Pancreatic iron and glucose dysregulation in thalassemia major

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Pancreatic iron overload and diabetes mellitus (DM) are common in thalassemia major patients. However, the relationship between iron stores and glucose disturbances is not well defined. We used a frequently sampled oral glucose tolerance test (OGTT), coupled with mathematical modeling, and magnetic resonance imaging (MRI) to examine the impact of pancreatic, cardiac, and hepatic iron overload on glucose regulation in 59 patients with thalassemia major. According to OGTT results, 11 patients had DM, 12 had impaired glucose tolerance (IGT), 8 had isolated impaired fasting glucose (IFG), and 28 patients had normal glucose tolerance (NGT). Patients with DM had significantly impaired insulin sensitivity and insulin release. Insulin resistance was most strongly associated with markers of inflammation and somatic iron overload, while disposition index (DI) (a measure of beta cell function) was most strongly correlated with pancreas R2*. Patients with DM and IGT had significantly worse DI than those with NGT or IFG, suggesting significant beta cell toxicity. One-third of patients having elevated pancreas R2* had normal glucose regulation (preclinical iron burden), but these patients were younger and had lower hepatic iron burdens. Our study indicates that pancreatic iron is the strongest predictor of beta cell toxicity, but total body iron burden, age, and body habitus also influence glucose regulation. We also demonstrate that MRI and fasting glucose/insulin are complementary screening tools, reducing the need for oral glucose tolerance testing, and identify high-risk patients before irreversible pancreatic damage. Am. J. Hematol. 87:155–160, 2012. © 2011 Wiley Periodicals, Inc.

Introduction

Iron overload remains a critical problem for thalassemia major patients, even in countries where chelation is widely available. Greater chelation options and magnetic resonance imaging (MRI) surveillance of cardiac iron have improved survival [1,2]; however, many patients still carry dangerous extrahepatic iron burdens. As patients live longer, cumulative iron-mediated toxicity compounded by natural aging makes diabetes a significant clinical problem. Pancreatic iron loading in thalassemia major patients begins in early childhood, and the overall prevalence of diabetes mellitus (DM) ranges from 6.4% to 14.1% on cross-sectional studies [3,4]. Both insulin resistance and decreased insulin secretion contribute to diabetes in thalassemia major patients. This has been shown by homeostatic model assessment [5], oral glucose tolerance test (OGTT) [6–10], euglycemic insulin clamp [11,12], and intravenous glucose tolerance test [13,14] studies. Diabetes prevalence in thalassemia has been shown to correlate with serum ferritin [9,15], with hepatitis C infection [15–18] and with pancreatic and cardiac iron [19–21].

MRI has become the gold standard for monitoring somatic iron stores. Liver iron concentration (LIC) is a good measure of total body iron, but disparate mechanisms of iron loading causes different kinetics of iron uptake and removal between the liver and the other organs [22–24]. Cardiac iron precedes overt cardiac dysfunction, providing a window for proactive therapy intensification. With routine cardiac iron assessment, congestive heart failure is now quite rare.

Unlike the heart, the link between MRI-detectable iron and pancreatic beta-cell dysfunction is not well characterized. Thus, our primary goal was to determine the functional significance of iron stores in thalassemia major by stratifying the risk of glucose dysregulation according to pancreas, liver, and heart iron burdens.

Materials and Methods

All patients with thalassemia receiving chronic transfusion therapy (>8 transfusions per year) for more than 7 years were invited to participate, regardless of their endocrine or cardiac history. We recruited 59 patients with thalassemia major between the ages of 10 and 49 to participate in a frequently sampled OGTT. This study was approved by the Children's Hospital Los Angeles (CHLA) Committee on Clinical Investigations (#CCI-07-01363). Informed consent was obtained by all subjects. MRI was used to assess patient liver, heart, and pancreas iron burdens. Liver R2 and R2*, heart R2*, and pancreas R2* were collected and analyzed as described previously [25–27]. A total of 43/59 patients had clinically indicated annual iron assessments, which did not coincide with the OGTT exam; linear interpolation was used to approximate organ iron burden at the time of study. Sixteen patients, who were not regularly seen at CHLA, had a single MRI on the same day as the study. All OGTT and MRI exams were performed at CHLA.

All patients were required to fast overnight (at least 12 hr). Paired baseline blood assessments (at −15 min and 0 min) of glucose, insulin, and C-peptide were performed to ensure stability of the baseline glycemic state. Proinsulin, vitamin D 25 OH, vitamin D 1-25, fructosamine, intact parathyroid hormone, and zinc were also collected at the −15 min time point. Following the baseline blood draws, patients were given 1.75 g/kg (maximum dose of 75 g) of glucose solution (Glucose Drink, Azer Scientific, Morgantown, PA). Glucose, insulin, and C-peptide levels were measured at 10, 20, 30, 45, 60, 90, 120, 150,

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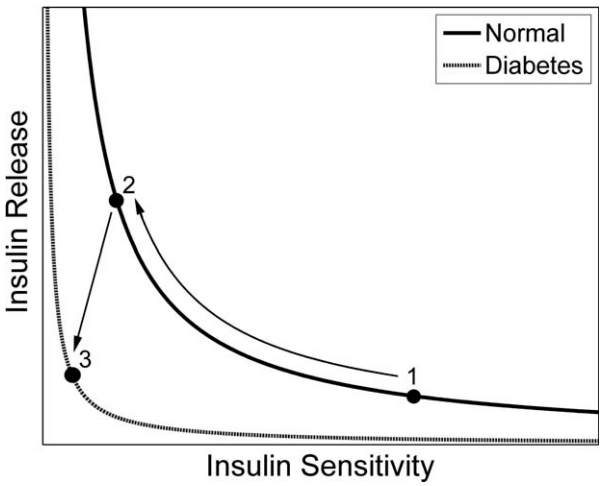

Figure 1. Schematic of DI. Insulin release is inversely proportional to insulin sensitivity, represented by the reciprocal curve. DI (double headed arrows) represents the shortest distance from the origin to the reciprocal curve. The solid line indicates insulin release/sensitivity trade-offs in a normal individual (large DI). Worsening DI is represented graphically by the curve being shifted closer to the origin (dashed line). The points on the graph represent three theoretical stages in the progression from normal to diabetes: (1) good insulin sensitivity and appropriate insulin release; (2) impaired insulin sensitivity, but still appropriate insulin release; and (3) impaired insulin release and diabetes; beta cells can no longer compensate for impaired insulin sensitivity.

and 180 min postglucose load. Point-of-care glucose (OneTouch Ultra Blood Glucose Meter, LifeScan, Milpitas, CA) was examined at 3 hr. If this value exceeded 126 mg/dL, a 4-hr sample was also collected to better document blood glucose recovery. Patients were classified as having either normal glucose tolerance (NGT), impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or DM, according to American Diabetes Association criteria [28]. A fasting glucose of less than 100 mg/dL and 2-hr glucose of less than 140 mg/dL was considered NGT. An abnormal fasting glucose and a 2-hr glucose $<$ 140 mg/dL represented isolated IFG; 2-hr glucose between 140 mg/dL and 200 mg/dL was considered IGT and a fasting glucose $>$ 126 mg/dL or 2-hr glucose greater than 200 mg/dL represented DM.

From the results of the OGTT, we estimated each patient's insulin release using first phase insulin release estimates validated by Stumvoll et al. [29]. Insulin sensitivity was determined using a minimal model approach adapted and validated for frequently sampled OGTT by Caumo et al. [30]. Insulin release is inversely proportional to insulin sensitivity as shown in Fig. 1. The shortest distance from the origin this curve is known as the Disposition Index (DI) and is marker of intrinsic beta cell function. DI is calculated as the product of insulin release and insulin sensitivity; worsening DI is represented graphically by curve being shifted closer to the origin [31]. Patients who lose insulin sensitivity, during pregnancy or obesity, compensate by secreting more insulin in response to a given glucose stimulus (Point 1 to Point 2) as long as their beta cells remain healthy. As beta cells become damaged or destroyed, DI declines (Point 2 to Point 3) and less insulin is secreted for any given insulin sensitivity.

Analysis of variance (ANOVA) was used to compare insulin release, insulin sensitivity, and DI across groups. The relationship between these variables and iron stores was examined using univariate and multivariate linear regression. Risk of abnormal OGTT was stratified using the presence or absence of cardiac and pancreatic iron and evaluated using chi-squared analysis. As LIC fluctuates much more rapidly than either pancreatic or heart R2*, we also included the product of age and LIC as a potential discriminating factor.

Five subjects had a history of diabetes (7.8 ± 4.0, range 1.4–15 years duration) before participation; two were controlled with oral hypoglycemic agents and three with insulin. Administration of oral hypoglycemic agents was delayed until after OGTT completion. For the three patients on bolus insulin, normal morning lente and NPH insulin were administered on the morning of OGTT but regular insulin was held. All patients were allowed to consume the glucose load only if his or her point-of-care glucose was $<$ 150 at the test initiation; one patient failed. Following the OGTT, the study endocrinologist (SM) recommended glucose based on point of care testing glucose values. Two additional patients had incomplete OGTT exams because of disrupted venous access and insulin sensitivity could not be accurately determined. All statistics were evaluated with and without results from the three insulin-dependent diabetic subjects; no appreciable differences were observed, and these patients were included in the final data.

Results

Patients ranged from 10 to 49 years of age (23.3 ± 9.8 years) and were well balanced by sex (29 female, 30 male). The patient cohort had a body mass index (BMI) of 21.3 ± 3.3 kg/m^2; six patients were overweight by World Health Organization criteria (BMI 25–30 kg/m^2) but none were obese (BMI $>$ 30 kg/m^2). All subjects received blood transfusions every 2–4 weeks and were on appropriate chelation therapy as indicated by their physician. A total of 81% of patients were on deferasirox (DFX) monotherapy; other patients were on either deferoxamine (DFO), deferiprone (DFP), or on combination therapy with DFX + DFO, DFX + DFP, or DFO + DFP. This cohort had started transfusions at an age of 2.7 ± 4.4 years and was moderately iron overloaded with a ferritin of 2697 ± 3017 ng/mL (median 1,480 ng/mL) and LIC of 13.5 ± 16.5 mg/g (median 5.6 mg/g). Cardiac R2* was 73.4 ± 70.8 Hz (median 35.9 Hz), and 21 patients had detectable cardiac iron (R2* $>$ 50 Hz). Pancreas R2* was 260 ± 273 Hz (median 169 Hz), and 39 patients had pancreas R2* values $>$ 100 Hz (which has been previously associated with increased risk of cardiac iron) [27].

More than half of the subjects had some degree of glucose dysregulation; 11 patients had DM, 12 had IGT, and 8 had isolated IFG, compared with 28 patients having NGT. Defects in both insulin release and insulin sensitivity were present. Figure 2 (left) demonstrates that insulin release was severely impaired in DM patients ($P = 0.004$) but normal for all other groups. Insulin sensitivity (Fig. 2, right) was also decreased in DM patients ($P = 0.01$) and trended low ($P = 0.09$) in IGT patients, while remaining completely normal in IFG patients. OGTT result and insulin sensitivity were independent of age; however, insulin release and DI declined weakly with age ($r^2 = 0.12$ and 0.09, respectively, $P < 0.03$).

Figure 3 (left) demonstrates the reciprocal relationship between insulin release and insulin sensitivity by OGTT result; the curve fits are given by the equation: Insulin Release = DI/Insulin Sensitivity. Using the given equation, DI can also be calculated in individual patients using the product of insulin release and insulin sensitivity. Figure 3 (right) demonstrates that the individual DI values varied with respect to disease severity (ANOVA $P < 0.001$). Mean DI values declined proportionally to the severity of glucose dysregulation, reaching statistical significance in patients with DM and IGT ($P < 0.0001$ and $P = 0.005$, respectively, using Dunnett's post hoc correction).

DI was negatively correlated with all metrics of iron burden including pancreatic R2*, cardiac R2*, LIC, and ferritin ($r^2 = 0.28$, 0.23, 0.19, and 0.20, respectively, $P < 0.001$). DI was also negatively correlated with age, $r^2 = 0.09$, $P = 0.03$. The relationship between pancreas R2* and DI is shown in Fig. 4; DI axis was log transformed. Vertical line indicates a pancreas R2* value of 100 Hz [27]; glucose dysregulation was present in 28/39 patients having pancreas R2* $>$ 100 Hz, compared with only 3/20 having pancreas R2* $<$ 100 Hz ($P < 0.0001$ by Fischer Exact test). Receiver operator characteristic (ROC) analysis confirmed that pancreas R2* of 100 Hz, in addition to being predictive of significant cardiac iron, was the optimal cutoff for predicting an abnormal OGTT, with an area under the curve (AUC) of 0.77 (not shown).

We used multivariate regression to better identify potential predictors of the components of pancreas dysfunction:

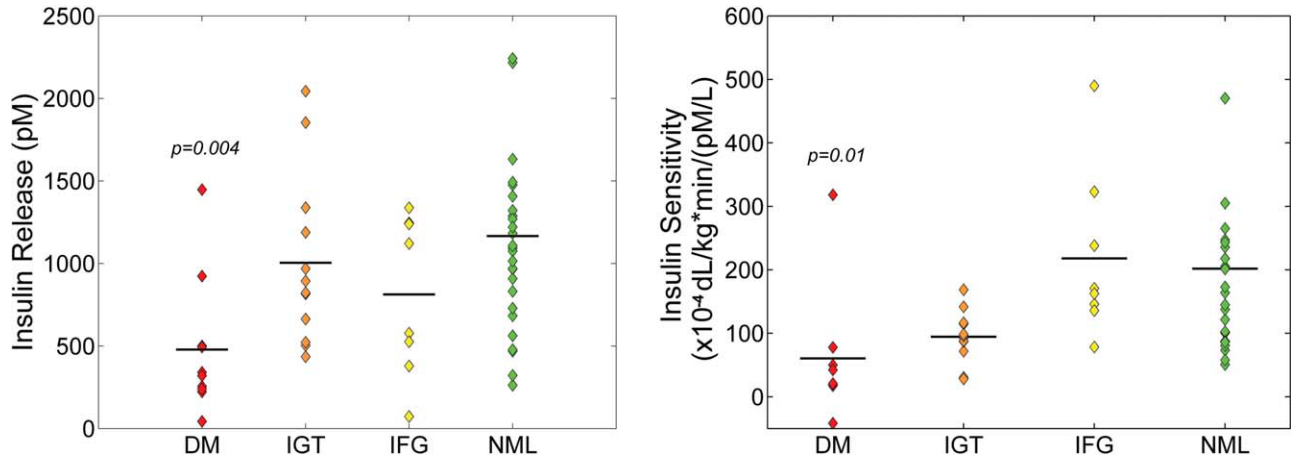

Figure 2. Insulin release and insulin sensitivity by diagnosis. Distributions for insulin release (left) and insulin sensitivity (right) are shown for the four classification groups (DM in red, IGT in orange, IFG in yellow, and normal glucose tolerance (NGT) in green). The *P*-value for significant difference with NGT is indicated above the respective group.

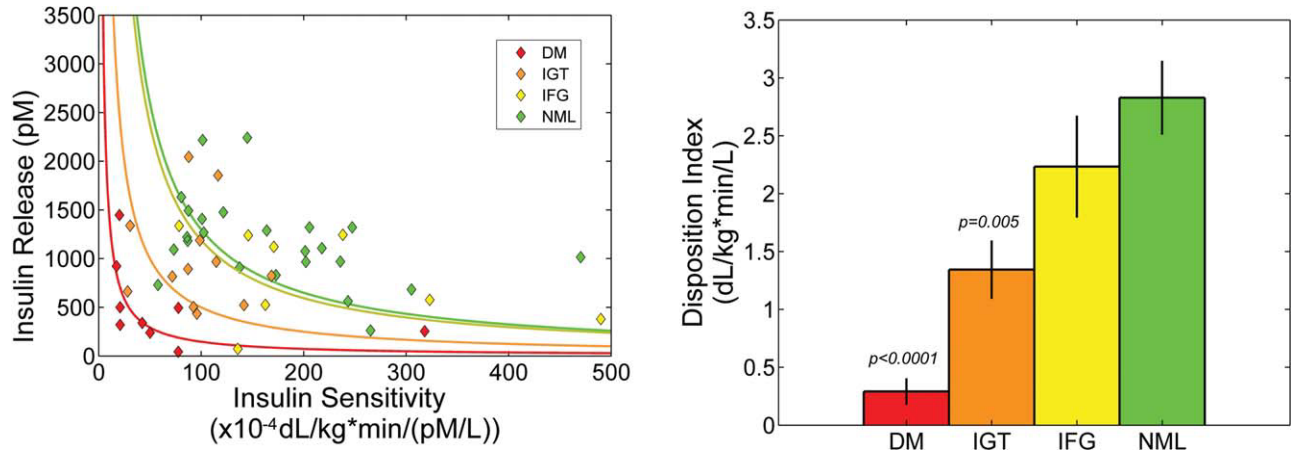

Figure 3. DI for the population. Insulin sensitivity versus insulin release is plotted for each patient, separated by classification group (DM in red, IGT in orange, IFG in yellow, and normal glucose tolerance (NGT) in green). Reciprocal relationships are shown in corresponding colors, representing isoclines of constant DI (left). The right shows a distribution of DI for the individuals, the *P*-value for significant difference with NGT is indicated above the respective group.

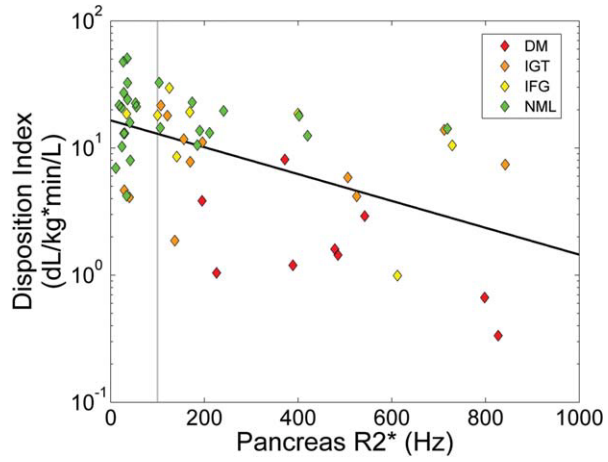

Figure 4. DI versus pancreas R2*. Pancreas R2* is shown on the *x*-axis, DI is shown on the log transformed *y*-axis. Patients with DM are shown in red, IGT in orange, IFG in yellow, and normal glucose tolerance (NGT) in green. A cutoff at pancreatic R2* of 100 Hz is shown to indicate significant pancreatic iron overload. DI and pancreas R2* had a significant negative correlation.

insulin sensitivity, insulin release, and DI. Independent variables included age, ferritin, BMI, LIC, pancreas R2*, and cardiac R2*. Ferritin, BMI, and LIC were the strongest predictors of insulin sensitivity (r^2 of 0.17, 0.08, and 0.04, respectively), consistent with oxidative stress from inflammation, metabolic factors, and iron overload. Age and LIC were the strongest predictors of impaired insulin release (r^2 of 0.14 and 0.05, respectively), suggesting that the chronicity of iron overload is crucial. Pancreas R2*, BMI, and ferritin emerged as the strongest contributors of DI ($r^2 = 0.18$, 0.07, and 0.03, respectively).

The relationship between cardiac and pancreatic R2* provides further insight into MRIs ability to predict risk for pancreas dysfunction. Figure 5 (left) shows cardiac R2* versus pancreas R2* for the population; patients are organized using color-coding for glucose handling classification. A cardiac R2* $>$ 50 Hz and a pancreas R2* $>$ 100 Hz were used to represent significant cardiac and pancreatic iron [27]. The presence of both pancreas and cardiac iron was highly specific for glucose abnormalities, with 17/21 patients having abnormal glucose regulation, including 10 of the 11 patients with DM. Of the four false positives, two had severe liver iron overload (36.1 and 42.7 mg/g dry weight) but were young (ages 10 and 15.8 years), suggesting inadequate time for toxic manifestations.

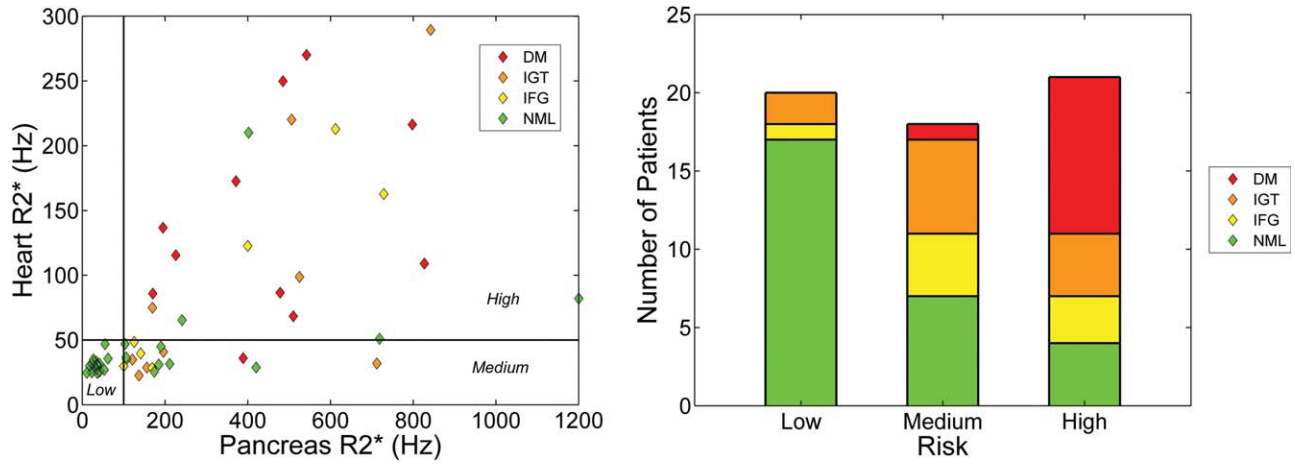

Figure 5. Risk of pancreas dysfunction based on pancreatic and cardiac iron. The left shows the relationship between pancreas R2* and cardiac R2* for the population. Patients with DM are shown in red, IGT in orange, IFG in yellow, and normal glucose tolerance (NGT) in green. Cutoffs at pancreatic R2* of 100 Hz and cardiac R2* of 50 Hz are shown to indicate significant iron overload. Pancreatic R2* and cardiac R2* had a significant correlation (not shown). Low, medium, and high risk grading is indicated in the corresponding quadrant on the graph. Shown on the right is the distribution of patients among the different risk groups.

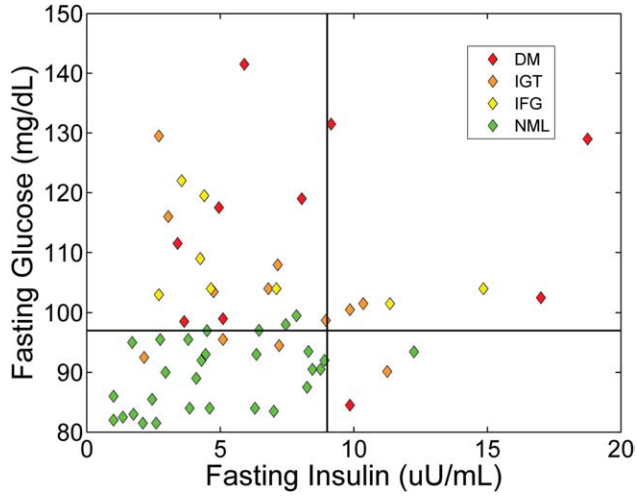

Figure 6. Fasting insulin and glucose. Fasting insulin is on the *x*-axis, fasting glucose on the *y*-axis. Patients with DM are shown in red, IGT in orange, IFG in yellow, and normal glucose tolerance (NGT) in green. Cutoffs for normal values for the population are shown (fasting insulin of 9 uU/mL and fasting glucose of 97 mg/dL).

Combining the predictive power of pancreatic and cardiac R2* yields an intuitive grading scheme (Fig. 5, right). Patients with isolated pancreas iron deposition (pancreas R2* $>$ 100 Hz and cardiac R2* $<$ 50 Hz) were at intermediate risk, with 11 out of 18 having glucose dysregulation. The subset ($\sim$ one-third) of patients who had pancreas R2* $>$ 100 Hz and NGT were generally either young, with severe liver siderosis, or older with excellent control of somatic iron stores. Their product of LIC and age was 191 ± 195 mg/g year versus 432 ± 501 mg/g year for those with elevated pancreas iron and abnormal function ($P = 0.04$); this suggests that intensity of hepatic iron loading combined with the duration of exposure increases risk for glucose disturbance independently of pancreatic iron burden. Three out of 20 patients in the low risk group had IGT or IFG, reinforcing that many factors interfere with glucose regulation.

We also evaluated the diagnostic sensitivity of fasting glucose and insulin (Fig. 6). Typically, a fasting insulin cutoff of 17 uU/mL is considered normal; however, we determined fasting insulin of 9 uU/mL to be a better cutoff for our population (based on ROC analysis). This likely reflects the lower BMI in our patients compared with population norms. Nine out of 10 patients with fasting insulin $>$ 9 uU/mL had glucose dysregulation. Similarly, fasting glucose $<$ 97 mg/dL was the optimal cutoff (instead of 100 mg/dL). Combining these cutoffs yielded a sensitivity of 89% and specificity of 90% for glucose dysregulation by OGTT. Two of the three patients missed by a pancreas R2* cutoff of 100 Hz would have been identified by fasting glucose levels $>$ 97 mg/dL, and 6/7 patients missed by fasting glucose alone had a pancreas R2* $>$ 100 Hz. This suggests that combined screening was more effective than either one alone.

Other serum tests collected were relatively uninformative. Proinsulin/insulin ratio trended lower in diabetic patients ($P = 0.06$). Fructosamine levels were increased in 3/11 DM and 1/11 IGT but none of the other subjects. No association was observed between glucose dysregulation and vitamin D 25 OH, vitamin D 1-25, intact parathyroid hormone or plasma zinc levels.

Discussion

The primary purpose of this study was to explore the relationship between pancreas iron and pancreas function in thalassemia major to identify predictors of preclinical glucose dysregulation. We demonstrate that pancreas R2* is the strongest overall predictor of glucose dysregulation. Cardiac R2* is more specific for glucose dysregulation, because it implies increased pancreatic iron for a long duration [27], but cardiac R2* is insensitive to milder glucose dysregulation. Furthermore, as chelation therapy is often intensified when cardiac iron is present, this observation is unlikely to change patient management except to heighten surveillance for glucose dysregulation. Pancreas R2* is abnormal much earlier in the disease spectrum; $\sim$ one-third of patients with pancreas iron have normal glucose handing. However, rather than being a limitation, this represents a strength, because it means that clinicians may modify therapy before the advent of clinical disease. It is entirely analogous to the normal left ventricular ejection fraction observed in many patients having T2* $<$ 10 ms [32]; most clinicians would intensify chelation for a cardiac T2* of 6 ms even if the ejection fraction was completely normal.

So should chelation therapy be escalated based on increased pancreas R2 for primary prevention of glucose dysregulation, in the absence of detectable heart iron? Some studies document improvement of glucose handling following

iron removal therapies in thalassemia, hemochromatosis, and metabolic syndrome patients [33–35]. However, we argue that response to the MRI findings is important, rather than waiting for OGTT abnormalities, because IGT is associated with 50% loss of beta cell function (Fig. 3). Unlike cardiac dysfunction, beta cell damage is not 100% reversible. Even if insulin sensitivity and euglycemia are achieved by intensive chelation therapy [36], response is often incomplete and patients remain vulnerable to diabetes as they age.

OGTT has been used in several thalassemia major studies [6–10], but our finer sampling interval and longer study duration allowed us to use minimal model estimates of insulin sensitivity and DI, which accurately stratified OGTT outcome (Fig. 3). As pancreatic function reflects a complicated interplay between insulin sensitivity and insulin release, these methodologies also allowed us to better probe the mechanisms of iron-mediated glucose dysregulation.

Glucose dysregulation was surprisingly common in our study population. Five out of 59 patients were known to have diabetes (8.5%), consistent with published figures. However, OGTT identified 23 patients (39%) with either DM or IGT, suggesting that disease prevalence may be underappreciated by patients and their practitioners. Although aging and obesity are strong predictors of diabetes in the normal population, our thalassemia patients were a relatively young and lean cohort. In fact, the patients with impaired function (DM, IGT, or IFG) were statistically identical in age and BMI to those with normal pancreas function. Nonetheless, age and BMI still contributed to glucose dysregulation on multivariate analysis.

Impaired insulin release and insulin sensitivity both contribute to diabetes in thalassemia major, similar to Type II diabetes in the normal population [31]. Our results are also consistent with a large body of prior work in thalassemia [5–14]. The delicate balance between insulin sensitivity and insulin release is represented by DI, an important marker of intrinsic beta cell functional capacity and diabetes reversibility [31,37]. DI is modulated by a number of genetic and environmental factors [38]. Low DI implies intrinsic beta cell dysfunction or death and may be associated with irreversible damage. Iron chelation therapy improves insulin sensitivity and glycemic control [36], but its ability to stabilize or increase DI is unknown. DI was most strongly associated with pancreas R2*, BMI, and ferritin level. Chronic oxidative stress is toxic to pancreatic beta cells [39] whether it results from iron, free fatty acids, hyperglycemia, or inflammation, and represents the most logical link between the MRI and OGTT findings.

Insulin resistance was most strongly influenced by surrogates of liver iron overload, inflammation, and body habitus, rather than pancreatic or cardiac iron burden. This suggests that hepatic toxicity may be a key mediator of insulin resistance in thalassemia, rather than skeletal muscle glucose handling but more sophisticated analyses such as glucose tracer studies are needed to specifically address this question. Nine of our patients had prior hepatitis C infection, which is a known risk factor for diabetes in thalassemia major [15–18], but only one had detectable viral mRNA.

MRI screening of heart and liver iron is rapidly becoming the standard of care in major thalassemia centers. Pancreas R2* can be measured during the same imaging session for no significant increase in exam time or cost. While pancreas R2* can be difficult to measure in patients having fatty gland infiltration and has greater variability than liver or cardiac R2* [27], it trends cardiac risk better than hepatic iron measurements and is a risk surrogate for other endocrine glands as well.

Published thalassemia guidelines recommend annual OGTT as the standard of care [40,41]. However, it is unpopular with patients and compliance with this screening regimen in our cohort was extremely poor (1/59 patients). We found that fasting glucose $>$ 97 mg/dL and insulin $>$ 9 uU/mL accurately identified an abnormal OGTT result (89% sensitivity, 90% specificity); lower cutoffs than population norms reflect the need for increased sensitivity in screening tests. Patients with glucose or insulin values outside these ranges should be sent for confirmatory OGTT. Insulin cutoffs may vary across institutions and assays, but fasting glucose levels were diagnostically more powerful, had lower measurement variability [42], and were more consistent with established cutoffs [28]. While fasting glucose/insulin and MRI were equally effective in predicting abnormal OGTT, the primary advantage of the MRI its identification of dangerous trends before irreversible damage has occurred. Both measurements were synergistic, with combined screening identifying 30/31 patients with glucose dysregulation on OGTT. Thus, we propose that annual fasting glucose/measurements coupled with trends in pancreas R2* measurement obtained in patients undergoing routine MRI assessment of liver iron can greatly reduce the need for OGTT and identify patients at high risk of glucose dysregulation in the future.

One limitation of this study was the use oral, rather than intravenous, glucose tolerance test to estimate insulin release, insulin sensitivity, and DI. Euglycemia and hyperglycemia clamp studies have also traditionally been used to assess insulin sensitivity [43]. However, these exams entail more patient risk and discomfort, particularly in a population with notoriously poor intravenous access, and patient acceptance would have been lower. Frequently sampled OGTTs, coupled with mathematical modeling, are gaining increasing acceptance for evaluation of glucose dysregulation in metabolic syndromes because they are easy, sufficiently accurate, and more closely approximate clinical practice [29,30,44].

Our patient population was relatively young and heavily iron overloaded. As a result, age was a relatively weak correlate of disease risk. However, patient age may be a stronger risk factor for glucose dysregulation in older, better-chelated, patient cohorts.

Finally, while this cross-sectional study offers insight into the risks of glucose dysregulation, longitudinal studies are necessary to understand how hepatic, pancreatic, and cardiac iron burden prospectively modulate the evolution of diabetes in thalassemia major. Unfortunately, given the complex pathophysiology of diabetes, these studies will require large networks collecting data over many years to adequately estimate prospective risk of pancreas R2* measurements.

In summary, overt and preclinical glucose dysregulation was extremely common in our thalassemia cohort. Pancreatic beta cell function was most closely correlated with pancreas iron (R2*) while insulin resistance was more strongly associated with somatic iron balance (serum ferritin, LIC). Glucose dysregulation was ubiquitous in patients with detectable cardiac iron. Isolated pancreas R2* $>$ 100 Hz had a 50% positive predictive value for glucose dysregulation, but risk was also modulated by the severity and chronicity of liver iron overload. Fasting glucose and insulin levels were appropriate screening tools for glucose dysregulation and were complementary to the MRI findings. Chelation therapy escalation in response to pancreatic, hepatic, or cardiac iron burden is prudent for primary prevention of DM, but longitudinal studies will be required to stratify the prospective risk associated with each of these metrics.

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Author Contributions

L.J.N. performed research, analyzed data and wrote the article; S.D.M. assisted in study design and data analysis; R.M.W. assisted in data analysis; T.D.C. collected data and assisted in writing; J.C.W. designed research, performed research, analyzed data, and wrote the article.

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