III data merged with the National Death Index is a prospective cohort study that passively followed up on the participants in the NHANES III. The linked mortality file uses a probabilistic matching method.[23] We limited our study to individuals 40 years old and older at baseline, the time of their NHANES III interview. The National Death Index involves searching national databases containing information about mortality and causes of death. Mortality status was ascertained by computerized matching to national databases and evaluation of the resulting matches. Persons not found to be deceased were assumed alive for analytic purposes. All living survey participants had been observed for at least 146 months, and our survival analysis was carried out to December 31, 2006.

Previously Diagnosed Diabetes

The NHANES III assessed participants for diagnosed diabetes using the questions, "Have you ever been told by a doctor that you have diabetes or sugar diabetes?", "Were you pregnant when you were told that you had diabetes?" and "Other than during pregnancy, has a doctor ever told you that you have diabetes or sugar diabetes?" We defined participants as having diagnosed diabetes if they answered "yes" to ever having been told they had diabetes, excluding pregnancy. Individuals with previously diagnosed diabetes were removed from the analysis. We also removed individuals with an HbA1c of 6.5% or greater, to account for undiagnosed diabetes.

Prediabetes

We defined prediabetes among individuals without previously diagnosed diabetes using HbA1c ranges as specified by the American Diabetes Association, 5.7%–6.4% (39-46 mmol/mol).[1] This range has been shown in a meta-analysis to be predictive of progression to diabetes.[18] We excluded individuals with previously diagnosed diabetes because the current glycemic status of those patients may simply represent diabetes control. Prediabetes status was missing for 1,123 of the NHANES respondents over the age of 40.

Transferrin Saturation

Serum iron and total iron-binding capacity (TIBC) were measured in serum, and calculated by dividing serum iron by TIBC and multiplying by 100. For the analyses, TS was categorized as: <50% and ≥50%. Despite the lack of universal agreement on the upper and lower limits of normal TS, this cut point has been used in several studies evaluating diabetes, TS and mortality.[21,24] Data was missing for transferrin saturation level for 1,288 of the NHANES respondents over the age of 40.

Serum Ferritin

Serum ferritin was used as a measure of body iron stores and was measured using the QuantImune Ferritin IRMA kit. Serum ferritin was categorized for the analyses as <898.80 SI units and ≥898.80 SI units (400 ng/mL). Data was missing for serum ferritin level for 1,288 of the NHANES respondents over the age of 40.

Elevated Iron

Individuals with both elevated serum ferritin and TS levels were considered to have elevated iron. Data was missing for elevated iron for 1,288 of the NHANES respondents over the age of 40.

Mortality

Mortality was measured as all-cause mortality. Mortality status was ascertained solely by computerized matching to national databases and evaluation of the resulting matches. Mortality status was missing for 15 of the NHANES respondents over the age of 40.

Covariates

Covariates used in our analyses included: age at baseline in the NHANES III, gender, race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, and other race), health insurance status, obesity (Body Mass Index computed in the exam of ≥30), previous diagnosis of a heart attack, previous diagnosis of a stroke, previous diagnosis of hypertension, previous diagnosis of hypercholesterolemia, previous diagnosis of cancer, family history of diabetes, family history of myocardial infarction before age 50, and current smoking status. Respondents were considered non-smokers if they reported

smoking at least100 cigarettes in their life or if they had smoked more than 100 cigarettes and were not currently smoking.

Analysis

In an effort to control for potential misclassification of persons who were very ill at baseline thereby affecting mortality risk of prediabetes, we left-censored the analysis to exclude any mortality events that occurred in the first three years following the individuals examination for the first three years of the cohort. For the analyses of mortality, we used sampling weights (specifically, the total MEC and Home examined weight) to calculate prevalence estimates for the civilian noninstitutionalized US population. Because of the complex sampling design of the survey, we performed statistical analyses using the statistical software package SUDAAN (Research Triangle Institute, Raleigh, NC), as recommended by the National Center for Health Statistics. Using SAS (Cary, NC), we computed Kaplan-Meier plots to show graphically the unadjusted relationship between all-cause mortality and prediabetes.

To accomplish our goals of examining a possible synergistic effect of having elevated iron with prediabetes we classified the population into 4 groups based upon prediabetes or normoglycemia and normal and elevated TS. We also classified the population into 4 groups based upon prediabetes or normoglycemia and normal and elevated iron based on levels of TS and ferritin.

We performed Cox proportional hazards analyses to measure the associations between all-cause mortality and prediabetes alone and for prediabetes controlling for all of the studied covariates using listwise deletion to account for missing data. In these

models, survival time was a continuous variable measured in 1-month increments from the date of the exam.

We also performed unadjusted Cox proportional hazards analysis with all-cause mortality for prediabetes in the 4 part variables with TS, ferritin and iron overload. We then conducted analyses adjusting for the aforementioned covariates.

We evaluated the proportionality of the hazards through examination of the Schoenfeld residuals.

RESULTS

A total of 8,041 (unweighted) individuals were over 40 years old and had HbA1c levels under 6.4%. Baseline characteristics for the sample are shown in Table 1. Table 1 indicates that 23.0% of the weighted sample had prediabetes, less than 7.0% of the sample exhibited elevated serum ferritin, and less than 4.0% had elevated TS. Elevated iron was indicated in 1.4% of the sample.

Table 2 shows the results of unadjusted and adjusted Cox proportional hazards models for prediabetes. The Kaplan Meier curve of the survival and prediabetes over the length of the time under observation is shown in Fig 1.

Table 3 presents results of the analyses combining prediabetes with iron markers. In models that examined the impact of a prediabetes state combined with markers of low iron, the hazard ratios were similar to that of prediabetes alone. However, when combined with prediabetes, there was an increased mortality risk among individuals with TS $\geq$50%, and even higher when individuals had TS >50% and serum ferritin >898.80 SI units (400 ng/mL). Figure 2 represents the relationship of survival of the four groups over the 12 years under observation. Individuals with prediabetes in the presence of elevated

iron have lower survival probabilities than other groups. An examination of the Schoenfeld residuals suggested proportionality of hazards and appropriateness of the statistical model for these analyses.

DISCUSSION

The results of this study in a nationally representative cohort that followed individuals for 12 years confirm that the mortality risk of prediabetes is probably low. This is not unexpected based on the mixed results from previous studies, several of which found either no future mortality risk or risk that was not robust across measures. However, we found that the presence of markers of elevated iron is associated with increased mortality risk of individuals with prediabetes. Among individuals with normal iron levels, those with prediabetes had low mortality risk levels similar to the adjusted risk of prediabetes alone. On the other hand, in adjusted survival analyses, individuals with prediabetes who also had elevated iron levels had more than twice the mortality risk. These findings extend previous work on iron markers and diabetes to the previously uninvestigated area of prediabetes.

These results suggest that additional stratification of individuals with prediabetes on the basis of iron markers would be useful to identify those with higher risk and who might benefit from iron lowering therapies. Previous data has indicated that elevated iron markers are associated with the development of diabetes and that among individuals with diabetes the co-occurrence of elevated TS increases those patients' mortality risk. Early identification of individuals with both conditions (prediabetes, elevated iron) may help in both slowing the development of diabetes as well as decreasing mortality risk. It is important for early identification of these individuals because much like individuals with

prediabetes, the vast majority of individuals with elevated iron do not know it.[25] These individuals need to be identified to mitigate the increased risk posed by elevated iron in combination with prediabetes. Such individuals would be targets for intensive interventions to reduce risk, including typical lifestyle interventions shown to help avoid the onset of diabetes in people at high risk.[26] Although more research is needed into the ability of interventions on iron in prediabetes to affect development of diabetes and mortality risk, some data suggest that reduction of TS improves HbA1c and glucose control.[27]

These associations of TS and ferritin with mortality in the context of prediabetes are not surprising especially if elevations of these parameters are interpreted in light of current understanding of iron toxicity.[28] Iron, whether absorbed as iron salts or in dietary heme is processed by enterocytes and released into the plasma where it is transported in a non-reactive state bound to transferrin. Iron that is bound to transferrin is in the Fe^{+3} state and is not reactive and, therefore, not toxic. However, when TS is above 40 to 50%, free iron or so-called non-transferrin-bound iron (NTBI) is released into the plasma as the buffering ability of transferrin is exceeded.[29] Labile plasma iron (LPI) is a highly reactive subspecies of NTBI that interacts with hydrogen peroxide through Fenton chemistry to form the extremely powerful oxidants, hydroxyl radical and singlet oxygen. These are the free radicals that ultimately directly damage protein and DNA. Perhaps more importantly, NTBI/LPI species are able to enter cells via ion channels. These channels, unlike the transferrin receptor, are not regulated so this reactive iron freely enters the cytoplasm of the pancreas, pituitary, and heart.

The current results suggest that exposure to excessive free iron is dangerous in the context of prediabetes. Furthermore, elevated ferritin and TS predict poor diabetes control and phlebotomy to reduce iron even over short periods of time improve HbA1c in parallel with changes in TS, even though ferritin is not changed,[27] putting further emphasis on the fact that NTBI/LPI reflected by TS is the proximal cause of the toxicity . Reduction in TS by phlebotomy has been shown to improve measures of diabetic control.[27] Furthermore, correction of severe iron overload can significantly improve glucose tolerance.[30] Thus, the finding that a baseline measure of high TS as point measure of toxic free iron plus elevation of ferritin, evidence of elevated cytosolic iron over a longer period of time, predicts increased risk of mortality among individuals with prediabetes supports the premise that toxic free iron is a health risk.

This study has several limitations. First, although we have a nationally representative, population-based cohort followed through the National Death Index, the biomarkers are measured only at baseline. There is the possibility that either the hyperglycemia or elevated iron measures were identified and interventions were implemented to lower these biomarkers. If that were the case and a substantial number of individuals did drop their levels due to interventions thereby decreasing the potential mortality risk, the observed adjusted risk of 2.28 for individuals elevated at baseline is even more concerning. Second, we were only able to follow these individuals for a little more than 12 years. It is possible that this time frame may have been too short to adequately see an effect for a biomarker like prediabetes. However, we did censor the first three years of mortality so that any deaths in that time frame would not be attributed

to prediabetes. The model still found a substantial mortality risk for the prediabetes plus iron markers in this length of time.

In conclusion, this study representative of the population of the United States helps to clarify the current evidence on the mortality risk of prediabetes and provides further support for the role of elevated iron markers in health risk. Future screening and intervention programs for prediabetes may benefit from additional strategies to recognize and treat iron elevations.

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CONTRIBUTION STATEMENT

AGM: conception, design, analysis, and drafting of manuscript

RJT: design, analysis, and drafting of manuscript

TDC: conception, design, and drafting of manuscript

RB: design and analysis

COMPETING INTERESTS

The authors report no competing interests.

DATA SHARING STATEMENT

Data from this study are publicly available on the Internet through the National Center for Health Statistics.

REFERENCES

1. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2012;35:S64–S71.

2. Bullard KM, Saydah SH, Imperatore G, et al. Secular changes in U.S. Prediabetes prevalence defined by hemoglobin A1c and fasting plasma glucose: National Health and Nutrition Examination Surveys, 1999-2010. *Diabetes Care* 2013;36:2286–93.

3. Mainous AG 3rd, Tanner RJ, Baker R, et al. Prevalence of prediabetes in England from 2003 to 2011: population-based, cross-sectional study. *BMJ Open* 2014 Jun 9;4(6):e005002. doi: 10.1136/bmjopen-2014-005002. Accessed 29 June, 2014.

4. Centers for Disease Control and Prevention (CDC). Awareness of prediabetes--United States, 2005-2010. MMWR Morb Mortal Wkly Rep. 2013 Mar 22;62:209-12.

5. de Vegt F, Dekker JM, Ruhé HG, et al. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia* 1999;42:926–31.

6. Fuller JH, Shipley MJ, Rose G, et al. Coronary-heart-disease risk and impaired glucose tolerance. The Whitehall study. *Lancet* 1980;1:1373–76.

7. Valdés S, Botas P, Delgado E, Cadórniga FD. Mortality risk in Spanish adults with diagnosed diabetes, undiagnosed diabetes, or pre-diabetes. The asturias study 1998-2004. *Rev Esp Cardiol (Engl Ed)* 2009;62:528–34.

8. Deedwania P, Patel K, Fonarow GC, et al. Prediabetes is not an independent risk factor for incident heart failure, other cardiovascular events or mortality in older adults: findings from a population-based cohort study. *Int J Cardiol* 2013;168:3616–22.

9. Kowall B, Rathmann W, Heier M, et al. Categories of glucose tolerance and continuous glycemic measures and mortality. *Eur J Epidemiol* 2011;26:637–45.

10. Zhou XH, Qiao Q, Zethelius B, et al. Diabetes, prediabetes and cancer mortality. *Diabetologia* 2010;53:1867–76.

11. Barr EL, Zimmet PZ, Welborn TA, et al. Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance: the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). *Circulation* 2007;116:151–57.

12. Orban E, Schwab S, Thorand B, Huth C. Association of iron indices and type 2 diabetes: a meta-analysis of observational studies. *Diabetes Metab Res Rev* 2014;30:372–94.

13. Bao W, Rong Y, Rong S, Liu L. Dietary iron intake, body iron stores, and the risk of type 2 diabetes: a systematic review and meta-analysis. *BMC Med* 2012;10:119. doi: 10.1186/1741-7015-10-119. Accessed 8 July, 2014.

14. Ellervik C, Mandrup-Poulsen T, Tybjærg-Hansen A, Nordestgaard BG. Total and cause-specific mortality by elevated transferrin saturation and hemochromatosis genotype in individuals with diabetes: two general population studies. *Diabetes Care* 2014;37:444–52.

15. Ellervik C, Tybjaerg-Hansen A, Nordestgaard BG. Total mortality by transferrin saturation levels: Two general population studies and a meta analysis. *Clin Chem* 2011;57:459–466.

16. Kim KS, Son HG, Hong NS, Lee DH. Associations of serum ferritin and transferrin % saturation with all-cause, cancer, and cardiovascular disease mortality: Third National

Health and Nutrition Examination Survey follow-up study. *J Prev Med Public Health* 2012;45:196-203.

17. Mainous AG 3rd, Gill JM, Carek PJ. Elevated transferrin saturation and mortality. *Ann Fam Med* 2004;2:133–138.

18. Mainous AG 3rd, Wells B, Carek PJ, et al. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. *Ann Fam Med* 2004;2:139-44.

19. Stack AG, Mutwali AI, Nguyen HT, et al. Transferrin saturation ratio and risk of total and cardiovascular mortality in the general population. *QJM.* 2014;107:623-33.

20. Wells BJ, Mainous AG 3rd, King DE, et al. The combined effect of transferrin saturation and low density lipoprotein on mortality. *Fam Med* 2004;36:324-9.

21. Ponikowska B, Suchocki T, Paleczny B, et al. Iron status and survival in diabetic patients with coronary artery disease. *Diabetes Care* 2013;36:4147–56.

22. Morris DH, Khunti K, Achana F, et al. Progression rates from HbA(1c) 6.0-6.4% and other prediabetes definitions to type 2 diabetes: a meta-analysis. *Diabetologia* 2013;56:1489–93.

23. National Center for Health Statistics. Office of Analysis and Epidemiology, NCHS 2011 Linked Mortality Files Matching Methodology, September, 2013. Hyattsville, Maryland. http://www.cdc.gov/nchs/data/datalinkage/2011_linked_mortality_file_matching_methodology.pdf. Accessed 20 August, 2014.

24. Ellervik C, Andersen HU, Tybjærg-Hansen A, et al. Total mortality by elevated transferrin saturation in patients with diabetes. *Diabetes Care* 2013;36:2646–54.

25. Mainous AG 3rd, Wright RU, Hulihan MM, et al. Elevated transferrin saturation, health-related quality of life and telomere length. *Biometals* 2014;27:135–41

26. Portero McLellan KC, Wyne K, Villagomez ET, Hsueh WA. Therapeutic interventions to reduce the risk of progression from prediabetes to type 2 diabetes mellitus. *Ther Clin Risk Manag* 2014;10:173–88.

27. Fernández-Real JM, Peñarroja G, Castro A, García-Bragado F, Hernández-Aguado I, Ricart W. Blood letting in high-ferritin type 2 diabetes: effects on insulin sensitivity and beta-cell function. *Diabetes* 2002;51:1000–4.

28. Hansen JB, Moen IW, Mandrup-Poulsen T. Iron: the hard player in diabetes pathophysiology. *Acta Physiol* 2014;210:717–32.

29. Coates TD. Physiology and pathophysiology of iron in hemoglobin-associated diseases. *Free Radic Biol Med* 2014;72C:23–40.

30. Farmaki K, Tzoumari I, Pappa C, et al. Normalisation of total body iron load with very intensive combined chelation reverses cardiac and endocrine complications of thalassaemia major. *Br J Haematol* 2010;148:466–75.

Table 1: Baseline Characteristics of the Cohort

	All Respondents (%)
Unweighted Sample Size	8,041
Weighted Sample Size	81,152,997
Age, years	
40-54	49.7
55-69	31.2
70+	19.2
Sex, male	46.3
Race/Ethnicity	
Non-Hispanic White	82.1
Non-Hispanic Black	8.3
Hispanic	6.6
Asian/Other	2.9
Has health insurance	93.5
Obese	23.5
Current Smoker, %	23.5
Has diagnosed hypertension, %	31.3
Ever had heart attack, %	2.8
Ever had stroke, %	5.3
Ever had cancer, %	12.3
Relative with diabetes, %	41.0
Relative with heart attack before age 50,%	15.4
High Transferrin Saturation, %	3.9
High Serum Ferritin, %	6.6
Elevated Iron, %	1.4
Prediabetes, %	23.0
Assumed deceased, %	30.7

Table 2: Unadjusted and Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes

	Hazard Ratio	95% Confidence Interval
Prediabetes		
Unadjusted Model		
Prediabetes	1.14	1.10-1.18
Normoglycemia	1.0	
Adjusted Model[a]		
Prediabetes	1.04	1.00-1.09
Normoglycemia	1.0	

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

Table 3: Unadjusted and Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes and Iron Markers

Unadjusted Model	**Hazard Ratio**	**95% Confidence Interval**
Normal TS/Normoglycemia	1.0	
Normal TS/Prediabetes	1.14	1.10-1.18
High TS/Normoglycemia	1.01	0.94-1.09
High TS/Prediabetes	1.39	1.09-1.77
Adjusted Model[a]		
Normal TS/Normoglycemia	1.0	
Normal TS/Prediabetes	1.04	1.00-1.08
High TS/Normoglycemia	1.05	0.93-1.18
High TS/Prediabetes	1.56	1.12-2.17
Unadjusted Model		
Normal TS+Ferritin/Normoglycemia	1.0	
Normal TS+Ferritin Iron/Prediabetes	1.14	1.10-1.18
Elevated TS+Ferritin/Normoglycemia	1.1	0.93-1.29
Elevated TS+Ferritin/Prediabetes	1.87	1.18-2.95
Adjusted Model[a]		
Normal TS+Ferritin/Normoglycemia	1.0	
Normal TS+Ferritin Iron/Prediabetes	1.04	1.00-1.08
Elevated TS+Ferritin/Normoglycemia	1.20	0.94-1.53
Elevated TS+Ferritin/Prediabetes	2.28	1.25-4.14

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension,

diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

Figure 1. Kaplan Meier curve of survival among individuals with prediabetes or normal glycemic levels.

——Normoglycemia

--- Prediabetes

+ Censored

Figure 2. Kaplan Meier curve of survival among individuals with prediabetes and elevated transferrin saturation and elevated ferritin

—Normoglycemia and Elevated Iron

--- Normoglycemia and Normal Iron

-·-· Prediabetes and Elevated Iron

····· Prediabetes and Normal Iron

+ Censored

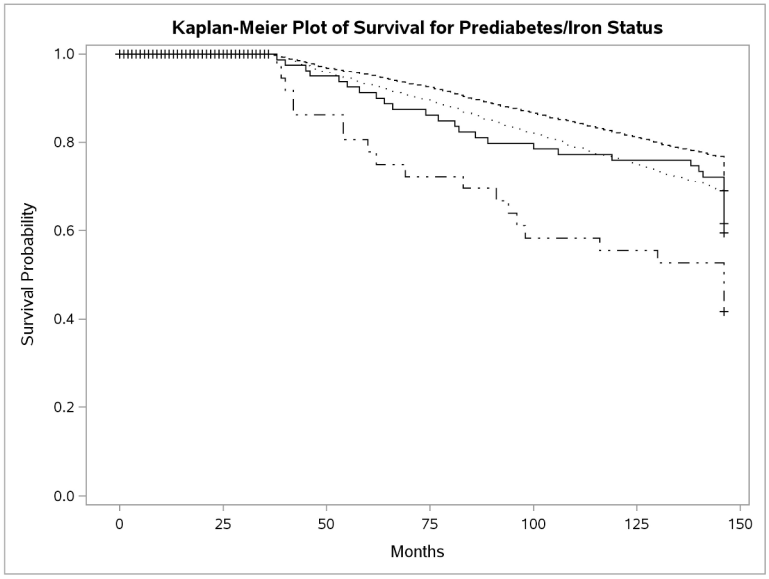

Figure 2. Kaplan Meier curve of survival among individuals with prediabetes and elevated transferrin saturation and elevated ferritin
------- Normoglycemia and Elevated Iron
--- Normoglycemia and Normal Iron
-••- Prediabetes and Elevated Iron
••••• Prediabetes and Normal Iron
+ Censored

267x350mm (300 x 300 DPI)

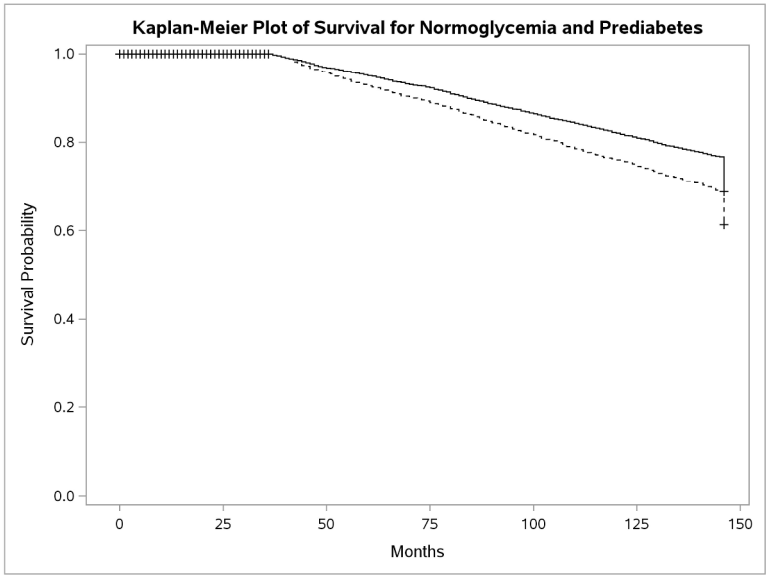

Figure 1¬¬¬. Kaplan Meier curve of survival among individuals with prediabetes or normal glycemic levels.
-------- Normoglycemia
--- Prediabetes
+ Censored

267x350mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page number
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4-5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-8
Bias	9	Describe any efforts to address potential sources of bias	9
Study size	10	Explain how the study size was arrived at	5-8
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8-9
		(*b*) Describe any methods used to examine subgroups and interactions	8
		(*c*) Explain how missing data were addressed	8
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	NA
		(*e*) Describe any sensitivity analyses	NA

Continued on next page

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5, 9
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9, 19
		(b) Indicate number of participants with missing data for each variable of interest	6-7
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	5
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	19
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-10 20-22
		(*b*) Report category boundaries when continuous variables were categorized	NA
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	10
Discussion			
Key results	18	Summarise key results with reference to study objectives	10-11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12-13
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12
Generalisability	21	Discuss the generalisability (external validity) of the study results	13
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Prediabetes, Elevated Iron, and All-Cause Mortality: A Cohort Study

Running Title: Prediabetes, Elevated Iron, and All-Cause Mortality

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ABSTRACT

Objectives: Data have indicated low to nonexistent increased mortality risk for individuals with prediabetes, but it is unclear if the risk is increased when the patient has elevated iron markers. Our purpose was to examine the mortality risk among adults with prediabetes in the context of co-existing elevated transferrin saturation (TS) or serum ferritin.

Setting: Data collected by the third National Health and Nutrition Examination Survey 1988-1994 (NHANES III) in the United States and by the National Center for Health Statistics for the National Death Index from 1988-2006.

Participants: Individuals age 40 and older who participated in the NHANES and provided a blood sample.

Primary Outcome Variable: Mortality was measured as all-cause mortality.

Results: Adjusted analyses show that prediabetes has a small increased mortality risk (HR =1.04; 95% CI, 1.00-1.08). Persons who had prediabetes and elevated serum ferritin had an increased hazard ratio for death (HR=1.14; 95% CI, 1.04-1.24) compared with those who had normal ferritin and normal glucose. Among persons with prediabetes who had elevated TS, they had an increased mortality risk (HR=1.86; 95% CI, 1.05-3.29) compared with those with normal TS levels and normal glucose. **Conclusion:** The mortality risk of prediabetes is low. However, among individuals who have co-existing elevated iron markers, particularly transferrin saturation, the risk rises substantially.

STRENGTHS AND LIMITATIONS OF STUDY

- This study utilizes a nationally representative population-based cohort that allows generalization of results to the population of the United States.
- Mortality was left-censored to control for the potential misclassification of people who were very ill at baseline.
- Biomarkers for elevated iron and prediabetes were available only at baseline.
- We were able to only observe individuals for 12 years, which may be insufficient to adequately see an effect on mortality for prediabetes.

INTRODUCTION

Prediabetes is defined by blood glucose concentrations that are higher than normal, but lower than established thresholds for diabetes.[1] Prediabetes is a high risk state for the development of not only diabetes, but also associated complications. Recent data have shown that in developed countries such as the United States and the United Kingdom more than a third of adults have prediabetes but the vast majority of these do not realize it.[2-4]

Several studies have shown that prediabetes is a mortality risk.[5,6] On the other hand, other studies have found that prediabetes is not a mortality risk.[7–10] Still other studies showed that the relationship was not very robust and was dependent on the measure of prediabetes.[11]

Three different meta-analyses of observational studies have concluded that elevated iron indices like serum ferritin and transferrin saturation (TS) are strongly associated with increased risk for developing diabetes.[12–14] Some evidence exists to indicate that pancreatic beta cells are killed in the presence of iron.[15] In addition to increased risk of diabetes, elevated TS or elevated ferritin is associated with increased mortality in the general population. [16-22] Further, recent evidence suggests that among patients with diabetes, mortality risk increases in the presence of elevated ferritin or elevated TS.[23,24] We therefore hypothesize that the mortality risk of individuals with prediabetes will be increased in the presence of elevated iron markers.

Considering that only about 10% of the US population with prediabetes realize that they have prediabetes, a better understanding of the potential mortality risk is warranted. Consequently, the purpose of this study was to evaluate the association

between prediabetes, elevated serum ferritin, elevated TS and mortality in a large, nationally representative cohort.

METHODS

We conducted longitudinal analyses of the third National Health and Nutrition Examination Survey, 1988-1994 (NHANES III) linked to mortality data collected through the National Death Index. Mortality data were available through December 31, 2006. The NHANES III survey provides population estimates of the United States and was conducted from October, 1988 through October, 1994. The NHANES III used complex, multi-stage, stratified, clustered samples of civilian, noninstitutionalized population, Of the 39,695 individuals eligible to participate, a total of 30,818 persons were examined in their homes or in mobile examination centers (MEC) which visited 89 communities across the United States (a participation rate of 77.6%). The health examination included collection of blood and urine specimens for the conduct of various laboratory analyses. The NHANES provides preexisting de-identified public use data which do not need specific approval from the National Center for Health Statistics.

The NHANES III data merged with the National Death Index is a prospective cohort study that passively followed up on the participants in the NHANES III. The linked mortality file uses a probabilistic matching method.[25] We limited our study to individuals 40 years old and older at baseline, the time of their NHANES III interview. The National Death Index involves searching national databases containing information about mortality and causes of death. Mortality status was ascertained by computerized matching to national databases and evaluation of the resulting matches. Persons not found to be deceased were assumed alive for analytic purposes. All living survey participants

had been observed for 146 months, and our survival analysis was carried out to December 31, 2006.

Previously Diagnosed Diabetes

The NHANES III assessed participants for diagnosed diabetes using the questions, "Have you ever been told by a doctor that you have diabetes or sugar diabetes?", "Were you pregnant when you were told that you had diabetes?" and "Other than during pregnancy, has a doctor ever told you that you have diabetes or sugar diabetes?" We defined participants as having diagnosed diabetes if they answered "yes" to ever having been told they had diabetes, excluding pregnancy. Individuals with previously diagnosed diabetes were removed from the analysis. We also removed individuals with an HbA1c of 6.5% or greater, to account for undiagnosed diabetes.

Normoglycemia & Prediabetes

We defined normoglycemia as an Hba1c level between 4.0%--5.6% (20-38 mmol/mol). To control for any potential effect of low HbA1c, we also removed individuals with an HbA1c below 4.0% (20 mmol/mol), a level associated with increased all-cause mortality in adults without diabetes.[26]

We defined prediabetes among individuals without previously diagnosed diabetes using HbA1c ranges as specified by the American Diabetes Association, 5.7%–6.4% (39-46 mmol/mol).[1] This range has been shown in a meta-analysis to be predictive of progression to diabetes.[19] We excluded individuals with previously diagnosed diabetes because the current glycemic status of those patients may simply represent diabetes control. Prediabetes status was missing for 1,637 of the NHANES respondents over the age of 40.

Transferrin Saturation

Serum iron and total iron-binding capacity (TIBC) were measured in serum, and calculated by dividing serum iron by TIBC and multiplying by 100. For the analyses, elevated transferrin saturation was categorized as TS ≥50%. Individuals with TS below 25% were removed from the analysis, as low TS has been linked to increased risk of mortality.[23] Despite the lack of universal agreement on the upper and lower limits of normal TS, these cut points has been used in several studies evaluating diabetes, TS and mortality.[23,27] Data were missing for transferrin saturation level for 536 of the NHANES respondents over the age of 40.

Serum Ferritin

Serum ferritin was used as a measure of body iron stores and was measured using the QuantImune Ferritin IRMA kit. Serum ferritin was categorized for the analyses as elevated if it was ≥674.1 pmol/l (300 ng/mL) for males and ≥449.4 pmol/l (200 ng/mL) for females.[28] Individuals with serum ferritin below 56.175 pmol/l (25 ng/mL) were removed from the analysis, as low ferritin has been linked to an increased risk of mortality.[23] Data were missing for serum ferritin level for 539 of the NHANES respondents over the age of 40.

Mortality

Mortality was measured as all-cause mortality. Mortality status was ascertained solely by computerized matching to national databases and evaluation of the resulting matches.

Covariates

Covariates used in our analyses included: age at baseline in the NHANES III, gender, race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, and other race), health insurance status, obesity (Body Mass Index computed in the exam of ≥30), previous diagnosis of a heart attack, previous diagnosis of a stroke, previous diagnosis of hypertension, previous diagnosis of hypercholesterolemia, previous diagnosis of cancer, family history of diabetes, family history of myocardial infarction before age 50, and current smoking status. Respondents were considered non-smokers if they reported smoking at least100 cigarettes in their life or if they had smoked more than 100 cigarettes and were not currently smoking.

In the analysis of serum ferritin, we also controlled for C-reactive protein. Ferritin is an acute phase reactant as well as an indicator of iron stores and as such may indicate inflammation. Consequently, we controlled for inflammation by adjusting for C-reactive protein. C-reactive protein was considered elevated at levels above 3.0 mg/l.[29]

Analysis

In an effort to control for potential misclassification of persons who were very ill at baseline thereby affecting mortality risk of prediabetes, we left-censored the analysis to exclude any mortality events that occurred in the first three years following the individuals examination for the first three years of the cohort. For the analyses of mortality, we used sampling weights (specifically, the total MEC and Home examined weight) to calculate prevalence estimates for the civilian noninstitutionalized US population. This strategy of basing the analyses on population estimates is a characteristic that makes the NHANES different from many other cohort designs that do not use weighted population estimates. However, this strategy does provide national

generalizability. Because of the complex sampling design of the survey, we performed statistical analyses using the statistical software package SUDAAN (Research Triangle Institute, Raleigh, NC), as recommended by the National Center for Health Statistics (NCHS). Using SAS (Cary, NC), we computed Kaplan-Meier plots to show graphically the unadjusted relationship between all-cause mortality and prediabetes.

All analyses were based on the population estimates and we followed the NCHS recommendations for assessing the reliability of estimates in the context of a limited sample size. If the standard error of an estimate was greater than 30% of an estimate it would be considered unreliable All estimates met the criteria for reliability.

To accomplish our goals of examining a possible synergistic effect of having elevated iron with prediabetes we classified the population into 4 groups based upon prediabetes or normoglycemia and normal and elevated TS. We also classified the population into 4 groups based upon prediabetes or normoglycemia and normal and elevated iron based on levels of TS and ferritin.

We performed Cox proportional hazards analyses to measure the associations between all-cause mortality and prediabetes controlling for all of the studied covariates using listwise deletion to account for missing data. In these models, survival time was a continuous variable measured in 1-month increments from the date of the exam.

We also performed adjusted Cox proportional hazards analysis with all-cause mortality for prediabetes in the 4 part variables with TS adjusting for the aforementioned covariates. For the adjusted Cox proportional hazards analysis with ferritin we adjusted for the aforementioned covariates and also C-reactive protein.

We evaluated the proportionality of the hazards through examination of the Schoenfeld residuals.

RESULTS

A total of 8,003 (unweighted) individuals were over 40 years old and had HbA1c levels between 4.0 and 6.4%. Baseline characteristics for the sample are shown in Table 1. Table 1 indicates that 23.2% of the weighted sample had prediabetes, 15.6% of the sample exhibited elevated serum ferritin, and 3.3% had elevated TS.

Of the respondents that had prediabetes, 38.8% died within 12 years, compared to 23.4% of respondents with normal HbA1c levels. Among individuals with normal TS and normoglycemia, 23.1% died, compared to 23.7% of those with elevated TS, 37.5% of those with normal TS and prediabetes, and 44.7% of those with elevated TS and prediabetes. Among individuals with normal ferritin and normoglycemia, 34.3% died, compared with 38.8% of those with normal ferritin and prediabetes, 29.2% of those with elevated ferritin and normoglycemia, and 38.8% of those with elevated ferritin and prediabetes.

Table 2 shows the results the adjusted Cox proportional hazards model for prediabetes. Table 2 indicates prediabetes alone has a small increased mortality risk. The Kaplan Meier curve of the survival and prediabetes over the length of the time under observation is shown in Fig 1.

Table 3 presents results of the analyses combining prediabetes with iron markers. In models that examined the impact of a prediabetes state combined with markers of low iron, the hazard ratios were similar to that of prediabetes alone. However, when combined with prediabetes, there was an increased mortality risk among individuals with

TS ≥50, as well as with individuals who had increased ferritin. The risk was most increased when individuals had elevated ferritin and elevated transferrin saturation together. Figure 2 represents the relationship of survival of the four groups over the 12 years under observation. Individuals with prediabetes in the presence of elevated iron have lower survival probabilities than other groups. An examination of the Schoenfeld residuals suggested proportionality of hazards and appropriateness of the statistical model for these analyses.

DISCUSSION

The results of this study in a nationally representative cohort that followed individuals for 12 years confirm that the mortality risk of prediabetes is probably low. This is not unexpected based on the mixed results from previous studies, several of which found either no future mortality risk or risk that was not robust across measures. However, we found that the presence of transferrin saturation and serum ferritin is associated with increased mortality risk of individuals with prediabetes. Among individuals with normal iron levels, those with prediabetes had low mortality risk levels similar to the adjusted risk of prediabetes alone. On the other hand, in adjusted survival analyses, individuals with prediabetes who also had elevated transferrin saturation had substantially increased mortality risk. These findings extend previous work on iron markers and diabetes to the previously uninvestigated area of prediabetes.

These results suggest that additional stratification of individuals with prediabetes on the basis of iron markers would be useful to identify those with higher risk and who might benefit from iron lowering therapies. Previous data has indicated that elevated iron markers are associated with the development of diabetes and that among individuals with

diabetes the co-occurrence of elevated TS increases those patients' mortality risk. Early identification of individuals with both conditions (prediabetes, elevated iron) may help in both slowing the development of diabetes as well as decreasing mortality risk. It is important for early identification of these individuals because much like individuals with prediabetes, the vast majority of individuals with elevated iron do not know it.[30] These individuals need to be identified to mitigate the increased risk posed by elevated iron in combination with prediabetes. Such individuals would be targets for intensive interventions to reduce risk, including typical lifestyle interventions shown to help avoid the onset of diabetes in people at high risk.[31] Although more research is needed into the ability of interventions on iron in prediabetes to affect development of diabetes and mortality risk, some data suggest that reduction of TS improves HbA1c and glucose control.[32]

These associations of TS and ferritin with mortality in the context of prediabetes are not surprising especially if elevations of these parameters are interpreted in light of current understanding of iron toxicity.[33] Iron, whether absorbed as iron salts or in dietary heme is processed by enterocytes and released into the plasma where it is transported in a non-reactive state bound to transferrin. Iron that is bound to transferrin is in the Fe^{+3} state and is not reactive and, therefore, not toxic. However, when TS is above 40 to 50%, free iron or so-called non-transferrin-bound iron (NTBI) is released into the plasma as the buffering ability of transferrin is exceeded.[34] Labile plasma iron (LPI) is a highly reactive subspecies of NTBI that interacts with hydrogen peroxide through Fenton chemistry to form the extremely powerful oxidants, hydroxyl radical and singlet oxygen. These are the free radicals that ultimately directly damage protein and DNA. Perhaps

more importantly, NTBI/LPI species are able to enter cells via ion channels. These channels, unlike the transferrin receptor, are not regulated so this reactive iron freely enters the cytoplasm of the pancreas, pituitary, and heart.

The current results suggest that exposure to excessive free iron is dangerous in the context of prediabetes. Furthermore, elevated ferritin and TS predict poor diabetes control and phlebotomy to reduce iron even over short periods of time improve HbA1c in parallel with changes in TS, even though ferritin is not changed,[32] putting further emphasis on the fact that NTBI/LPI reflected by TS is the proximal cause of the toxicity. Several strategies are available to decrease iron, including chelation therapy and phlebotomy. Phlebotomy is an easy, inexpensive, and well-tolerated intervention. Reduction in TS by phlebotomy has been shown to improve measures of diabetic control.[32] Furthermore, correction of severe iron overload can significantly improve glucose tolerance.[35] Thus, the finding that a baseline measure of high TS as point measure of toxic free iron plus elevation of ferritin, evidence of elevated cytosolic iron over a longer period of time, predicts increased risk of mortality among individuals with prediabetes supports the premise that toxic free iron is a health risk.

The normoglycemic group with elevated iron markers did not show increased mortality risk in comparison to the reference group of normoglycemic and normal iron marker levels. This may seem inconsistent with other data on the increased mortality risk due to elevated TS by itself. However, there is the potential that the effect of TS on mortality is modified by the presence of other variables.[36,37] This effect has been shown in the past. Rather than being inconsistent with the TS alone and mortality findings, these new findings enhance our understanding of elevated iron markers and morbidity and

mortality and allow us to consider the more complex, but real, situation of patients by considering multiple variables together rather than independently.

This study has several limitations. First, although we have a nationally representative, population-based cohort followed through the National Death Index, the biomarkers are measured only at baseline. There is the possibility that either the hyperglycemia or elevated iron measures were identified and interventions were implemented to lower these biomarkers. If that were the case and a substantial number of individuals did drop their levels due to interventions thereby decreasing the potential mortality risk, the observed adjusted risk individuals elevated at baseline is even more concerning. Second, we were only able to follow these individuals for 12 years. It is possible that this time frame may have been too short to adequately see an effect for a biomarker like prediabetes. However, we did censor the first three years of mortality so that any deaths in that time frame would not be attributed to prediabetes. The model still found a substantial mortality risk for the prediabetes plus iron markers in this length of time. Third, we were unable to evaluate the relationship between being elevated on both transferrin saturation and serum ferritin with prediabetes on the risk of mortality. We attempted such an analysis but the number of individuals in the group with prediabetes and elevation on both iron markers was small and the population estimates were deemed unreliable.

In conclusion, this study representative of the population of the United States helps to clarify the current evidence on the mortality risk of prediabetes and provides further support for the role of elevated iron markers in health risk. Future screening and

intervention programs for prediabetes may benefit from additional strategies to recognize and treat iron elevations, particularly transferrin saturation.

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CONTRIBUTION STATEMENT

AGM: conception, design, analysis, and drafting of manuscript

RJT: design, analysis, and drafting of manuscript

TDC: conception, design, and drafting of manuscript

RB: design and analysis

COMPETING INTERESTS

The authors report no competing interests.

DATA SHARING STATEMENT

Data from this study are publicly available on the Internet through the National Center for Health Statistics.

FIGURE LEGENDS

Figure 1. Kaplan Meier curve of survival among individuals with prediabetes or normal glycemic levels.

———Normoglycemia

– – – – – –Prediabetes

Figure 2. Kaplan Meier curve of survival among individuals with prediabetes and elevated transferrin saturation

Normoglycemia and Normal Transferrin Saturation

Normoglycemia and Elevated Transferrin Saturation

Prediabetes and Normal Transferrin Saturation

Prediabetes and Elevated Transferrin Saturation

REFERENCES

1. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2012;35:S64–S71.

2. Bullard KM, Saydah SH, Imperatore G, et al. Secular changes in U.S. Prediabetes prevalence defined by hemoglobin A1c and fasting plasma glucose: National Health and Nutrition Examination Surveys, 1999-2010. *Diabetes Care* 2013;36:2286–93.

3. Mainous AG 3rd, Tanner RJ, Baker R, et al. Prevalence of prediabetes in England from 2003 to 2011: population-based, cross-sectional study. *BMJ Open* 2014 Jun 9;4(6):e005002. doi: 10.1136/bmjopen-2014-005002. Accessed 29 June, 2014.

4. Centers for Disease Control and Prevention (CDC). Awareness of prediabetes--United States, 2005-2010. MMWR Morb Mortal Wkly Rep. 2013 Mar 22;62:209-12.

5. de Vegt F, Dekker JM, Ruhé HG, et al. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia* 1999;42:926–31.

6. Fuller JH, Shipley MJ, Rose G, et al. Coronary-heart-disease risk and impaired glucose tolerance. The Whitehall study. *Lancet* 1980;1:1373–76.

7. Valdés S, Botas P, Delgado E, Cadórniga FD. Mortality risk in Spanish adults with diagnosed diabetes, undiagnosed diabetes, or pre-diabetes. The asturias study 1998-2004. *Rev Esp Cardiol (Engl Ed)* 2009;62:528–34.

8. Deedwania P, Patel K, Fonarow GC, et al. Prediabetes is not an independent risk factor for incident heart failure, other cardiovascular events or mortality in older adults: findings from a population-based cohort study. *Int J Cardiol* 2013;168:3616–22.

9. Kowall B, Rathmann W, Heier M, et al. Categories of glucose tolerance and continuous glycemic measures and mortality. *Eur J Epidemiol* 2011;26:637–45.

10. Zhou XH, Qiao Q, Zethelius B, et al. Diabetes, prediabetes and cancer mortality. *Diabetologia* 2010;53:1867–76.

11. Barr EL, Zimmet PZ, Welborn TA, et al. Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance: the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). *Circulation* 2007;116:151–57.

12. Orban E, Schwab S, Thorand B, Huth C. Association of iron indices and type 2 diabetes: a meta-analysis of observational studies. *Diabetes Metab Res Rev* 2014;30:372–94.

13. Bao W, Rong Y, Rong S, Liu L. Dietary iron intake, body iron stores, and the risk of type 2 diabetes: a systematic review and meta-analysis. *BMC Med* 2012;10:119. doi: 10.1186/1741-7015-10-119. Accessed 8 July, 2014.

14. Ellervik C, Mandrup-Poulsen T, Tybjærg-Hansen A, Nordestgaard BG. Total and cause-specific mortality by elevated transferrin saturation and hemochromatosis genotype in individuals with diabetes: two general population studies. *Diabetes Care* 2014;37:444–52.

15. Masuda Y, Ichii H, Vaziri ND. At pharmacologically relevant concentrations intravenous iron preparations cause pancreatic beta cell death. Am J Transl Res. 2013;6:64-70.

16. Ellervik C, Tybjaerg-Hansen A, Nordestgaard BG. Total mortality by transferrin saturation levels: Two general population studies and a meta analysis. *Clin Chem* 2011;57:459–466.

17. Kim KS, Son HG, Hong NS, Lee DH. Associations of serum ferritin and transferrin % saturation with all-cause, cancer, and cardiovascular disease mortality: Third National Health and Nutrition Examination Survey follow-up study. *J Prev Med Public Health* 2012;45:196-203.

18. Mainous AG 3rd, Gill JM, Carek PJ. Elevated transferrin saturation and mortality. *Ann Fam Med* 2004;2:133–138.

19. Mainous AG 3rd, Wells B, Carek PJ, et al. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. *Ann Fam Med* 2004;2:139-44.

20. Stack AG, Mutwali AI, Nguyen HT, et al. Transferrin saturation ratio and risk of total and cardiovascular mortality in the general population. *QJM.* 2014;107:623-33.

21. Wells BJ, Mainous AG 3rd, King DE, et al. The combined effect of transferrin saturation and low density lipoprotein on mortality. *Fam Med* 2004;36:324-9.

22. Ellervik C, Marott JL, Tybjærg-Hansen A, Schnohr P, Nordestgaard BG. Total and Cause-Specific Mortality by Moderately and Markedly Increased Ferritin Concentrations: General Population Study and Metaanalysis. Clin Chem. 2014 Aug 25. pii: clinchem.2014.229013.

23. Ponikowska B, Suchocki T, Paleczny B, et al. Iron status and survival in diabetic patients with coronary artery disease. *Diabetes Care* 2013;36:4147–56.

24. Morris DH, Khunti K, Achana F, et al. Progression rates from HbA(1c) 6.0-6.4% and other prediabetes definitions to type 2 diabetes: a meta-analysis. *Diabetologia* 2013;56:1489–93.

25. National Center for Health Statistics. Office of Analysis and Epidemiology, NCHS 2011 Linked Mortality Files Matching Methodology, September, 2013. Hyattsville, Maryland. http://www.cdc.gov/nchs/data/datalinkage/2011_linked_mortality_file_matching_methodology.pdf. Accessed 20 August, 2014.

26. Carson AP1, Fox CS, McGuire DK, Levitan EB, Laclaustra M, Mann DM, Muntner P. Low hemoglobin A1c and risk of all-cause mortality among US adults without diabetes. Circ Cardiovasc Qual Outcomes. 2010 Nov;3(6):661-7.

27. Ellervik C, Andersen HU, Tybjærg-Hansen A, et al. Total mortality by elevated transferrin saturation in patients with diabetes. *Diabetes Care* 2013;36:2646–54.

28. Adams PC, Reboussin DM, Barton JC, et al. Hemochromatosis and Iron Overload Screening (HEIRS) Study Research Investigators. Hemochromatosis and iron-overload screening in a racially diverse population. N Engl J Med. 2005;352:1769-78.

29. Pearson TA, Mensah GA, Alexander RW, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice. A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 2003;107(3):499 – 511.

30. Mainous AG 3rd, Wright RU, Hulihan MM, et al. Elevated transferrin saturation, health-related quality of life and telomere length. *Biometals* 2014;27:135–41

31. Portero McLellan KC, Wyne K, Villagomez ET, Hsueh WA. Therapeutic interventions to reduce the risk of progression from prediabetes to type 2 diabetes mellitus. *Ther Clin Risk Manag* 2014;10:173–88.

32. Fernández-Real JM, Peñarroja G, Castro A, García-Bragado F, Hernández-Aguado I, Ricart W. Blood letting in high-ferritin type 2 diabetes: effects on insulin sensitivity and beta-cell function. *Diabetes* 2002;51:1000–4.

33. Hansen JB, Moen IW, Mandrup-Poulsen T. Iron: the hard player in diabetes pathophysiology. *Acta Physiol* 2014;210:717–32.

34. Coates TD. Physiology and pathophysiology of iron in hemoglobin-associated diseases. *Free Radic Biol Med* 2014;72C:23–40.

35. Farmaki K, Tzoumari I, Pappa C, et al. Normalisation of total body iron load with very intensive combined chelation reverses cardiac and endocrine complications of thalassaemia major. *Br J Haematol* 2010;148:466–75.

36. Mainous AG 3rd, Wells B, Carek PJ, Gill JM, Geesey ME. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. Ann Fam Med. 2004;2:139-44.

37. Wells BJ, Mainous AG 3rd, King DE, Gill JM, Carek PJ, Geesey ME. The combined effect of transferrin saturation and low density lipoprotein on mortality. Fam Med. 2004;36(5):324-9.

Table 1: Baseline Characteristics of the Sample

	Study Sample (%)
Unweighted Sample Size	8,003
Population Estimate	80,653,788
Age, years	
40-54	49.6
55-69	31.2
70+	19.2
Sex, male	46.1
Race/Ethnicity	
Non-Hispanic White	82.2
Non-Hispanic Black	8.3
Hispanic	6.7
Asian/Other	2.9
Has health insurance	93.6
Obese	23.3
Current Smoker	23.6
Has diagnosed high cholesterol	40.1
Has diagnosed hypertension	31.3
Ever diagnosed with heart attack	5.3
Ever diagnosed with stroke	2.8
Ever diagnosed with cancer	12.3
Relative with diabetes	41.2
Relative with heart attack before age 50	15.4
Elevated C-reactive protein	1.2
Elevated Transferrin Saturation	3.3
Elevated Ferritin	15.6
Prediabetes	23.2
Assumed deceased	27.0

Table 2: Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes

	Hazard Ratio	95% Confidence Interval
Adjusted Model[a]		
Prediabetes	1.04	1.00-1.08
Normoglycemia	1.0	--

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

Table 3: Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes and Iron Markers

	Hazard Ratio	95% Confidence Interval
Adjusted Model[a]		
Normal Transferrin Saturation/ Normoglycemia	1.00	--
Normal Transferrin Saturation/ Prediabetes	1.13	1.09-1.18
Elevated Transferrin Saturation/ Normoglycemia	.99	.90-1.08
Elevated Transferrin Saturation/ Prediabetes	1.86	1.05-3.29
Adjusted Model[b]		
Normal Ferritin/ Normoglycemia	1.00	--
Normal Ferritin/ Prediabetes	1.15	1.09-1.20
Elevated Ferritin/ Normoglycemia	1.05	1.00-1.12
Elevated Ferritin/ Prediabetes	1.14	1.04-1.24

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

[b]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, family history of early heart attack, and elevated C-reactive protein.

Prediabetes, Elevated Iron, and All-Cause Mortality: A Cohort Study

Running Title: Prediabetes, Elevated Iron, and All-Cause Mortality

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ABSTRACT

Objectives: Data have indicated low to nonexistent increased mortality risk for individuals with prediabetes, but it is unclear if the risk is increased when the patient has elevated iron markers. Our purpose was to examine the mortality risk among adults with prediabetes in the context of co-existing elevated transferrin saturation (TS) or serum ferritin.

Setting: Data collected by the third National Health and Nutrition Examination Survey 1988-1994 (NHANES III) in the United States and by the National Center for Health Statistics for the National Death Index from 1988-2006.

Participants: Individuals age 40 and older who participated in the NHANES and provided a blood sample.

Primary Outcome Variable: Mortality was measured as all-cause mortality.

Results: Adjusted analyses show that prediabetes has a small increased mortality risk (HR =1.04; 95% CI, 1.00-1.08). Persons who had prediabetes and elevated serum ferritin had an increased hazard ratio for death (HR=1.14; 95% CI, 1.04-1.24) compared with those who had normal ferritin and normal glucose. Among persons with prediabetes who had elevated TS, they had an increased mortality risk (HR=1.86; 95% CI, 1.05-3.29) compared with those with normal TS levels and normal glucose. **Conclusion:** The mortality risk of prediabetes is low. However, among individuals who have co-existing elevated iron markers, particularly transferrin saturation, the risk rises substantially.

STRENGTHS AND LIMITATIONS OF STUDY

- This study utilizes a nationally representative population-based cohort that allows generalization of results to the population of the United States.
- Mortality was left-censored to control for the potential misclassification of people who were very ill at baseline.
- Biomarkers for elevated iron and prediabetes were available only at baseline.
- We were able to only observe individuals for 12 years, which may be insufficient to adequately see an effect on mortality for prediabetes.

INTRODUCTION

Prediabetes is defined by blood glucose concentrations that are higher than normal, but lower than established thresholds for diabetes.[1] Prediabetes is a high risk state for the development of not only diabetes, but also associated complications. Recent data have shown that in developed countries such as the United States and the United Kingdom more than a third of adults have prediabetes but the vast majority of these do not realize it.[2-4]

Several studies have shown that prediabetes is a mortality risk.[5,6] On the other hand, other studies have found that prediabetes is not a mortality risk.[7–10] Still other studies showed that the relationship was not very robust and was dependent on the measure of prediabetes.[11]

Three different meta-analyses of observational studies have concluded that elevated iron indices like serum ferritin and transferrin saturation (TS) are strongly associated with increased risk for developing diabetes.[12–14] Some evidence exists to indicate that pancreatic beta cells are killed in the presence of iron.[15] In addition to increased risk of diabetes, elevated TS or elevated ferritin is associated with increased mortality in the general population. [16-22] Further, recent evidence suggests that among patients with diabetes, mortality risk increases in the presence of elevated ferritin or elevated TS.[23,24] We therefore hypothesize that the mortality risk of individuals with prediabetes will be increased in the presence of elevated iron markers.

Considering that only about 10% of the US population with prediabetes realize that they have prediabetes, a better understanding of the potential mortality risk is warranted. Consequently, the purpose of this study was to evaluate the association

between prediabetes, elevated serum ferritin, elevated TS and mortality in a large, nationally representative cohort.

METHODS

We conducted longitudinal analyses of the third National Health and Nutrition Examination Survey, 1988-1994 (NHANES III) linked to mortality data collected through the National Death Index. Mortality data were available through December 31, 2006. The NHANES III survey provides population estimates of the United States and was conducted from October, 1988 through October, 1994. The NHANES III used complex, multi-stage, stratified, clustered samples of civilian, noninstitutionalized population, Of the 39,695 individuals eligible to participate, a total of 30,818 persons were examined in their homes or in mobile examination centers (MEC) which visited 89 communities across the United States (a participation rate of 77.6%). The health examination included collection of blood and urine specimens for the conduct of various laboratory analyses. The NHANES provides preexisting de-identified public use data which do not need specific approval from the National Center for Health Statistics.

The NHANES III data merged with the National Death Index is a prospective cohort study that passively followed up on the participants in the NHANES III. The linked mortality file uses a probabilistic matching method.[25] We limited our study to individuals 40 years old and older at baseline, the time of their NHANES III interview. The National Death Index involves searching national databases containing information about mortality and causes of death. Mortality status was ascertained by computerized matching to national databases and evaluation of the resulting matches. Persons not found to be deceased were assumed alive for analytic purposes. All living survey participants

had been observed for 146 months, and our survival analysis was carried out to December 31, 2006.

Previously Diagnosed Diabetes

The NHANES III assessed participants for diagnosed diabetes using the questions, "Have you ever been told by a doctor that you have diabetes or sugar diabetes?", "Were you pregnant when you were told that you had diabetes?" and "Other than during pregnancy, has a doctor ever told you that you have diabetes or sugar diabetes?" We defined participants as having diagnosed diabetes if they answered "yes" to ever having been told they had diabetes, excluding pregnancy. Individuals with previously diagnosed diabetes were removed from the analysis. We also removed individuals with an HbA1c of 6.5% or greater, to account for undiagnosed diabetes.

Normoglycemia & Prediabetes

We defined normoglycemia as an Hba1c level between 4.0%--5.6% (20-38 mmol/mol). To control for any potential effect of low HbA1c, we also removed individuals with an HbA1c below 4.0% (20 mmol/mol), a level associated with increased all-cause mortality in adults without diabetes.[26]

We defined prediabetes among individuals without previously diagnosed diabetes using HbA1c ranges as specified by the American Diabetes Association, 5.7%–6.4% (39-46 mmol/mol).[1] This range has been shown in a meta-analysis to be predictive of progression to diabetes.[19] We excluded individuals with previously diagnosed diabetes because the current glycemic status of those patients may simply represent diabetes control. Prediabetes status was missing for 1,637 of the NHANES respondents over the age of 40.

Transferrin Saturation

Serum iron and total iron-binding capacity (TIBC) were measured in serum, and calculated by dividing serum iron by TIBC and multiplying by 100. For the analyses, elevated transferrin saturation was categorized as TS ≥50%. Individuals with TS below 25% were removed from the analysis, as low TS has been linked to increased risk of mortality.[23] Despite the lack of universal agreement on the upper and lower limits of normal TS, these cut points has been used in several studies evaluating diabetes, TS and mortality.[23,27] Data were missing for transferrin saturation level for 536 of the NHANES respondents over the age of 40.

Serum Ferritin

Serum ferritin was used as a measure of body iron stores and was measured using the QuantImune Ferritin IRMA kit. Serum ferritin was categorized for the analyses as elevated if it was ≥674.1 pmol/l (300 ng/mL) for males and ≥449.4 pmol/l (200 ng/mL) for females.[28] Individuals with serum ferritin below 56.175 pmol/l (25 ng/mL) were removed from the analysis, as low ferritin has been linked to an increased risk of mortality.[23] Data were missing for serum ferritin level for 539 of the NHANES respondents over the age of 40.

Mortality

Mortality was measured as all-cause mortality. Mortality status was ascertained solely by computerized matching to national databases and evaluation of the resulting matches.

Covariates

Covariates used in our analyses included: age at baseline in the NHANES III, gender, race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, and other race), health insurance status, obesity (Body Mass Index computed in the exam of ≥30), previous diagnosis of a heart attack, previous diagnosis of a stroke, previous diagnosis of hypertension, previous diagnosis of hypercholesterolemia, previous diagnosis of cancer, family history of diabetes, family history of myocardial infarction before age 50, and current smoking status. Respondents were considered non-smokers if they reported smoking at least100 cigarettes in their life or if they had smoked more than 100 cigarettes and were not currently smoking.

In the analysis of serum ferritin, we also controlled for C-reactive protein. Ferritin is an acute phase reactant as well as an indicator of iron stores and as such may indicate inflammation. Consequently, we controlled for inflammation by adjusting for C-reactive protein. C-reactive protein was considered elevated at levels above 3.0 mg/l.[29]

Analysis

In an effort to control for potential misclassification of persons who were very ill at baseline thereby affecting mortality risk of prediabetes, we left-censored the analysis to exclude any mortality events that occurred in the first three years following the individuals examination for the first three years of the cohort. For the analyses of mortality, we used sampling weights (specifically, the total MEC and Home examined weight) to calculate prevalence estimates for the civilian noninstitutionalized US population. This strategy of basing the analyses on population estimates is a characteristic that makes the NHANES different from many other cohort designs that do not use weighted population estimates. However, this strategy does provide national

generalizability. Because of the complex sampling design of the survey, we performed statistical analyses using the statistical software package SUDAAN (Research Triangle Institute, Raleigh, NC), as recommended by the National Center for Health Statistics (NCHS). Using SAS (Cary, NC), we computed Kaplan-Meier plots to show graphically the unadjusted relationship between all-cause mortality and prediabetes.

All analyses were based on the population estimates and we followed the NCHS recommendations for assessing the reliability of estimates in the context of a limited sample size. If the standard error of an estimate was greater than 30% of an estimate it would be considered unreliable All estimates met the criteria for reliability.

To accomplish our goals of examining a possible synergistic effect of having elevated iron with prediabetes we classified the population into 4 groups based upon prediabetes or normoglycemia and normal and elevated TS. We also classified the population into 4 groups based upon prediabetes or normoglycemia and normal and elevated iron based on levels of TS and ferritin.

We performed Cox proportional hazards analyses to measure the associations between all-cause mortality and prediabetes controlling for all of the studied covariates using listwise deletion to account for missing data. In these models, survival time was a continuous variable measured in 1-month increments from the date of the exam.

We also performed adjusted Cox proportional hazards analysis with all-cause mortality for prediabetes in the 4 part variables with TS adjusting for the aforementioned covariates. For the adjusted Cox proportional hazards analysis with ferritin we adjusted for the aforementioned covariates and also C-reactive protein.

We evaluated the proportionality of the hazards through examination of the Schoenfeld residuals.

RESULTS

A total of 8,003 (unweighted) individuals were over 40 years old and had HbA1c levels between 4.0 and 6.4%. Baseline characteristics for the sample are shown in Table 1. Table 1 indicates that 23.2% of the weighted sample had prediabetes, 15.6% of the sample exhibited elevated serum ferritin, and 3.3% had elevated TS.

Of the respondents that had prediabetes, 38.8% died within 12 years, compared to 23.4% of respondents with normal HbA1c levels. Among individuals with normal TS and normoglycemia, 23.1% died, compared to 23.7% of those with elevated TS, 37.5% of those with normal TS and prediabetes, and 44.7% of those with elevated TS and prediabetes. Among individuals with normal ferritin and normoglycemia, 34.3% died, compared with 38.8% of those with normal ferritin and prediabetes, 29.2% of those with elevated ferritin and normoglycemia, and 38.8% of those with elevated ferritin and prediabetes.

Table 2 shows the results the adjusted Cox proportional hazards model for prediabetes. Table 2 indicates prediabetes alone has a small increased mortality risk. The Kaplan Meier curve of the survival and prediabetes over the length of the time under observation is shown in Fig 1.

Table 3 presents results of the analyses combining prediabetes with iron markers. In models that examined the impact of a prediabetes state combined with markers of low iron, the hazard ratios were similar to that of prediabetes alone. However, when combined with prediabetes, there was an increased mortality risk among individuals with

TS ≥50, as well as with individuals who had increased ferritin. The risk was most increased when individuals had elevated ferritin and elevated transferrin saturation together. Figure 2 represents the relationship of survival of the four groups over the 12 years under observation. Individuals with prediabetes in the presence of elevated iron have lower survival probabilities than other groups. An examination of the Schoenfeld residuals suggested proportionality of hazards and appropriateness of the statistical model for these analyses.

DISCUSSION

The results of this study in a nationally representative cohort that followed individuals for 12 years confirm that the mortality risk of prediabetes is probably low. This is not unexpected based on the mixed results from previous studies, several of which found either no future mortality risk or risk that was not robust across measures. However, we found that the presence of transferrin saturation and serum ferritin is associated with increased mortality risk of individuals with prediabetes. Among individuals with normal iron levels, those with prediabetes had low mortality risk levels similar to the adjusted risk of prediabetes alone. On the other hand, in adjusted survival analyses, individuals with prediabetes who also had elevated transferrin saturation had substantially increased mortality risk. These findings extend previous work on iron markers and diabetes to the previously uninvestigated area of prediabetes.

These results suggest that additional stratification of individuals with prediabetes on the basis of iron markers would be useful to identify those with higher risk and who might benefit from iron lowering therapies. Previous data has indicated that elevated iron markers are associated with the development of diabetes and that among individuals with

diabetes the co-occurrence of elevated TS increases those patients' mortality risk. Early identification of individuals with both conditions (prediabetes, elevated iron) may help in both slowing the development of diabetes as well as decreasing mortality risk. It is important for early identification of these individuals because much like individuals with prediabetes, the vast majority of individuals with elevated iron do not know it.[30] These individuals need to be identified to mitigate the increased risk posed by elevated iron in combination with prediabetes. Such individuals would be targets for intensive interventions to reduce risk, including typical lifestyle interventions shown to help avoid the onset of diabetes in people at high risk.[31] Although more research is needed into the ability of interventions on iron in prediabetes to affect development of diabetes and mortality risk, some data suggest that reduction of TS improves HbA1c and glucose control.[32]

These associations of TS and ferritin with mortality in the context of prediabetes are not surprising especially if elevations of these parameters are interpreted in light of current understanding of iron toxicity.[33] Iron, whether absorbed as iron salts or in dietary heme is processed by enterocytes and released into the plasma where it is transported in a non-reactive state bound to transferrin. Iron that is bound to transferrin is in the Fe^{+3} state and is not reactive and, therefore, not toxic. However, when TS is above 40 to 50%, free iron or so-called non-transferrin-bound iron (NTBI) is released into the plasma as the buffering ability of transferrin is exceeded.[34] Labile plasma iron (LPI) is a highly reactive subspecies of NTBI that interacts with hydrogen peroxide through Fenton chemistry to form the extremely powerful oxidants, hydroxyl radical and singlet oxygen. These are the free radicals that ultimately directly damage protein and DNA. Perhaps

more importantly, NTBI/LPI species are able to enter cells via ion channels. These channels, unlike the transferrin receptor, are not regulated so this reactive iron freely enters the cytoplasm of the pancreas, pituitary, and heart.

The current results suggest that exposure to excessive free iron is dangerous in the context of prediabetes. Furthermore, elevated ferritin and TS predict poor diabetes control and phlebotomy to reduce iron even over short periods of time improve HbA1c in parallel with changes in TS, even though ferritin is not changed,[32] putting further emphasis on the fact that NTBI/LPI reflected by TS is the proximal cause of the toxicity. Several strategies are available to decrease iron, including chelation therapy and phlebotomy. Phlebotomy is an easy, inexpensive, and well-tolerated intervention. Reduction in TS by phlebotomy has been shown to improve measures of diabetic control.[32] Furthermore, correction of severe iron overload can significantly improve glucose tolerance.[35] Thus, the finding that a baseline measure of high TS as point measure of toxic free iron plus elevation of ferritin, evidence of elevated cytosolic iron over a longer period of time, predicts increased risk of mortality among individuals with prediabetes supports the premise that toxic free iron is a health risk.

The normoglycemic group with elevated iron markers did not show increased mortality risk in comparison to the reference group of normoglycemic and normal iron marker levels. This may seem inconsistent with other data on the increased mortality risk due to elevated TS by itself. However, there is the potential that the effect of TS on mortality is modified by the presence of other variables.[36,37] This effect has been shown in the past. Rather than being inconsistent with the TS alone and mortality findings, these new findings enhance our understanding of elevated iron markers and morbidity and

mortality and allow us to consider the more complex, but real, situation of patients by considering multiple variables together rather than independently.

This study has several limitations. First, although we have a nationally representative, population-based cohort followed through the National Death Index, the biomarkers are measured only at baseline. There is the possibility that either the hyperglycemia or elevated iron measures were identified and interventions were implemented to lower these biomarkers. If that were the case and a substantial number of individuals did drop their levels due to interventions thereby decreasing the potential mortality risk, the observed adjusted risk individuals elevated at baseline is even more concerning. Second, we were only able to follow these individuals for 12 years. It is possible that this time frame may have been too short to adequately see an effect for a biomarker like prediabetes. However, we did censor the first three years of mortality so that any deaths in that time frame would not be attributed to prediabetes. The model still found a substantial mortality risk for the prediabetes plus iron markers in this length of time. Third, we were unable to evaluate the relationship between being elevated on both transferrin saturation and serum ferritin with prediabetes on the risk of mortality. We attempted such an analysis but the number of individuals in the group with prediabetes and elevation on both iron markers was small and the population estimates were deemed unreliable.

In conclusion, this study representative of the population of the United States helps to clarify the current evidence on the mortality risk of prediabetes and provides further support for the role of elevated iron markers in health risk. Future screening and

intervention programs for prediabetes may benefit from additional strategies to recognize and treat iron elevations, particularly transferrin saturation.

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CONTRIBUTION STATEMENT

AGM: conception, design, analysis, and drafting of manuscript

RJT: design, analysis, and drafting of manuscript

TDC: conception, design, and drafting of manuscript

RB: design and analysis

COMPETING INTERESTS

The authors report no competing interests.

REFERENCES

1. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2012;35:S64–S71.

2. Bullard KM, Saydah SH, Imperatore G, et al. Secular changes in U.S. Prediabetes prevalence defined by hemoglobin A1c and fasting plasma glucose: National Health and Nutrition Examination Surveys, 1999-2010. *Diabetes Care* 2013;36:2286–93.

3. Mainous AG 3rd, Tanner RJ, Baker R, et al. Prevalence of prediabetes in England from 2003 to 2011: population-based, cross-sectional study. *BMJ Open* 2014 Jun 9;4(6):e005002. doi: 10.1136/bmjopen-2014-005002. Accessed 29 June, 2014.

4. Centers for Disease Control and Prevention (CDC). Awareness of prediabetes--United States, 2005-2010. MMWR Morb Mortal Wkly Rep. 2013 Mar 22;62:209-12.

5. de Vegt F, Dekker JM, Ruhé HG, et al. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia* 1999;42:926–31.

6. Fuller JH, Shipley MJ, Rose G, et al. Coronary-heart-disease risk and impaired glucose tolerance. The Whitehall study. *Lancet* 1980;1:1373–76.

7. Valdés S, Botas P, Delgado E, Cadórniga FD. Mortality risk in Spanish adults with diagnosed diabetes, undiagnosed diabetes, or pre-diabetes. The asturias study 1998-2004. *Rev Esp Cardiol (Engl Ed)* 2009;62:528–34.

8. Deedwania P, Patel K, Fonarow GC, et al. Prediabetes is not an independent risk factor for incident heart failure, other cardiovascular events or mortality in older adults: findings from a population-based cohort study. *Int J Cardiol* 2013;168:3616–22.

9. Kowall B, Rathmann W, Heier M, et al. Categories of glucose tolerance and continuous glycemic measures and mortality. *Eur J Epidemiol* 2011;26:637–45.

10. Zhou XH, Qiao Q, Zethelius B, et al. Diabetes, prediabetes and cancer mortality. *Diabetologia* 2010;53:1867–76.

11. Barr EL, Zimmet PZ, Welborn TA, et al. Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance: the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). *Circulation* 2007;116:151–57.

12. Orban E, Schwab S, Thorand B, Huth C. Association of iron indices and type 2 diabetes: a meta-analysis of observational studies. *Diabetes Metab Res Rev* 2014;30:372–94.

13. Bao W, Rong Y, Rong S, Liu L. Dietary iron intake, body iron stores, and the risk of type 2 diabetes: a systematic review and meta-analysis. *BMC Med* 2012;10:119. doi: 10.1186/1741-7015-10-119. Accessed 8 July, 2014.

14. Ellervik C, Mandrup-Poulsen T, Tybjærg-Hansen A, Nordestgaard BG. Total and cause-specific mortality by elevated transferrin saturation and hemochromatosis genotype in individuals with diabetes: two general population studies. *Diabetes Care* 2014;37:444–52.

15. Masuda Y, Ichii H, Vaziri ND. At pharmacologically relevant concentrations intravenous iron preparations cause pancreatic beta cell death. Am J Transl Res. 2013;6:64-70.

16. Ellervik C, Tybjaerg-Hansen A, Nordestgaard BG. Total mortality by transferrin saturation levels: Two general population studies and a meta analysis. *Clin Chem* 2011;57:459–466.

17. Kim KS, Son HG, Hong NS, Lee DH. Associations of serum ferritin and transferrin % saturation with all-cause, cancer, and cardiovascular disease mortality: Third National Health and Nutrition Examination Survey follow-up study. *J Prev Med Public Health* 2012;45:196-203.

18. Mainous AG 3rd, Gill JM, Carek PJ. Elevated transferrin saturation and mortality. *Ann Fam Med* 2004;2:133–138.

19. Mainous AG 3rd, Wells B, Carek PJ, et al. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. *Ann Fam Med* 2004;2:139-44.

20. Stack AG, Mutwali AI, Nguyen HT, et al. Transferrin saturation ratio and risk of total and cardiovascular mortality in the general population. *QJM.* 2014;107:623-33.

21. Wells BJ, Mainous AG 3rd, King DE, et al. The combined effect of transferrin saturation and low density lipoprotein on mortality. *Fam Med* 2004;36:324-9.

22. Ellervik C, Marott JL, Tybjærg-Hansen A, Schnohr P, Nordestgaard BG. Total and Cause-Specific Mortality by Moderately and Markedly Increased Ferritin Concentrations: General Population Study and Metaanalysis. Clin Chem. 2014 Aug 25. pii: clinchem.2014.229013.

23. Ponikowska B, Suchocki T, Paleczny B, et al. Iron status and survival in diabetic patients with coronary artery disease. *Diabetes Care* 2013;36:4147–56.

24. Morris DH, Khunti K, Achana F, et al. Progression rates from HbA(1c) 6.0-6.4% and other prediabetes definitions to type 2 diabetes: a meta-analysis. *Diabetologia* 2013;56:1489–93.

25. National Center for Health Statistics. Office of Analysis and Epidemiology, NCHS 2011 Linked Mortality Files Matching Methodology, September, 2013. Hyattsville, Maryland. http://www.cdc.gov/nchs/data/datalinkage/2011_linked_mortality_file_matching_methodology.pdf. Accessed 20 August, 2014.

26. Carson AP1, Fox CS, McGuire DK, Levitan EB, Laclaustra M, Mann DM, Muntner P. Low hemoglobin A1c and risk of all-cause mortality among US adults without diabetes. Circ Cardiovasc Qual Outcomes. 2010 Nov;3(6):661-7.

27. Ellervik C, Andersen HU, Tybjærg-Hansen A, et al. Total mortality by elevated transferrin saturation in patients with diabetes. *Diabetes Care* 2013;36:2646–54.

28. Adams PC, Reboussin DM, Barton JC, et al. Hemochromatosis and Iron Overload Screening (HEIRS) Study Research Investigators. Hemochromatosis and iron-overload screening in a racially diverse population. N Engl J Med. 2005;352:1769-78.

29. Pearson TA, Mensah GA, Alexander RW, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice. A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 2003;107(3):499 – 511.

30. Mainous AG 3rd, Wright RU, Hulihan MM, et al. Elevated transferrin saturation, health-related quality of life and telomere length. *Biometals* 2014;27:135–41

31. Portero McLellan KC, Wyne K, Villagomez ET, Hsueh WA. Therapeutic interventions to reduce the risk of progression from prediabetes to type 2 diabetes mellitus. *Ther Clin Risk Manag* 2014;10:173–88.

32. Fernández-Real JM, Peñarroja G, Castro A, García-Bragado F, Hernández-Aguado I, Ricart W. Blood letting in high-ferritin type 2 diabetes: effects on insulin sensitivity and beta-cell function. *Diabetes* 2002;51:1000–4.

33. Hansen JB, Moen IW, Mandrup-Poulsen T. Iron: the hard player in diabetes pathophysiology. *Acta Physiol* 2014;210:717–32.

34. Coates TD. Physiology and pathophysiology of iron in hemoglobin-associated diseases. *Free Radic Biol Med* 2014;72C:23–40.

35. Farmaki K, Tzoumari I, Pappa C, et al. Normalisation of total body iron load with very intensive combined chelation reverses cardiac and endocrine complications of thalassaemia major. *Br J Haematol* 2010;148:466–75.

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Race/Ethnicity	
Non-Hispanic White	82.2
Non-Hispanic Black	8.3
Hispanic	6.7
Asian/Other	2.9
Has health insurance	93.6
Obese	23.3
Current Smoker	23.6
Has diagnosed high cholesterol	40.1
Has diagnosed hypertension	31.3
Ever diagnosed with heart attack	5.3
Ever diagnosed with stroke	2.8
Ever diagnosed with cancer	12.3
Relative with diabetes	41.2
Relative with heart attack before age 50	15.4
Elevated C-reactive protein	1.2
Elevated Transferrin Saturation	3.3
Elevated Ferritin	15.6
Prediabetes	23.2
Assumed deceased	27.0

Table 2: Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes

	Hazard Ratio	95% Confidence Interval
Adjusted Model[a]		
Prediabetes	1.04	1.00-1.08
Normoglycemia	1.0	--

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

Table 3: Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes and Iron Markers

	Hazard Ratio	95% Confidence Interval
Adjusted Model[a]		
Normal Transferrin Saturation/ Normoglycemia	1.00	--
Normal Transferrin Saturation/ Prediabetes	1.13	1.09-1.18
Elevated Transferrin Saturation/ Normoglycemia	.99	.90-1.08
Elevated Transferrin Saturation/ Prediabetes	1.86	1.05-3.29
Adjusted Model[b]		
Normal Ferritin/ Normoglycemia	1.00	--
Normal Ferritin/ Prediabetes	1.15	1.09-1.20
Elevated Ferritin/ Normoglycemia	1.05	1.00-1.12
Elevated Ferritin/ Prediabetes	1.14	1.04-1.24

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

[b]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, family history of early heart attack, and elevated C-reactive protein.

Figure 1. Kaplan Meier curve of survival among individuals with prediabetes or normal glycemic levels.

——Normoglycemia

——Prediabetes

Figure 2. Kaplan Meier curve of survival among individuals with prediabetes and elevated transferrin saturation

——Normoglycemia and Normal Transferrin Saturation

——Normoglycemia and Elevated Transferrin Saturation

——Prediabetes and Normal Transferrin Saturation

——Prediabetes and Elevated Transferrin Saturation

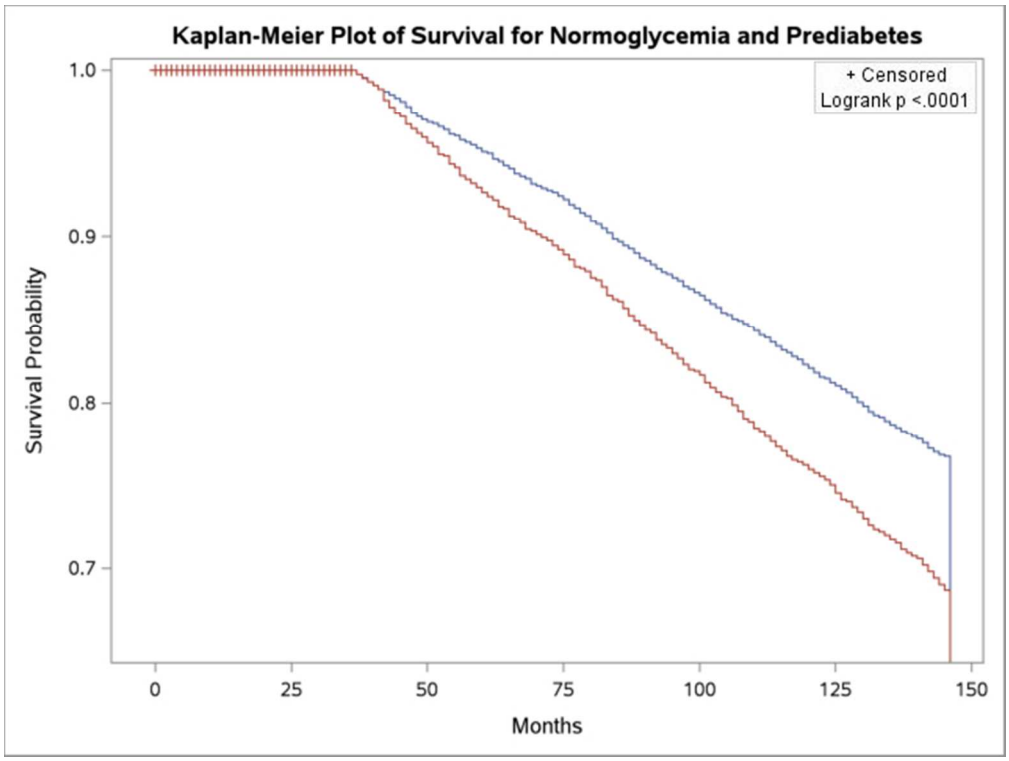

Figure 1¬¬¬. Kaplan Meier curve of survival among individuals with prediabetes or normal glycemic levels.
blue line = Normoglycemia
red line = Prediabetes

54x40mm (300 x 300 DPI)

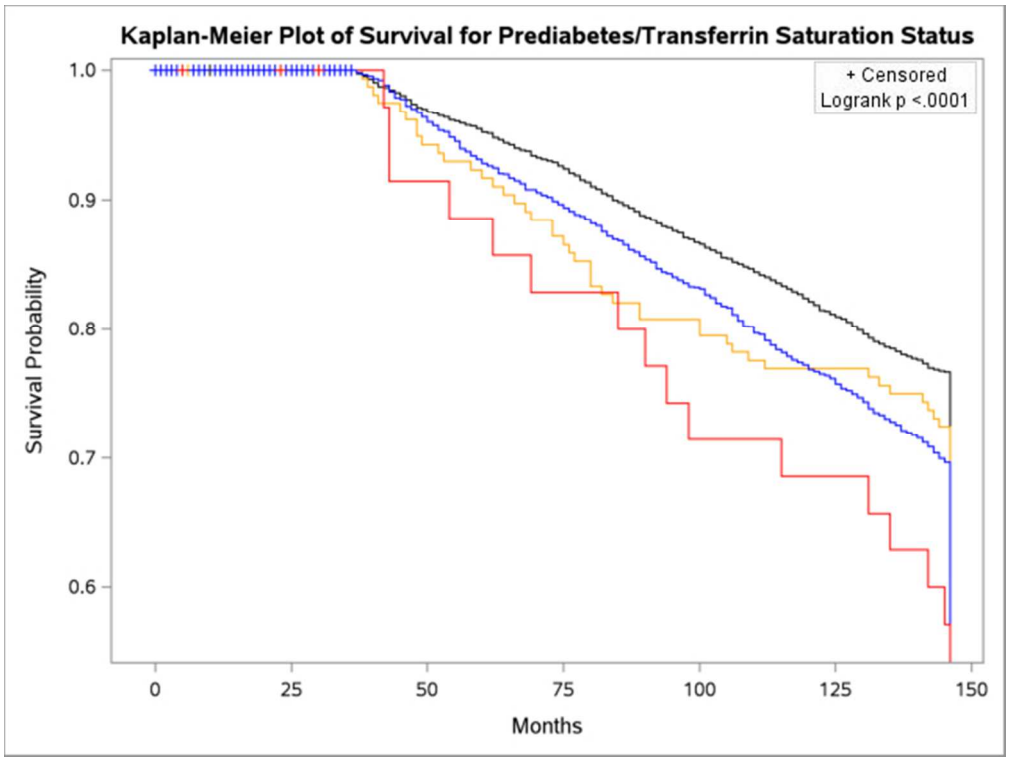

Figure 2. Kaplan Meier curve of survival among individuals with prediabetes and elevated transferrin saturation
black line = Normoglycemia and Normal Transferrin Saturation
yellow line = Normoglycemia and Elevated Transferrin Saturation
blue line = Prediabetes and Normal Transferrin Saturation
red line = Prediabetes and Elevated Transferrin Saturation

54x40mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page number
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4-5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-8
Bias	9	Describe any efforts to address potential sources of bias	9
Study size	10	Explain how the study size was arrived at	5-8
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8-9
		(*b*) Describe any methods used to examine subgroups and interactions	8
		(*c*) Explain how missing data were addressed	8
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	NA
		(*e*) Describe any sensitivity analyses	NA

Continued on next page

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5, 9
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9, 19
		(b) Indicate number of participants with missing data for each variable of interest	6-7
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	5
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	19
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-10 20-22
		(*b*) Report category boundaries when continuous variables were categorized	NA
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	10
Discussion			
Key results	18	Summarise key results with reference to study objectives	10-11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12-13
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12
Generalisability	21	Discuss the generalisability (external validity) of the study results	13
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Prediabetes, Elevated Iron, and All-Cause Mortality: A Cohort Study

Running Title: Prediabetes, Elevated Iron, and All-Cause Mortality

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ABSTRACT

Objectives: Data have indicated low to nonexistent increased mortality risk for individuals with prediabetes, but it is unclear if the risk is increased when the patient has elevated iron markers. Our purpose was to examine the mortality risk among adults with prediabetes in the context of co-existing elevated transferrin saturation (TS) or serum ferritin.

Setting: Data collected by the third National Health and Nutrition Examination Survey 1988-1994 (NHANES III) in the United States and by the National Center for Health Statistics for the National Death Index from 1988-2006.

Participants: Individuals age 40 and older who participated in the NHANES and provided a blood sample.

Primary Outcome Variable: Mortality was measured as all-cause mortality.

Results: Adjusted analyses show that prediabetes has a small increased mortality risk (HR =1.04; 95% CI, 1.00-1.08). Persons who had prediabetes and elevated serum ferritin had an increased hazard ratio for death (HR=1.14; 95% CI, 1.04-1.24) compared with those who had normal ferritin and normal glucose. Among persons with prediabetes who had elevated TS, they had an increased mortality risk (HR=1.88; 95% CI, 1.06-3.30) compared with those with normal TS levels and normal glucose.

Conclusion: The mortality risk of prediabetes is low. However, among individuals who have co-existing elevated iron markers, particularly transferrin saturation, the risk rises substantially.

STRENGTHS AND LIMITATIONS OF STUDY

- This study utilizes a nationally representative population-based cohort that allows generalization of results to the noninstitutionalized civilian population of the United States.
- Mortality was left-censored to control for the potential misclassification of people who were very ill at baseline.
- Biomarkers for elevated iron and prediabetes were available only at baseline.
- We were able to only observe individuals for 12 years, which may be insufficient to adequately see an effect on mortality for prediabetes.

INTRODUCTION

Prediabetes is defined by blood glucose concentrations that are higher than normal, but lower than established thresholds for diabetes.[1] Prediabetes is a high risk state for the development of not only diabetes, but also associated complications. Recent data have shown that in developed countries such as the United States and the United Kingdom more than a third of adults have prediabetes but the vast majority of these do not realize it.[2-4]

Several studies have shown that prediabetes is a mortality risk.[5,6] On the other hand, other studies have found that prediabetes is not a mortality risk.[7–10] Still other studies showed that the relationship was not very robust and was dependent on the measure of prediabetes.[11]

Three different meta-analyses of observational studies have concluded that elevated iron indices like serum ferritin and transferrin saturation (TS) are strongly associated with increased risk for developing diabetes.[12–14] Some evidence exists to indicate that pancreatic beta cells are killed in the presence of iron.[15] In addition to increased risk of diabetes, elevated TS or elevated ferritin is associated with increased mortality in the general population. [16-22] Further, recent evidence suggests that among patients with diabetes, mortality risk increases in the presence of elevated ferritin or elevated TS.[23,24] We therefore hypothesize that the mortality risk of individuals with prediabetes will be increased in the presence of elevated iron markers.

Considering that only about 10% of the US population with prediabetes realize that they have prediabetes, a better understanding of the potential mortality risk is warranted. Consequently, the purpose of this study was to evaluate the association

between prediabetes, elevated serum ferritin, elevated TS and mortality in a large, nationally representative cohort.

METHODS

We conducted longitudinal analyses of the third National Health and Nutrition Examination Survey, 1988-1994 (NHANES III) linked to mortality data collected through the National Death Index. Mortality data were available through December 31, 2006. The NHANES III survey provides population estimates of the United States and was conducted from October, 1988 through October, 1994.

The NHANES III used complex, multi-stage, stratified, clustered samples of civilian, noninstitutionalized population and is designed and conducted for the purpose of making health-related prevalence estimates that are nationally generalizable. To make accurate population estimates, analysis of the NHANES requires the use of weight and design variables that account for this complex design. The use of sampling weights is necessary to account for differences in probability of selection for each participant and also accounts for noncoverage and nonresponse.[25] The NHANES III oversampled different groups, including older individuals, African Americans, and Mexican-Americans. The application of sampling weights allows us to conduct analyses on the individuals who were sampled in the NHANES and extrapolate those results to the population at large. According to the technical reports provided by the National Center for Health Statistics, without the use of sampling weights, misinterpretation of population estimates based on NHANES III is likely. This strategy of basing the analyses on population estimates is a characteristic that makes the NHANES different from many other cohort designs that do not use weighted population estimates, and provides national generalizability.

Of the 39,695 individuals eligible to participate, a total of 30,818 persons of all ages were examined in their homes or in mobile examination centers (MEC) which visited 89 communities across the United States (a participation rate of 77.6%). The health examination included collection of blood and urine specimens for the conduct of various laboratory analyses.

The NHANES III data merged with the National Death Index is a prospective cohort study that passively followed up on the participants in the NHANES III. The linked mortality file uses a probabilistic matching method.[26] The National Death Index involves searching national databases containing information about mortality and causes of death. Mortality status was ascertained by computerized matching to national databases and evaluation of the resulting matches. Persons not found to be deceased were assumed alive for analytic purposes.

The NHANES III is preexisting de-identified public use data which do not need specific approval from the National Center for Health Statistics.

We limited our study to individuals 40 years old and older at baseline, the time of their NHANES III interview. All analyses were based on the population estimates generated by applying variables accounting for the design and sampling methodology of the NHANES. The results presented here are generalizable to the noninstitutionalized civilian population of the United States aged 40 and older from 1998-1994.

Previously Diagnosed Diabetes

The NHANES III assessed participants for diagnosed diabetes using the questions, “Have you ever been told by a doctor that you have diabetes or sugar diabetes?”, “Were you pregnant when you were told that you had diabetes?” and “Other

than during pregnancy, has a doctor ever told you that you have diabetes or sugar diabetes?" We defined participants as having diagnosed diabetes if they answered "yes" to ever having been told they had diabetes, excluding pregnancy. Individuals with previously diagnosed diabetes were removed from the analysis. We also removed individuals with an HbA1c of 6.5% or greater, to account for undiagnosed diabetes.

Normoglycemia & Prediabetes

We defined normoglycemia as an Hba1c level between 4.0%--5.6% (20-38 mmol/mol). To control for any potential effect of low HbA1c, we also removed individuals with an HbA1c below 4.0% (20 mmol/mol), a level associated with increased all-cause mortality in adults without diabetes.[27]

We defined prediabetes among individuals without previously diagnosed diabetes using HbA1c ranges as specified by the American Diabetes Association, 5.7%–6.4% (39-46 mmol/mol).[1] This range has been shown in a meta-analysis to be predictive of progression to diabetes.[19] We excluded individuals with previously diagnosed diabetes because the current glycemic status of those patients may simply represent diabetes control. Prediabetes status was missing for 1,637 of the NHANES respondents over the age of 40.

Transferrin Saturation

Serum iron and total iron-binding capacity (TIBC) were measured in serum, and calculated by dividing serum iron by TIBC and multiplying by 100. For the analyses, elevated transferrin saturation was categorized as TS $\geq$50%. Individuals with TS below 25% were removed from the analysis, as low TS has been linked to increased risk of mortality.[23] Despite the lack of universal agreement on the upper and lower limits of

normal TS, these cut points has been used in several studies evaluating diabetes, TS and mortality.[23,28] Data were missing for transferrin saturation level for 536 of the NHANES respondents over the age of 40.

Serum Ferritin

Serum ferritin was used as a measure of body iron stores and was measured using the QuantImune Ferritin IRMA kit. Serum ferritin was categorized for the analyses as elevated if it was ≥674.1 pmol/l (300 ng/mL) for males and ≥449.4 pmol/l (200 ng/mL) for females.[29] Individuals with serum ferritin below 56.175 pmol/l (25 ng/mL) were removed from the analysis, as low ferritin has been linked to an increased risk of mortality.[23] Data were missing for serum ferritin level for 539 of the NHANES respondents over the age of 40.

Mortality

Mortality was measured as all-cause mortality. Mortality status was ascertained solely by computerized matching to national databases and evaluation of the resulting matches. All living survey participants examined in this study had been observed for 146 months, and our survival analysis was carried out to December 31, 2006.

Covariates

Covariates used in our analyses included: age at baseline in the NHANES III, gender, race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican-American, and other), health insurance status, obesity (Body Mass Index computed in the exam of ≥30), previous diagnosis of a heart attack, previous diagnosis of a stroke, previous diagnosis of hypertension, previous diagnosis of hypercholesterolemia, previous diagnosis of cancer, family history of diabetes, family history of myocardial infarction before age 50, and

current smoking status. Respondents were considered non-smokers if they reported smoking less than 100 cigarettes in their life or if they had smoked more than 100 cigarettes and were not currently smoking.

In the analysis of serum ferritin, we also controlled for C-reactive protein. Ferritin is an acute phase reactant as well as an indicator of iron stores and as such may indicate inflammation. Consequently, we controlled for inflammation by adjusting for C-reactive protein. C-reactive protein was considered elevated at levels above 3.0 mg/l.[30]

Analysis

In an effort to control for potential misclassification of persons who were very ill at baseline thereby affecting mortality risk of prediabetes, we left-censored the analysis to exclude any mortality events that occurred in the first three years following the individuals examination for the first three years of the cohort. Because of the complex sampling design of the survey, we performed statistical analyses using the statistical software package SUDAAN (Research Triangle Institute, Raleigh, NC), as recommended by the National Center for Health Statistics (NCHS). Using SAS (Cary, NC), we computed Kaplan-Meier plots to show graphically the unadjusted relationship between all-cause mortality and prediabetes.

We followed the NCHS recommendations for assessing the reliability of estimates in the context of a limited sample size. If the standard error of an estimate was greater than 30% of an estimate it would be considered unreliable All estimates met the criteria for reliability.

To accomplish our goals of examining a possible synergistic effect of having elevated iron with prediabetes we classified the population into 4 groups based upon

prediabetes or normoglycemia and normal or elevated TS. The population was also classified into 4 groups based upon prediabetes or normoglycemia and normal or elevated serum ferritin.

We performed Cox proportional hazards analyses to measure the associations between all-cause mortality and prediabetes controlling for all of the studied covariates using listwise deletion to account for missing data. In these models, survival time was a continuous variable measured in 1-month increments from the date of the exam.

We also performed adjusted Cox proportional hazards analysis with all-cause mortality for prediabetes in the 4 part variables with TS adjusting for the aforementioned covariates. For the adjusted Cox proportional hazards analysis with ferritin we adjusted for the aforementioned covariates and also C-reactive protein.

We evaluated the proportionality of the hazards through examination of the Schoenfeld residuals.

RESULTS

A total of 8,003 (unweighted) individuals were over 40 years old and had HbA1c levels between 4.0 and 6.4%, or 80,653,788 individuals nationally. Baseline characteristics for the sample are shown in Table 1. Table 1 indicates that 23.2% of the weighted sample had prediabetes, 15.6% of the sample exhibited elevated serum ferritin, and 3.3% had elevated TS.

Of the respondents that had prediabetes, 38.8% died within 12 years (723,702 died; 11,431,597 survived), compared to 23.4% of respondents with normal HbA1c levels (14,527,028 died; 47,458,061 survived). Among individuals with normal TS and normoglycemia, 23.1% died (10,724,279 died; 35,649,283 survived), compared to 23.7%

of those with elevated TS and normoglycemia (412,237 died; 1,327,253 survived), 37.5% of those with normal TS and prediabetes (5,137,131 died; 8,572,762 survived), and 44.7% of those with elevated TS and prediabetes (126,633 died; 156,790 survived). Among individuals with normal ferritin and normoglycemia, 24.3% died (10,967,486 died; 34,132,718 survived), compared with 38.8% of those with normal ferritin and prediabetes (5,465,483 died; 8,614,683 survived), 29.2% of those with elevated ferritin and normoglycemia (2,333,436 died; 5,662,576 survived), and 38.8% of those with elevated ferritin and prediabetes (1,150,647died; 1,818,565 survived).

Table 2 shows the results the adjusted Cox proportional hazards model for prediabetes. Table 2 indicates prediabetes alone has a small increased mortality risk. The Kaplan Meier curve of the survival and prediabetes over the length of the time under observation is shown in Fig 1.

Table 3 presents results of the analyses combining prediabetes with iron markers. In models that examined the impact of a prediabetes state combined with markers of low iron, the hazard ratios were similar to that of prediabetes alone. However, when combined with prediabetes, there was an increased mortality risk among individuals with TS $\geq$50, as well as with individuals who had increased ferritin. The risk was most increased when individuals had elevated ferritin and elevated transferrin saturation together. Figure 2 represents the relationship of survival of the four groups over the 12 years under observation. Individuals with prediabetes in the presence of elevated iron have lower survival probabilities than other groups. An examination of the Schoenfeld residuals suggested proportionality of hazards and appropriateness of the statistical model for these analyses.

DISCUSSION

The results of this study in a nationally representative cohort that followed individuals for 12 years confirm that the mortality risk of prediabetes is probably low. This is not unexpected based on the mixed results from previous studies, several of which found either no future mortality risk or risk that was not robust across measures. However, we found that the presence of transferrin saturation and serum ferritin is associated with increased mortality risk of individuals with prediabetes. Among individuals with normal iron levels, those with prediabetes had low mortality risk levels similar to the adjusted risk of prediabetes alone. On the other hand, in adjusted survival analyses, individuals with prediabetes who also had elevated transferrin saturation had substantially increased mortality risk. These findings extend previous work on iron markers and diabetes to the previously uninvestigated area of prediabetes.

These results suggest that additional stratification of individuals with prediabetes on the basis of iron markers would be useful to identify those with higher risk and who might benefit from iron lowering therapies. Previous data has indicated that elevated iron markers are associated with the development of diabetes and that among individuals with diabetes the co-occurrence of elevated TS increases those patients' mortality risk. Early identification of individuals with both conditions (prediabetes, elevated iron) may help in both slowing the development of diabetes as well as decreasing mortality risk. It is important for early identification of these individuals because much like individuals with prediabetes, the vast majority of individuals with elevated iron do not know it.[31] These individuals need to be identified to mitigate the increased risk posed by elevated iron in combination with prediabetes. Such individuals would be targets for intensive

interventions to reduce risk, including typical lifestyle interventions shown to help avoid the onset of diabetes in people at high risk.[32] Although more research is needed into the ability of interventions on iron in prediabetes to affect development of diabetes and mortality risk, some data suggest that reduction of TS improves HbA1c and glucose control.[33]

These associations of TS and ferritin with mortality in the context of prediabetes are not surprising especially if elevations of these parameters are interpreted in light of current understanding of iron toxicity.[34] Iron, whether absorbed as iron salts or in dietary heme is processed by enterocytes and released into the plasma where it is transported in a non-reactive state bound to transferrin. Iron that is bound to transferrin is in the Fe^{+3} state and is not reactive and, therefore, not toxic. However, when TS is above 40 to 50%, free iron or so-called non-transferrin-bound iron (NTBI) is released into the plasma as the buffering ability of transferrin is exceeded.[35] Labile plasma iron (LPI) is a highly reactive subspecies of NTBI that interacts with hydrogen peroxide through Fenton chemistry to form the extremely powerful oxidants, hydroxyl radical and singlet oxygen. These are the free radicals that ultimately directly damage protein and DNA. Perhaps more importantly, NTBI/LPI species are able to enter cells via ion channels. These channels, unlike the transferrin receptor, are not regulated so this reactive iron freely enters the cytoplasm of the pancreas, pituitary, and heart.

The current results suggest that exposure to excessive free iron is dangerous in the context of prediabetes. Furthermore, elevated ferritin and TS predict poor diabetes control and phlebotomy to reduce iron even over short periods of time improve HbA1c in parallel with changes in TS, even though ferritin is not changed,[33] putting further

emphasis on the fact that NTBI/LPI reflected by TS is the proximal cause of the toxicity. Several strategies are available to decrease iron, including chelation therapy and phlebotomy. Phlebotomy is an easy, inexpensive, and well-tolerated intervention. Reduction in TS by phlebotomy has been shown to improve measures of diabetic control.[33] Furthermore, correction of severe iron overload can significantly improve glucose tolerance.[36] Thus, the finding that a baseline measure of high TS as point measure of toxic free iron plus elevation of ferritin, evidence of elevated cytosolic iron over a longer period of time, predicts increased risk of mortality among individuals with prediabetes supports the premise that toxic free iron is a health risk.

The normoglycemic group with elevated iron markers did not show increased mortality risk in comparison to the reference group of normoglycemic and normal iron marker levels. This may seem inconsistent with other data on the increased mortality risk due to elevated TS by itself. However, there is the potential that the effect of TS on mortality is modified by the presence of other variables.[37,38] This effect has been shown in the past. Rather than being inconsistent with the TS alone and mortality findings, these new findings enhance our understanding of elevated iron markers and morbidity and mortality and allow us to consider the more complex, but real, situation of patients by considering multiple variables together rather than independently.

This study has several limitations. First, although we have a nationally representative, population-based cohort followed through the National Death Index, the biomarkers are measured only at baseline. There is the possibility that either the hyperglycemia or elevated iron measures were identified and interventions were implemented to lower these biomarkers. If that were the case and a substantial number of

individuals did drop their levels due to interventions thereby decreasing the potential mortality risk, the observed adjusted risk individuals elevated at baseline is even more concerning. Second, we were only able to follow these individuals for 12 years. It is possible that this time frame may have been too short to adequately see an effect for a biomarker like prediabetes. However, we did censor the first three years of mortality so that any deaths in that time frame would not be attributed to prediabetes. The model still found a substantial mortality risk for the prediabetes plus iron markers in this length of time. Third, we were unable to evaluate the relationship between being elevated on both transferrin saturation and serum ferritin with prediabetes on the risk of mortality. We attempted such an analysis but the number of individuals in the group with prediabetes and elevation on both iron markers was small and the population estimates were deemed unreliable.

In conclusion, this study representative of the population of the United States helps to clarify the current evidence on the mortality risk of prediabetes and provides further support for the role of elevated iron markers in health risk. Future screening and intervention programs for prediabetes may benefit from additional strategies to recognize and treat iron elevations, particularly transferrin saturation.

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CONTRIBUTION STATEMENT

AGM: conception, design, analysis, and drafting of manuscript

RJT: design, analysis, and drafting of manuscript

TDC: conception, design, and drafting of manuscript

RB: design and analysis

COMPETING INTERESTS

The authors report no competing interests.

DATA SHARING STATEMENT

Data from this study are publicly available on the Internet through the National Center for Health Statistics.

REFERENCES

1. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2012;35:S64–S71.

2. Bullard KM, Saydah SH, Imperatore G, et al. Secular changes in U.S. Prediabetes prevalence defined by hemoglobin A1c and fasting plasma glucose: National Health and Nutrition Examination Surveys, 1999-2010. *Diabetes Care* 2013;36:2286–93.

3. Mainous AG 3rd, Tanner RJ, Baker R, et al. Prevalence of prediabetes in England from 2003 to 2011: population-based, cross-sectional study. *BMJ Open* 2014 Jun 9;4(6):e005002. doi: 10.1136/bmjopen-2014-005002. Accessed 29 June, 2014.

4. Centers for Disease Control and Prevention (CDC). Awareness of prediabetes--United States, 2005-2010. MMWR Morb Mortal Wkly Rep. 2013 Mar 22;62:209-12.

5. de Vegt F, Dekker JM, Ruhé HG, et al. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia* 1999;42:926–31.

6. Fuller JH, Shipley MJ, Rose G, et al. Coronary-heart-disease risk and impaired glucose tolerance. The Whitehall study. *Lancet* 1980;1:1373–76.

7. Valdés S, Botas P, Delgado E, Cadórniga FD. Mortality risk in Spanish adults with diagnosed diabetes, undiagnosed diabetes, or pre-diabetes. The asturias study 1998-2004. *Rev Esp Cardiol (Engl Ed)* 2009;62:528–34.

8. Deedwania P, Patel K, Fonarow GC, et al. Prediabetes is not an independent risk factor for incident heart failure, other cardiovascular events or mortality in older adults: findings from a population-based cohort study. *Int J Cardiol* 2013;168:3616–22.

9. Kowall B, Rathmann W, Heier M, et al. Categories of glucose tolerance and continuous glycemic measures and mortality. *Eur J Epidemiol* 2011;26:637–45.

10. Zhou XH, Qiao Q, Zethelius B, et al. Diabetes, prediabetes and cancer mortality. *Diabetologia* 2010;53:1867–76.

11. Barr EL, Zimmet PZ, Welborn TA, et al. Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance: the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). *Circulation* 2007;116:151–57.

12. Orban E, Schwab S, Thorand B, Huth C. Association of iron indices and type 2 diabetes: a meta-analysis of observational studies. *Diabetes Metab Res Rev* 2014;30:372–94.

13. Bao W, Rong Y, Rong S, Liu L. Dietary iron intake, body iron stores, and the risk of type 2 diabetes: a systematic review and meta-analysis. *BMC Med* 2012;10:119. doi: 10.1186/1741-7015-10-119. Accessed 8 July, 2014.

14. Ellervik C, Mandrup-Poulsen T, Tybjærg-Hansen A, Nordestgaard BG. Total and cause-specific mortality by elevated transferrin saturation and hemochromatosis genotype in individuals with diabetes: two general population studies. *Diabetes Care* 2014;37:444–52.

15. Masuda Y, Ichii H, Vaziri ND. At pharmacologically relevant concentrations intravenous iron preparations cause pancreatic beta cell death. Am J Transl Res. 2013;6:64-70.

16. Ellervik C, Tybjaerg-Hansen A, Nordestgaard BG. Total mortality by transferrin saturation levels: Two general population studies and a meta analysis. *Clin Chem* 2011;57:459–466.

17. Kim KS, Son HG, Hong NS, Lee DH. Associations of serum ferritin and transferrin % saturation with all-cause, cancer, and cardiovascular disease mortality: Third National Health and Nutrition Examination Survey follow-up study. *J Prev Med Public Health* 2012;45:196-203.

18. Mainous AG 3rd, Gill JM, Carek PJ. Elevated transferrin saturation and mortality. *Ann Fam Med* 2004;2:133–138.

19. Mainous AG 3rd, Wells B, Carek PJ, et al. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. *Ann Fam Med* 2004;2:139-44.

20. Stack AG, Mutwali AI, Nguyen HT, et al. Transferrin saturation ratio and risk of total and cardiovascular mortality in the general population. *QJM.* 2014;107:623-33.

21. Wells BJ, Mainous AG 3rd, King DE, et al. The combined effect of transferrin saturation and low density lipoprotein on mortality. *Fam Med* 2004;36:324-9.

22. Ellervik C, Marott JL, Tybjærg-Hansen A, Schnohr P, Nordestgaard BG. Total and Cause-Specific Mortality by Moderately and Markedly Increased Ferritin Concentrations: General Population Study and Metaanalysis. Clin Chem. 2014 Aug 25. pii: clinchem.2014.229013.

23. Ponikowska B, Suchocki T, Paleczny B, et al. Iron status and survival in diabetic patients with coronary artery disease. *Diabetes Care* 2013;36:4147–56.

24. Morris DH, Khunti K, Achana F, et al. Progression rates from HbA(1c) 6.0-6.4% and other prediabetes definitions to type 2 diabetes: a meta-analysis. *Diabetologia* 2013;56:1489–93.

25. National Center for Health Statistics. Plan and operation of the Third National Health and Nutrition Examination Survey, 1988-1994. Vital Health Stat. 1994:1(32). http://www.cdc.gov/nchs/data/series/sr_01/sr01_032.pdf. Accessed 15 October 2014.

26. National Center for Health Statistics. Office of Analysis and Epidemiology, NCHS 2011 Linked Mortality Files Matching Methodology, September, 2013. Hyattsville, Maryland. http://www.cdc.gov/nchs/data/datalinkage/2011_linked_mortality_file_matching_methodology.pdf. Accessed 20 August, 2014.

27. Carson AP1, Fox CS, McGuire DK, Levitan EB, Laclaustra M, Mann DM, Muntner P. Low hemoglobin A1c and risk of all-cause mortality among US adults without diabetes. Circ Cardiovasc Qual Outcomes. 2010 Nov;3(6):661-7.

28. Ellervik C, Andersen HU, Tybjærg-Hansen A, et al. Total mortality by elevated transferrin saturation in patients with diabetes. *Diabetes Care* 2013;36:2646–54.

29. Adams PC, Reboussin DM, Barton JC, et al. Hemochromatosis and Iron Overload Screening (HEIRS) Study Research Investigators. Hemochromatosis and iron-overload screening in a racially diverse population. N Engl J Med. 2005;352:1769-78.

30. Pearson TA, Mensah GA, Alexander RW, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice. A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 2003;107(3):499 – 511.

31. Mainous AG 3rd, Wright RU, Hulihan MM, et al. Elevated transferrin saturation, health-related quality of life and telomere length. *Biometals* 2014;27:135–41

32. Portero McLellan KC, Wyne K, Villagomez ET, Hsueh WA. Therapeutic interventions to reduce the risk of progression from prediabetes to type 2 diabetes mellitus. *Ther Clin Risk Manag* 2014;10:173–88.

33. Fernández-Real JM, Peñarroja G, Castro A, García-Bragado F, Hernández-Aguado I, Ricart W. Blood letting in high-ferritin type 2 diabetes: effects on insulin sensitivity and beta-cell function. *Diabetes* 2002;51:1000–4.

34. Hansen JB, Moen IW, Mandrup-Poulsen T. Iron: the hard player in diabetes pathophysiology. *Acta Physiol* 2014;210:717–32.

35. Coates TD. Physiology and pathophysiology of iron in hemoglobin-associated diseases. *Free Radic Biol Med* 2014;72C:23–40.

36. Farmaki K, Tzoumari I, Pappa C, et al. Normalisation of total body iron load with very intensive combined chelation reverses cardiac and endocrine complications of thalassaemia major. *Br J Haematol* 2010;148:466–75.

37. Mainous AG 3rd, Wells B, Carek PJ, Gill JM, Geesey ME. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. Ann Fam Med. 2004;2:139-44.

38. Wells BJ, Mainous AG 3rd, King DE, Gill JM, Carek PJ, Geesey ME. The combined effect of transferrin saturation and low density lipoprotein on mortality. Fam Med. 2004;36(5):324-9.

Figure 1. Kaplan Meier curve of survival among individuals with prediabetes or normal glycemic levels.

Normoglycemia

Prediabetes

Figure 2. Kaplan Meier curve of survival among individuals with prediabetes and elevated transferrin saturation

Normoglycemia and Normal Transferrin Saturation

Normoglycemia and Elevated Transferrin Saturation

Prediabetes and Normal Transferrin Saturation

Prediabetes and Elevated Transferrin Saturation

Table 1: Baseline Characteristics of the Sample

	Study Sample (%)
Unweighted Sample Size	8,003
Population Estimate	80,653,788
Age, years	
40-54	49.6
55-69	31.2
70+	19.2
Sex, male	46.1
Race/Ethnicity	
Non-Hispanic White	82.2
Non-Hispanic Black	8.3
Mexican-American	3.2
Other	6.4
Has health insurance	93.6
Obese	23.3
Current Smoker	23.6
Has diagnosed high cholesterol	40.1
Has diagnosed hypertension	31.3
Ever diagnosed with heart attack	5.3
Ever diagnosed with stroke	2.8
Ever diagnosed with cancer	12.3
Relative with diabetes	41.2
Relative with heart attack before age 50	15.4
Elevated C-reactive protein	1.2
Elevated Transferrin Saturation	3.3
Elevated Ferritin	15.6
Prediabetes	23.2
Assumed deceased	27.0

Table 2: Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes

	Hazard Ratio	95% Confidence Interval
Adjusted Model[a]		
Prediabetes	1.04	1.00-1.08
Normoglycemia	1.0	--

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

Table 3: Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes and Iron Markers

	Hazard Ratio	95% Confidence Interval
Adjusted Model[a]		
Normal Transferrin Saturation/ Normoglycemia	1.00	--
Normal Transferrin Saturation/ Prediabetes	1.13	1.08-1.19
Elevated Transferrin Saturation/ Normoglycemia	.98	.90-1.07
Elevated Transferrin Saturation/ Prediabetes	1.88	1.06-3.30
Adjusted Model[b]		
Normal Ferritin/ Normoglycemia	1.00	--
Normal Ferritin/ Prediabetes	1.15	1.09-1.20
Elevated Ferritin/ Normoglycemia	1.05	1.00-1.12
Elevated Ferritin/ Prediabetes	1.14	1.04-1.24

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

[b]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, family history of early heart attack, and elevated C-reactive protein.

Prediabetes, Elevated Iron, and All-Cause Mortality: A Cohort Study

Running Title: Prediabetes, Elevated Iron, and All-Cause Mortality

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ABSTRACT

Objectives: Data have indicated low to nonexistent increased mortality risk for individuals with prediabetes, but it is unclear if the risk is increased when the patient has elevated iron markers. Our purpose was to examine the mortality risk among adults with prediabetes in the context of co-existing elevated transferrin saturation (TS) or serum ferritin.

Setting: Data collected by the third National Health and Nutrition Examination Survey 1988-1994 (NHANES III) in the United States and by the National Center for Health Statistics for the National Death Index from 1988-2006.

Participants: Individuals age 40 and older who participated in the NHANES and provided a blood sample.

Primary Outcome Variable: Mortality was measured as all-cause mortality.

Results: Adjusted analyses show that prediabetes has a small increased mortality risk (HR =1.04; 95% CI, 1.00-1.08). Persons who had prediabetes and elevated serum ferritin had an increased hazard ratio for death (HR=1.14; 95% CI, 1.04-1.24) compared with those who had normal ferritin and normal glucose. Among persons with prediabetes who had elevated TS, they had an increased mortality risk (HR=1.88; 95% CI, 1.06-3.30) compared with those with normal TS levels and normal glucose.

Conclusion: The mortality risk of prediabetes is low. However, among individuals who have co-existing elevated iron markers, particularly transferrin saturation, the risk rises substantially.

STRENGTHS AND LIMITATIONS OF STUDY

- This study utilizes a nationally representative population-based cohort that allows generalization of results to the noninstitutionalized civilian population of the United States.
- Mortality was left-censored to control for the potential misclassification of people who were very ill at baseline.
- Biomarkers for elevated iron and prediabetes were available only at baseline.
- We were able to only observe individuals for 12 years, which may be insufficient to adequately see an effect on mortality for prediabetes.

INTRODUCTION

Prediabetes is defined by blood glucose concentrations that are higher than normal, but lower than established thresholds for diabetes.[1] Prediabetes is a high risk state for the development of not only diabetes, but also associated complications. Recent data have shown that in developed countries such as the United States and the United Kingdom more than a third of adults have prediabetes but the vast majority of these do not realize it.[2-4]

Several studies have shown that prediabetes is a mortality risk.[5,6] On the other hand, other studies have found that prediabetes is not a mortality risk.[7–10] Still other studies showed that the relationship was not very robust and was dependent on the measure of prediabetes.[11]

Three different meta-analyses of observational studies have concluded that elevated iron indices like serum ferritin and transferrin saturation (TS) are strongly associated with increased risk for developing diabetes.[12–14] Some evidence exists to indicate that pancreatic beta cells are killed in the presence of iron.[15] In addition to increased risk of diabetes, elevated TS or elevated ferritin is associated with increased mortality in the general population. [16-22] Further, recent evidence suggests that among patients with diabetes, mortality risk increases in the presence of elevated ferritin or elevated TS.[23,24] We therefore hypothesize that the mortality risk of individuals with prediabetes will be increased in the presence of elevated iron markers.

Considering that only about 10% of the US population with prediabetes realize that they have prediabetes, a better understanding of the potential mortality risk is warranted. Consequently, the purpose of this study was to evaluate the association

between prediabetes, elevated serum ferritin, elevated TS and mortality in a large, nationally representative cohort.

METHODS

We conducted longitudinal analyses of the third National Health and Nutrition Examination Survey, 1988-1994 (NHANES III) linked to mortality data collected through the National Death Index. Mortality data were available through December 31, 2006. The NHANES III survey provides population estimates of the United States and was conducted from October, 1988 through October, 1994.

The NHANES III used complex, multi-stage, stratified, clustered samples of civilian, noninstitutionalized population and is designed and conducted for the purpose of making health-related prevalence estimates that are nationally generalizable. To make accurate population estimates, analysis of the NHANES requires the use of weight and design variables that account for this complex design. The use of sampling weights is necessary to account for differences in probability of selection for each participant and also accounts for noncoverage and nonresponse.[25] The NHANES III oversampled different groups, including older individuals, African Americans, and Mexican-Americans. The application of sampling weights allows us to conduct analyses on the individuals who were sampled in the NHANES and extrapolate those results to the population at large. According to the technical reports provided by the National Center for Health Statistics, without the use of sampling weights, misinterpretation of population estimates based on NHANES III is likely. This strategy of basing the analyses on population estimates is a characteristic that makes the NHANES different from many other cohort designs that do not use weighted population estimates, and provides national generalizability.

Of the 39,695 individuals eligible to participate, a total of 30,818 persons of all ages were examined in their homes or in mobile examination centers (MEC) which visited 89 communities across the United States (a participation rate of 77.6%). The health examination included collection of blood and urine specimens for the conduct of various laboratory analyses.

The NHANES III data merged with the National Death Index is a prospective cohort study that passively followed up on the participants in the NHANES III. The linked mortality file uses a probabilistic matching method.[26] The National Death Index involves searching national databases containing information about mortality and causes of death. Mortality status was ascertained by computerized matching to national databases and evaluation of the resulting matches. Persons not found to be deceased were assumed alive for analytic purposes.

The NHANES III is preexisting de-identified public use data which do not need specific approval from the National Center for Health Statistics.

We limited our study to individuals 40 years old and older at baseline, the time of their NHANES III interview. All analyses were based on the population estimates generated by applying variables accounting for the design and sampling methodology of the NHANES. The results presented here are generalizable to the noninstitutionalized civilian population of the United States aged 40 and older from 1998-1994.

Previously Diagnosed Diabetes

The NHANES III assessed participants for diagnosed diabetes using the questions, “Have you ever been told by a doctor that you have diabetes or sugar diabetes?”, “Were you pregnant when you were told that you had diabetes?” and “Other

than during pregnancy, has a doctor ever told you that you have diabetes or sugar diabetes?" We defined participants as having diagnosed diabetes if they answered "yes" to ever having been told they had diabetes, excluding pregnancy. Individuals with previously diagnosed diabetes were removed from the analysis. We also removed individuals with an HbA1c of 6.5% or greater, to account for undiagnosed diabetes.

Normoglycemia & Prediabetes

We defined normoglycemia as an Hba1c level between 4.0%--5.6% (20-38 mmol/mol). To control for any potential effect of low HbA1c, we also removed individuals with an HbA1c below 4.0% (20 mmol/mol), a level associated with increased all-cause mortality in adults without diabetes.[27]

We defined prediabetes among individuals without previously diagnosed diabetes using HbA1c ranges as specified by the American Diabetes Association, 5.7%–6.4% (39-46 mmol/mol).[1] This range has been shown in a meta-analysis to be predictive of progression to diabetes.[19] We excluded individuals with previously diagnosed diabetes because the current glycemic status of those patients may simply represent diabetes control. Prediabetes status was missing for 1,637 of the NHANES respondents over the age of 40.

Transferrin Saturation

Serum iron and total iron-binding capacity (TIBC) were measured in serum, and calculated by dividing serum iron by TIBC and multiplying by 100. For the analyses, elevated transferrin saturation was categorized as TS $\geq$50%. Individuals with TS below 25% were removed from the analysis, as low TS has been linked to increased risk of mortality.[23] Despite the lack of universal agreement on the upper and lower limits of

normal TS, these cut points has been used in several studies evaluating diabetes, TS and mortality.[23,28] Data were missing for transferrin saturation level for 536 of the NHANES respondents over the age of 40.

Serum Ferritin

Serum ferritin was used as a measure of body iron stores and was measured using the QuantImune Ferritin IRMA kit. Serum ferritin was categorized for the analyses as elevated if it was ≥674.1 pmol/l (300 ng/mL) for males and ≥449.4 pmol/l (200 ng/mL) for females.[29] Individuals with serum ferritin below 56.175 pmol/l (25 ng/mL) were removed from the analysis, as low ferritin has been linked to an increased risk of mortality.[23] Data were missing for serum ferritin level for 539 of the NHANES respondents over the age of 40.

Mortality

Mortality was measured as all-cause mortality. Mortality status was ascertained solely by computerized matching to national databases and evaluation of the resulting matches. All living survey participants examined in this study had been observed for 146 months, and our survival analysis was carried out to December 31, 2006.

Covariates

Covariates used in our analyses included: age at baseline in the NHANES III, gender, race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican-American, and other), health insurance status, obesity (Body Mass Index computed in the exam of ≥30), previous diagnosis of a heart attack, previous diagnosis of a stroke, previous diagnosis of hypertension, previous diagnosis of hypercholesterolemia, previous diagnosis of cancer, family history of diabetes, family history of myocardial infarction before age 50, and

current smoking status. Respondents were considered non-smokers if they reported smoking less than 100 cigarettes in their life or if they had smoked more than 100 cigarettes and were not currently smoking.

In the analysis of serum ferritin, we also controlled for C-reactive protein. Ferritin is an acute phase reactant as well as an indicator of iron stores and as such may indicate inflammation. Consequently, we controlled for inflammation by adjusting for C-reactive protein. C-reactive protein was considered elevated at levels above 3.0 mg/l.[30]

Analysis

In an effort to control for potential misclassification of persons who were very ill at baseline thereby affecting mortality risk of prediabetes, we left-censored the analysis to exclude any mortality events that occurred in the first three years following the individuals examination for the first three years of the cohort. Because of the complex sampling design of the survey, we performed statistical analyses using the statistical software package SUDAAN (Research Triangle Institute, Raleigh, NC), as recommended by the National Center for Health Statistics (NCHS). Using SAS (Cary, NC), we computed Kaplan-Meier plots to show graphically the unadjusted relationship between all-cause mortality and prediabetes.

We followed the NCHS recommendations for assessing the reliability of estimates in the context of a limited sample size. If the standard error of an estimate was greater than 30% of an estimate it would be considered unreliable All estimates met the criteria for reliability.

To accomplish our goals of examining a possible synergistic effect of having elevated iron with prediabetes we classified the population into 4 groups based upon

prediabetes or normoglycemia and normal or elevated TS. The population was also classified into 4 groups based upon prediabetes or normoglycemia and normal or elevated serum ferritin.

We performed Cox proportional hazards analyses to measure the associations between all-cause mortality and prediabetes controlling for all of the studied covariates using listwise deletion to account for missing data. In these models, survival time was a continuous variable measured in 1-month increments from the date of the exam.

We also performed adjusted Cox proportional hazards analysis with all-cause mortality for prediabetes in the 4 part variables with TS adjusting for the aforementioned covariates. For the adjusted Cox proportional hazards analysis with ferritin we adjusted for the aforementioned covariates and also C-reactive protein.

We evaluated the proportionality of the hazards through examination of the Schoenfeld residuals.

RESULTS

A total of 8,003 (unweighted) individuals were over 40 years old and had HbA1c levels between 4.0 and 6.4%, or 80,653,788 individuals nationally. Baseline characteristics for the sample are shown in Table 1. Table 1 indicates that 23.2% of the weighted sample had prediabetes, 15.6% of the sample exhibited elevated serum ferritin, and 3.3% had elevated TS.

Of the respondents that had prediabetes, 38.8% died within 12 years (723,702 died; 11,431,597 survived), compared to 23.4% of respondents with normal HbA1c levels (14,527,028 died; 47,458,061 survived). Among individuals with normal TS and normoglycemia, 23.1% died (10,724,279 died; 35,649,283 survived), compared to 23.7%

of those with elevated TS and normoglycemia (412,237 died; 1,327,253 survived), 37.5% of those with normal TS and prediabetes (5,137,131 died; 8,572,762 survived), and 44.7% of those with elevated TS and prediabetes (126,633 died; 156,790 survived). Among individuals with normal ferritin and normoglycemia, 24.3% died (10,967,486 died; 34,132,718 survived), compared with 38.8% of those with normal ferritin and prediabetes (5,465,483 died; 8,614,683 survived), 29.2% of those with elevated ferritin and normoglycemia (2,333,436 died; 5,662,576 survived), and 38.8% of those with elevated ferritin and prediabetes (1,150,647died; 1,818,565 survived).

Table 2 shows the results the adjusted Cox proportional hazards model for prediabetes. Table 2 indicates prediabetes alone has a small increased mortality risk. The Kaplan Meier curve of the survival and prediabetes over the length of the time under observation is shown in Fig 1.

Table 3 presents results of the analyses combining prediabetes with iron markers. In models that examined the impact of a prediabetes state combined with markers of low iron, the hazard ratios were similar to that of prediabetes alone. However, when combined with prediabetes, there was an increased mortality risk among individuals with TS $\geq$50, as well as with individuals who had increased ferritin. The risk was most increased when individuals had elevated ferritin and elevated transferrin saturation together. Figure 2 represents the relationship of survival of the four groups over the 12 years under observation. Individuals with prediabetes in the presence of elevated iron have lower survival probabilities than other groups. An examination of the Schoenfeld residuals suggested proportionality of hazards and appropriateness of the statistical model for these analyses.

DISCUSSION

The results of this study in a nationally representative cohort that followed individuals for 12 years confirm that the mortality risk of prediabetes is probably low. This is not unexpected based on the mixed results from previous studies, several of which found either no future mortality risk or risk that was not robust across measures. However, we found that the presence of transferrin saturation and serum ferritin is associated with increased mortality risk of individuals with prediabetes. Among individuals with normal iron levels, those with prediabetes had low mortality risk levels similar to the adjusted risk of prediabetes alone. On the other hand, in adjusted survival analyses, individuals with prediabetes who also had elevated transferrin saturation had substantially increased mortality risk. These findings extend previous work on iron markers and diabetes to the previously uninvestigated area of prediabetes.

These results suggest that additional stratification of individuals with prediabetes on the basis of iron markers would be useful to identify those with higher risk and who might benefit from iron lowering therapies. Previous data has indicated that elevated iron markers are associated with the development of diabetes and that among individuals with diabetes the co-occurrence of elevated TS increases those patients' mortality risk. Early identification of individuals with both conditions (prediabetes, elevated iron) may help in both slowing the development of diabetes as well as decreasing mortality risk. It is important for early identification of these individuals because much like individuals with prediabetes, the vast majority of individuals with elevated iron do not know it.[31] These individuals need to be identified to mitigate the increased risk posed by elevated iron in combination with prediabetes. Such individuals would be targets for intensive

interventions to reduce risk, including typical lifestyle interventions shown to help avoid the onset of diabetes in people at high risk.[32] Although more research is needed into the ability of interventions on iron in prediabetes to affect development of diabetes and mortality risk, some data suggest that reduction of TS improves HbA1c and glucose control.[33]

These associations of TS and ferritin with mortality in the context of prediabetes are not surprising especially if elevations of these parameters are interpreted in light of current understanding of iron toxicity.[34] Iron, whether absorbed as iron salts or in dietary heme is processed by enterocytes and released into the plasma where it is transported in a non-reactive state bound to transferrin. Iron that is bound to transferrin is in the Fe^{+3} state and is not reactive and, therefore, not toxic. However, when TS is above 40 to 50%, free iron or so-called non-transferrin-bound iron (NTBI) is released into the plasma as the buffering ability of transferrin is exceeded.[35] Labile plasma iron (LPI) is a highly reactive subspecies of NTBI that interacts with hydrogen peroxide through Fenton chemistry to form the extremely powerful oxidants, hydroxyl radical and singlet oxygen. These are the free radicals that ultimately directly damage protein and DNA. Perhaps more importantly, NTBI/LPI species are able to enter cells via ion channels. These channels, unlike the transferrin receptor, are not regulated so this reactive iron freely enters the cytoplasm of the pancreas, pituitary, and heart.

The current results suggest that exposure to excessive free iron is dangerous in the context of prediabetes. Furthermore, elevated ferritin and TS predict poor diabetes control and phlebotomy to reduce iron even over short periods of time improve HbA1c in parallel with changes in TS, even though ferritin is not changed,[33] putting further

emphasis on the fact that NTBI/LPI reflected by TS is the proximal cause of the toxicity. Several strategies are available to decrease iron, including chelation therapy and phlebotomy. Phlebotomy is an easy, inexpensive, and well-tolerated intervention. Reduction in TS by phlebotomy has been shown to improve measures of diabetic control.[33] Furthermore, correction of severe iron overload can significantly improve glucose tolerance.[36] Thus, the finding that a baseline measure of high TS as point measure of toxic free iron plus elevation of ferritin, evidence of elevated cytosolic iron over a longer period of time, predicts increased risk of mortality among individuals with prediabetes supports the premise that toxic free iron is a health risk.

The normoglycemic group with elevated iron markers did not show increased mortality risk in comparison to the reference group of normoglycemic and normal iron marker levels. This may seem inconsistent with other data on the increased mortality risk due to elevated TS by itself. However, there is the potential that the effect of TS on mortality is modified by the presence of other variables.[37,38] This effect has been shown in the past. Rather than being inconsistent with the TS alone and mortality findings, these new findings enhance our understanding of elevated iron markers and morbidity and mortality and allow us to consider the more complex, but real, situation of patients by considering multiple variables together rather than independently.

This study has several limitations. First, although we have a nationally representative, population-based cohort followed through the National Death Index, the biomarkers are measured only at baseline. There is the possibility that either the hyperglycemia or elevated iron measures were identified and interventions were implemented to lower these biomarkers. If that were the case and a substantial number of

individuals did drop their levels due to interventions thereby decreasing the potential mortality risk, the observed adjusted risk individuals elevated at baseline is even more concerning. Second, we were only able to follow these individuals for 12 years. It is possible that this time frame may have been too short to adequately see an effect for a biomarker like prediabetes. However, we did censor the first three years of mortality so that any deaths in that time frame would not be attributed to prediabetes. The model still found a substantial mortality risk for the prediabetes plus iron markers in this length of time. Third, we were unable to evaluate the relationship between being elevated on both transferrin saturation and serum ferritin with prediabetes on the risk of mortality. We attempted such an analysis but the number of individuals in the group with prediabetes and elevation on both iron markers was small and the population estimates were deemed unreliable.

In conclusion, this study representative of the population of the United States helps to clarify the current evidence on the mortality risk of prediabetes and provides further support for the role of elevated iron markers in health risk. Future screening and intervention programs for prediabetes may benefit from additional strategies to recognize and treat iron elevations, particularly transferrin saturation.

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CONTRIBUTION STATEMENT

AGM: conception, design, analysis, and drafting of manuscript

RJT: design, analysis, and drafting of manuscript

TDC: conception, design, and drafting of manuscript

RB: design and analysis

COMPETING INTERESTS

The authors report no competing interests.

REFERENCES

1. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2012;35:S64–S71.

2. Bullard KM, Saydah SH, Imperatore G, et al. Secular changes in U.S. Prediabetes prevalence defined by hemoglobin A1c and fasting plasma glucose: National Health and Nutrition Examination Surveys, 1999-2010. *Diabetes Care* 2013;36:2286–93.

3. Mainous AG 3rd, Tanner RJ, Baker R, et al. Prevalence of prediabetes in England from 2003 to 2011: population-based, cross-sectional study. *BMJ Open* 2014 Jun 9;4(6):e005002. doi: 10.1136/bmjopen-2014-005002. Accessed 29 June, 2014.

4. Centers for Disease Control and Prevention (CDC). Awareness of prediabetes--United States, 2005-2010. MMWR Morb Mortal Wkly Rep. 2013 Mar 22;62:209-12.

5. de Vegt F, Dekker JM, Ruhé HG, et al. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia* 1999;42:926–31.

6. Fuller JH, Shipley MJ, Rose G, et al. Coronary-heart-disease risk and impaired glucose tolerance. The Whitehall study. *Lancet* 1980;1:1373–76.

7. Valdés S, Botas P, Delgado E, Cadórniga FD. Mortality risk in Spanish adults with diagnosed diabetes, undiagnosed diabetes, or pre-diabetes. The asturias study 1998-2004. *Rev Esp Cardiol (Engl Ed)* 2009;62:528–34.

8. Deedwania P, Patel K, Fonarow GC, et al. Prediabetes is not an independent risk factor for incident heart failure, other cardiovascular events or mortality in older adults: findings from a population-based cohort study. *Int J Cardiol* 2013;168:3616–22.

9. Kowall B, Rathmann W, Heier M, et al. Categories of glucose tolerance and continuous glycemic measures and mortality. *Eur J Epidemiol* 2011;26:637–45.

10. Zhou XH, Qiao Q, Zethelius B, et al. Diabetes, prediabetes and cancer mortality. *Diabetologia* 2010;53:1867–76.

11. Barr EL, Zimmet PZ, Welborn TA, et al. Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance: the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). *Circulation* 2007;116:151–57.

12. Orban E, Schwab S, Thorand B, Huth C. Association of iron indices and type 2 diabetes: a meta-analysis of observational studies. *Diabetes Metab Res Rev* 2014;30:372–94.

13. Bao W, Rong Y, Rong S, Liu L. Dietary iron intake, body iron stores, and the risk of type 2 diabetes: a systematic review and meta-analysis. *BMC Med* 2012;10:119. doi: 10.1186/1741-7015-10-119. Accessed 8 July, 2014.

14. Ellervik C, Mandrup-Poulsen T, Tybjærg-Hansen A, Nordestgaard BG. Total and cause-specific mortality by elevated transferrin saturation and hemochromatosis genotype in individuals with diabetes: two general population studies. *Diabetes Care* 2014;37:444–52.

15. Masuda Y, Ichii H, Vaziri ND. At pharmacologically relevant concentrations intravenous iron preparations cause pancreatic beta cell death. Am J Transl Res. 2013;6:64-70.

16. Ellervik C, Tybjaerg-Hansen A, Nordestgaard BG. Total mortality by transferrin saturation levels: Two general population studies and a meta analysis. *Clin Chem* 2011;57:459–466.

17. Kim KS, Son HG, Hong NS, Lee DH. Associations of serum ferritin and transferrin % saturation with all-cause, cancer, and cardiovascular disease mortality: Third National Health and Nutrition Examination Survey follow-up study. *J Prev Med Public Health* 2012;45:196-203.

18. Mainous AG 3rd, Gill JM, Carek PJ. Elevated transferrin saturation and mortality. *Ann Fam Med* 2004;2:133–138.

19. Mainous AG 3rd, Wells B, Carek PJ, et al. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. *Ann Fam Med* 2004;2:139-44.

20. Stack AG, Mutwali AI, Nguyen HT, et al. Transferrin saturation ratio and risk of total and cardiovascular mortality in the general population. *QJM.* 2014;107:623-33.

21. Wells BJ, Mainous AG 3rd, King DE, et al. The combined effect of transferrin saturation and low density lipoprotein on mortality. *Fam Med* 2004;36:324-9.

22. Ellervik C, Marott JL, Tybjærg-Hansen A, Schnohr P, Nordestgaard BG. Total and Cause-Specific Mortality by Moderately and Markedly Increased Ferritin Concentrations: General Population Study and Metaanalysis. Clin Chem. 2014 Aug 25. pii: clinchem.2014.229013.

23. Ponikowska B, Suchocki T, Paleczny B, et al. Iron status and survival in diabetic patients with coronary artery disease. *Diabetes Care* 2013;36:4147–56.

24. Morris DH, Khunti K, Achana F, et al. Progression rates from HbA(1c) 6.0-6.4% and other prediabetes definitions to type 2 diabetes: a meta-analysis. *Diabetologia* 2013;56:1489–93.

25. National Center for Health Statistics. Plan and operation of the Third National Health and Nutrition Examination Survey, 1988-1994. Vital Health Stat. 1994:1(32). http://www.cdc.gov/nchs/data/series/sr_01/sr01_032.pdf. Accessed 15 October 2014.

26. National Center for Health Statistics. Office of Analysis and Epidemiology, NCHS 2011 Linked Mortality Files Matching Methodology, September, 2013. Hyattsville, Maryland. http://www.cdc.gov/nchs/data/datalinkage/2011_linked_mortality_file_matching_methodology.pdf. Accessed 20 August, 2014.

27. Carson AP1, Fox CS, McGuire DK, Levitan EB, Laclaustra M, Mann DM, Muntner P. Low hemoglobin A1c and risk of all-cause mortality among US adults without diabetes. Circ Cardiovasc Qual Outcomes. 2010 Nov;3(6):661-7.

28. Ellervik C, Andersen HU, Tybjærg-Hansen A, et al. Total mortality by elevated transferrin saturation in patients with diabetes. *Diabetes Care* 2013;36:2646–54.

29. Adams PC, Reboussin DM, Barton JC, et al. Hemochromatosis and Iron Overload Screening (HEIRS) Study Research Investigators. Hemochromatosis and iron-overload screening in a racially diverse population. N Engl J Med. 2005;352:1769-78.

30. Pearson TA, Mensah GA, Alexander RW, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice. A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 2003;107(3):499 – 511.

31. Mainous AG 3rd, Wright RU, Hulihan MM, et al. Elevated transferrin saturation, health-related quality of life and telomere length. *Biometals* 2014;27:135–41

32. Portero McLellan KC, Wyne K, Villagomez ET, Hsueh WA. Therapeutic interventions to reduce the risk of progression from prediabetes to type 2 diabetes mellitus. *Ther Clin Risk Manag* 2014;10:173–88.

33. Fernández-Real JM, Peñarroja G, Castro A, García-Bragado F, Hernández-Aguado I, Ricart W. Blood letting in high-ferritin type 2 diabetes: effects on insulin sensitivity and beta-cell function. *Diabetes* 2002;51:1000–4.

34. Hansen JB, Moen IW, Mandrup-Poulsen T. Iron: the hard player in diabetes pathophysiology. *Acta Physiol* 2014;210:717–32.

35. Coates TD. Physiology and pathophysiology of iron in hemoglobin-associated diseases. *Free Radic Biol Med* 2014;72C:23–40.

36. Farmaki K, Tzoumari I, Pappa C, et al. Normalisation of total body iron load with very intensive combined chelation reverses cardiac and endocrine complications of thalassaemia major. *Br J Haematol* 2010;148:466–75.

37. Mainous AG 3rd, Wells B, Carek PJ, Gill JM, Geesey ME. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. Ann Fam Med. 2004;2:139-44.

38. Wells BJ, Mainous AG 3rd, King DE, Gill JM, Carek PJ, Geesey ME. The combined effect of transferrin saturation and low density lipoprotein on mortality. Fam Med. 2004;36(5):324-9.

Table 1: Baseline Characteristics of the Sample

	Study Sample (%)
Unweighted Sample Size	8,003
Population Estimate	80,653,788
Age, years	
40-54	49.6
55-69	31.2
70+	19.2
Sex, male	46.1
Race/Ethnicity	
Non-Hispanic White	82.2
Non-Hispanic Black	8.3
Mexican-American	3.2
Other	6.4
Has health insurance	93.6
Obese	23.3
Current Smoker	23.6
Has diagnosed high cholesterol	40.1
Has diagnosed hypertension	31.3
Ever diagnosed with heart attack	5.3
Ever diagnosed with stroke	2.8
Ever diagnosed with cancer	12.3
Relative with diabetes	41.2
Relative with heart attack before age 50	15.4
Elevated C-reactive protein	1.2
Elevated Transferrin Saturation	3.3
Elevated Ferritin	15.6
Prediabetes	23.2
Assumed deceased	27.0

Table 2: Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes

	Hazard Ratio	95% Confidence Interval
Adjusted Model[a]		
Prediabetes	1.04	1.00-1.08
Normoglycemia	1.0	--

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

Table 3: Adjusted Hazard Ratios from Cox Regression for Mortality Risk of Individuals with Prediabetes and Iron Markers

	Hazard Ratio	95% Confidence Interval
Adjusted Model[a]		
Normal Transferrin Saturation/ Normoglycemia	1.00	--
Normal Transferrin Saturation/ Prediabetes	1.13	1.08-1.19
Elevated Transferrin Saturation/ Normoglycemia	.98	.90-1.07
Elevated Transferrin Saturation/ Prediabetes	1.88	1.06-3.30
Adjusted Model[b]		
Normal Ferritin/ Normoglycemia	1.00	--
Normal Ferritin/ Prediabetes	1.15	1.09-1.20
Elevated Ferritin/ Normoglycemia	1.05	1.00-1.12
Elevated Ferritin/ Prediabetes	1.14	1.04-1.24

[a]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, and family history of early heart attack.

[b]Adjusted for: age, gender, race/ethnicity, health insurance status, obesity, current smoking status, diagnosed heart attack, diagnosed stroke, diagnosed hypertension, diagnosed high cholesterol, diagnosis of cancer, family history of diabetes, family history of early heart attack, and elevated C-reactive protein.

Figure 1. Kaplan Meier curve of survival among individuals with prediabetes or normal glycemic levels.

Normoglycemia

Prediabetes

Figure 2. Kaplan Meier curve of survival among individuals with prediabetes and elevated transferrin saturation

Normoglycemia and Normal Transferrin Saturation

Normoglycemia and Elevated Transferrin Saturation

Prediabetes and Normal Transferrin Saturation

Prediabetes and Elevated Transferrin Saturation

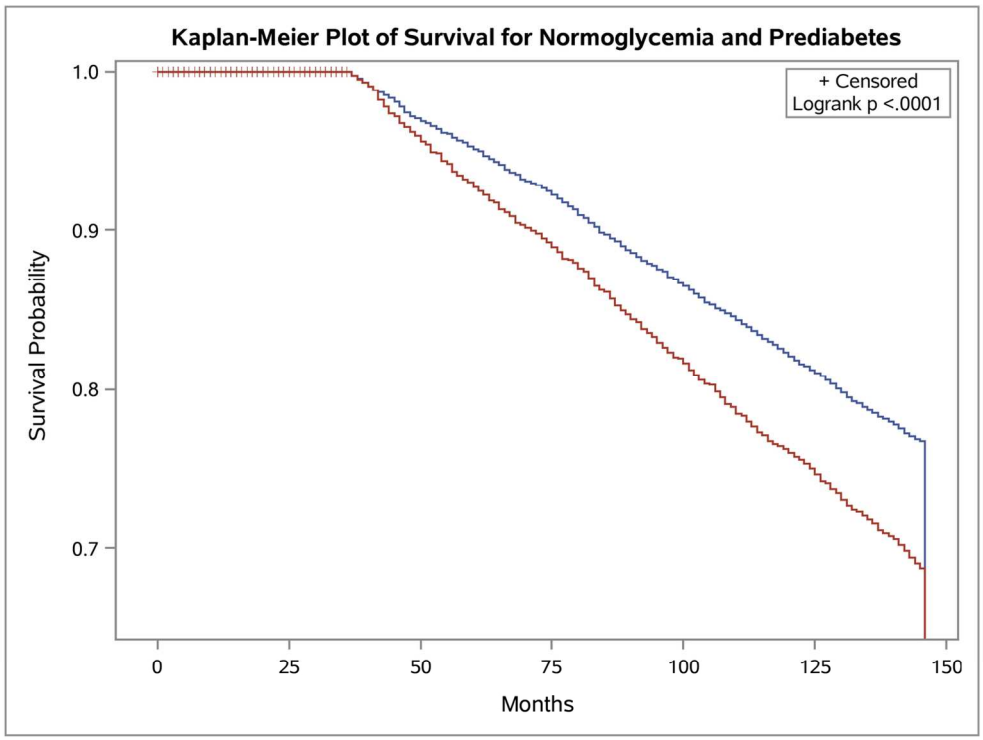

Figure 1¬¬¬. Kaplan Meier curve of survival among individuals with prediabetes or normal glycemic levels.

blue line = Normoglycemia
red line = Prediabetes
131x100mm (300 x 300 DPI)

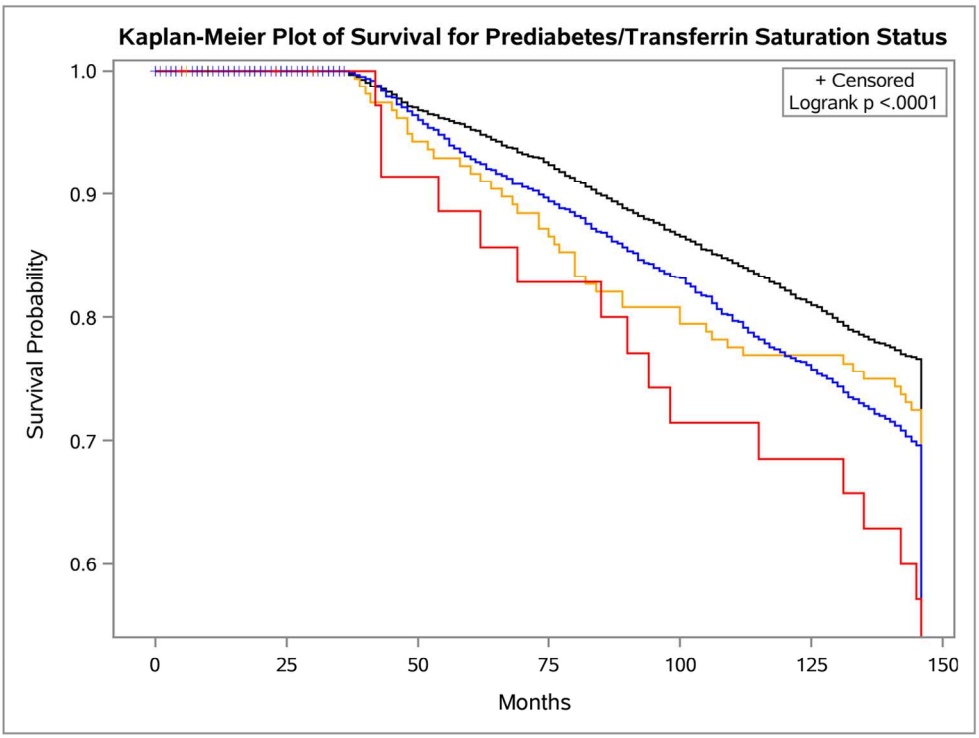

Figure 2. Kaplan Meier curve of survival among individuals with prediabetes and elevated transferrin saturation

black line = Normoglycemia and Normal Transferrin Saturation
yellow line = Normoglycemia and Elevated Transferrin Saturation
blue line = Prediabetes and Normal Transferrin Saturation
red line = Prediabetes and Elevated Transferrin Saturation
131x99mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page number
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4-5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-8
Bias	9	Describe any efforts to address potential sources of bias	9
Study size	10	Explain how the study size was arrived at	5-8
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8-9
		(*b*) Describe any methods used to examine subgroups and interactions	8
		(*c*) Explain how missing data were addressed	8
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	NA
		(*e*) Describe any sensitivity analyses	NA

Continued on next page

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5, 9
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9, 19
		(b) Indicate number of participants with missing data for each variable of interest	6-7
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	5
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	19
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-10 20-22
		(*b*) Report category boundaries when continuous variables were categorized	NA
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	10
Discussion			
Key results	18	Summarise key results with reference to study objectives	10-11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12-13
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12
Generalisability	21	Discuss the generalisability (external validity) of the study results	13
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.