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Effect of Iron Depletion in Carbohydrate-Intolerant Patients With Clinical Evidence of Nonalcoholic Fatty Liver Disease

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Background & Aims: **Increased body iron, genetic hemochromatosis (GH) mutations, and nonalcoholic fatty liver disease (NAFLD) tend to cluster in carbohydrate-intolerant patients. In an attempt to further clarify the interrelationships among these conditions, we studied 42 carbohydrate-intolerant patients who were free of the common GH mutations C282Y and H63D, and had a serum iron saturation lower than 50%.** ***Methods:*** **We measured body iron stores, and induced iron depletion to a level of near-iron deficiency (NID) by quantitative phlebotomy.** ***Results:*** **In the 17 patients with clinical evidence of NAFLD, we could not demonstrate supranormal levels of body iron (1.6 ± 0.2 vs. 1.4 ± 0.2 g; $P = 0.06$). However, at NID, there was a 40%–55% improvement ($P = 0.05–0.0001$) of both fasting and glucose-stimulated plasma insulin concentrations, and near-normalization of serum alanine aminotransferase activity (from 61 ± 5 to 32 ± 2 IU/L; $P < 0.001$).** ***Conclusions:*** **These results reflect the insulin-sparing effect of iron depletion and indicate a key role of iron and hyperinsulinemia in the pathogenesis of NAFLD.**

It was recently shown that the C282Y and H63D mutations in the HFE gene are common[1,2] in nonalcoholic fatty liver disease (NAFLD). In general, carriers of HFE mutations had greater hepatic iron concentration, an abnormality that positively correlated with the degree of fibrosis.[1] These, as well as similar findings from other studies,[3] lead to the opinion that iron might be an important factor in the progression from steatosis to more severe forms of NAFLD. Younossi et al.[4] were unable to detect increased hepatic iron in NAFLD. Albeit HFE mutations were not studied, Younossi et al. diagnosed NAFLD in many patients without iron overload, and concluded that NAFLD is frequently observed even when liver iron concentration is normal. Similar conclusions also came from a study by Bonkovsky et al.[5] who found a high prevalence of HFE mutations in NAFLD. Although in the latter 2 studies[4,5] patients were selected on the basis of histologic evidence of NAFLD, the other investigations considered individuals attending iron storage disorders clinics who had high serum or liver iron indexes.[1,2] Thus, it remains unknown whether HFE mutations and excess iron are as frequent when patients are selected from centers, such as diabetic clinics, where there is no particular diagnostic or therapeutic emphasis on either iron storage or liver disorders. Moreover, it remains unknown whether the pathogenetic importance of iron is confined to conditions of HFE mutation-related iron overloading or not. In an attempt to address these questions we screened 45 consecutive carbohydrate (CHO)-intolerant patients for genetic hemochromatosis (GH) by both a serum iron saturation index and genetic mutational analysis. Patients who carried 1 or 2 copies of the C282Y-H63D mutations or had a serum iron saturation ≥50% were excluded. All the remaining patients, presumably free of GH even at a heterozygous level, were subsequently venesected to accurately assess body iron burden.

Furthermore, to explore the role of iron in GH-unrelated NAFLD, we measured markers of liver inflammation before and after induction of iron depletion to near-iron deficiency (NID).

Materials and Methods

Patient Selection

To minimize the effect of potential confounders such as severe obesity, long-standing diabetes, and liver disease other than NAFLD, we only considered nonmorbidly obese (body mass index [BMI] <32 kg/m^2) patients who met the following criteria: (1) history of noninsulin-requiring type 2 diabe-

Abbreviations used in this paper: CHO, carbohydrate; GH, genetic hemochromatosis; HOMA, homeostasis model assessment; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; MCV, mean corpuscular cell volume; NAFLD, nonalcoholic fatty liver disease; NID, near-iron deficiency; NIDDM, type 2 diabetes; OGTT, oral glucose tolerance test.

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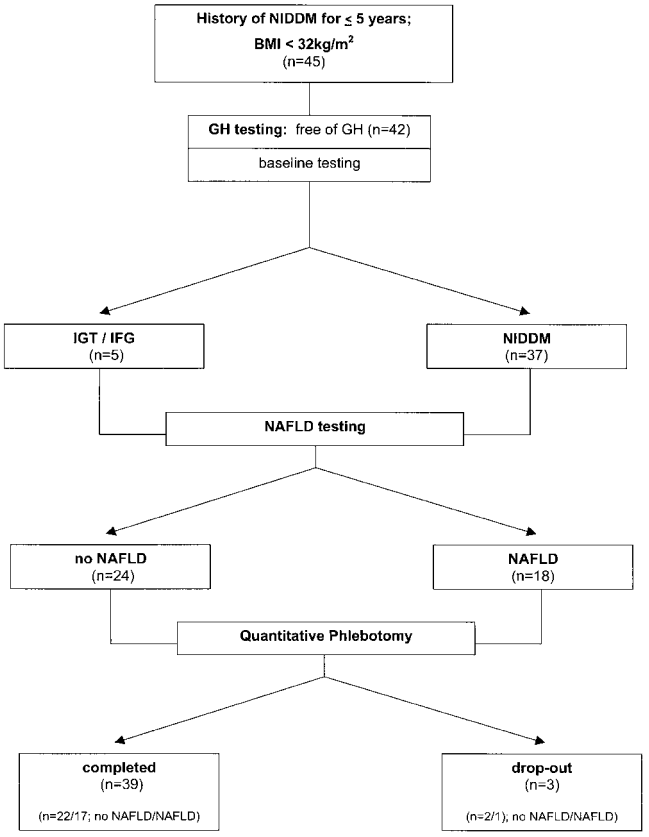

Figure 1. Flowchart summarizing selection, screening, and the final number of patients completing the experimental protocol.

tes[6] for no longer than 5 years; (2) ethanol intake lower than 20 g/day; (3) not consuming any medication known to cause hepatic steatosis[7]; (4) not affected by any other acute or chronic illness; (5) normal serum copper, alkaline phosphatase, ceruloplasmin, and α_1-antitrypsin concentrations; and (6) negative antinuclear antibodies and serologies for viral hepatitis (A, B, or C).

Forty-five consecutive patients fulfilling these criteria were subsequently screened for GH. Forty-two (93%) had a serum iron saturation lower than 50% and negative mutational DNA analysis for C282Y/H63D.[8] After giving their informed consent, these 42 patients entered the study protocol. The patient selection process is summarized in Figure 1.

Experimental Protocol

After overnight fasting, blood was drawn for measurement of serum aminotransferase activity,[9] plasma glucose,[10] insulin,[11] HgBA1c,[12] fructosamine,[13] iron,[14] transferrin,[15] and ferritin.[16] A 75-g, 3-hour oral glucose tolerance test was also performed. The insulin assay (Pharmacia Corp., Peapack, NJ) had a 41%, 0.18%, and <0.1% cross reactivity with proinsulin, c-peptide, and insulin-like growth factor (IGF) 1 and 2, respectively. At baseline testing, 5 of 42 patients met criteria[6] for impaired glucose tolerance (IGT) or impaired fasting glucose (IFG). Type 2 diabetes was confirmed in the remaining 37 individuals (Figure 1). Since IGT and IFG are also carbohydrate intolerant states the 5 patients were not excluded from the study. To estimate insulin sensitivity we used both plasma insulin concentration and the homeostasis model assessment (HOMA),[17] which, particularly in log form, was shown to better correlate with in vivo insulin-mediated glucose uptake in type 2 diabetes patients.[18] NAFLD was diagnosed by a serum alanine aminotransferase (ALT) activity over 30 IU/L in at least 2 separate occasions (during the previous 6 months) with bright liver on ultrasonography and no history of infectious hepatitis. With these criteria, there were 18 patients with NAFLD and 24 without. In CHO-intolerant patients without NAFLD, serum ALT was lower than 30 IU/L and liver echogenicity normal.

After baseline tests, quantitative phlebotomy[19] was initiated in both groups. Half-liter phlebotomies were performed monthly or bimonthly under topical anesthesia with 0.5–1.0 mL of 1% lidocaine, until all subjects reached NID. NID was arbitrarily defined as the anemia-free condition characterized by a serum ferritin concentration lower than or equal to 30 μg/L, serum iron saturation lower than or equal to 15% and a mean corpuscular cell volume (MCV) lower than or equal to 82 fl. At NID, baseline body iron stores were estimated as follows: (baseline Hct + NID Hct/2) × blood volume removed (mL) × 1 mg/mL.

Metabolic measurements were repeated in similar fashion after a 1-month time interval from the last phlebotomy. A pilot study showed that such an interval is sufficient for correction of those minimal degrees of hemodilution occasionally observed after phlebotomy.[20]

Statistics

Results were averaged, expressed as mean ± SEM, and frequency distribution was estimated for each variable. Unpaired Student *t* test was used for group comparison. Values at NID were compared with baseline by either paired (2-tailed) Student *t* test or the Wilcoxon-matched pairs test for normally distributed and nonparametric correlated variables, respectively. Nonparametric variables were serum fructosamine, ferritin, and insulin concentrations.

Statistical analysis was performed with a commercial software (Statsoft-Inc., Tulsa, OK) for the MacIntosh (mod.iMAC, Apple Computers, Cupertino, CA).

Results

Three of 42 (1 with and 2 without NAFLD) patients dropped out secondary to poor venous access and fatigue. Thirty-nine patients, 17 with and 22 without NAFLD, completed the study. The clinical characteristics of the 2 groups are summarized in Table 1 and Figure 1. Patients with NAFLD were neither older nor significantly heavier than patients without NAFLD (BMI = 29 ± 0.6 vs. 28 ± 0.9 kg/m^2; $P = 0.2$). Duration of diabetes, treatment modalities, and glucometa-

Table 1. Clinical and Demographic Characteristics of Patients With and Without NAFLD

Variable	NAFLD	No NAFLD
N	17	22
Age (*yr*)	49 ± 9	47 ± 8
Gender (M/F)	12/5	12/10
NIDDM/IGT-IFG	15/2	19/3
NIDDM (*yr*)	3 ± 1	3 ± 1
BMI (kg/m^2)	29 ± 1	28 ± 1
Diet Rx only	3	5
Sulfonylureas	10	12
Metformin	4	5

bolic control were also similar in the 2 groups (Tables 1–3). Compared with patients without NAFLD, patients with NAFLD had a 3-fold elevation of serum ALT (61 ± 5 vs. 19 ± 1 IU/L; $P < 0.001$), a 2.5-fold elevation of fasting insulin levels (258 ± 32 vs. 108 ± 19 pmol/L; $P < 0.001$), as well as higher storage iron (1.6 ± 0.2 vs. 1.4 ± 0.2 g; $P = 0.06$). At NID, depletion of body iron stores, as indicated by reductions of MCV, ferritin, and serum iron saturation, was comparable in both groups (Tables 2 and 3). As compared with baseline, at NID there was no significant change in body weight or in medication usage. Despite no change in body weight and medications, there were notable metabolic changes, particularly in patients with NAFLD. As illustrated in Figures 2 and 3 fasting insulin decreased ~40% (from 258 ± 32 to 152 ± 17 pmol/L; $P < 0.001$) and 3-hour oral glucose tolerance test (OGTT) insulin decreased >50% (from 1104 ± 144 to 498 ± 67 pmol/L; $P < 0.001$).

In patients without NAFLD (Figures 3 and 4) fasting insulin was unchanged (from 108 ± 19 to 96 ± 5; $P =$ NS), whereas 3-hour OGTT insulin decreased ~40% (from 468 ± 67 to 271 ± 47; $P < 0.001$).

In patients with NAFLD, the reductions in insulin concentrations were parallelled by statistically significant decreases in glucose levels (Figure 3), aspartate aminotransferase (AST) (from 27 ± 2 to 22 ± 1 IU/L; $P < 0.05$), and ALT (from 61 ± 5 to 32 ± 2 IU/L; $P < 0.001$). On the contrary, ALT and AST were unchanged in patients without NAFLD (from 19 ± 1 to 17 ± 1 IU/L; and from 14 ± 1 to 15 ± 1, respectively; $P =$ NS). Individual changes of serum ALT activity, from baseline to NID, are shown in Figure 5.

Table 2. Iron and Chronic Gluco-Metabolic Parameters at Baseline and NID: Patients With NAFLD

Variable	Baseline	NID	P
Ferritin (10–300 μg/L)	299 ± 41	15 ± 1	<0.001
Iron saturation (15%–55%)	28 ± 2	11 ± 1	<0.001
MCV (80–96 fl)	86 ± 2	80 ± 2	<0.001
Hct (38%–48%)	42 ± 1	40 ± 1	NS
HgbA1c (4.8%–6.0%)	7.9 ± 0.6	6.8 ± 0.4	<0.01
Fructosamine (0–265 μmol/L)	279 ± 11	261 ± 10	0.05

Table 3. Iron and Chronic Gluco-Metabolic Parameters at Baseline and NID: Patients Without NAFLD

Variable	Baseline	NID	P
Ferritin (10–300 μg/L)	220 ± 40	13 ± 1	<0.001
Iron saturation (15%–55%)	33 ± 3	9 ± 1	<0.001
MCV (80–96 fl)	90 ± 2	81 ± 0.5	<0.001
Hct (38%–48%)	42 ± 1	40 ± 1	NS
HgbA1c (4.8%–6.0%)	7.6 ± 0.5	6.9 ± 0.4	<0.05
Fructosamine (0–265 μmol/L)	303 ± 17	286 ± 16	NS

Discussion

Hepatic steatosis with or without inflammation is commonly observed in carbohydrate-intolerant individuals.[21–26] In this setting, when soft hepatomegaly and an otherwise unexplained 1.5–5-fold increase of serum ALT activity (in the absence of signs of chronic liver failure) are detected, diagnosis of NAFLD is generally unequivocal.[24,26] Apart from a greater prevalence of obesity, NAFLD and CHO-intolerance share other attributes, notably, impaired glucose metabolism, a hyperinsulinemic response to carbohydrates, and insulin resistance.[22–26] Similar features also characterize GH and recent studies indicated that 1 in 3 patients with NAFLD have increased liver iron[1–3] and up to 70% carry either 1 or 2 copies of the common HFE mutations C282Y or H63D.[5] In an attempt to further clarify the complex interrelationships among CHO-intolerance, NAFLD, and body iron stores, we studied consecutive patients from diabetic clinics in whom GH was excluded by sensitive screening. Hence, the following observations apply to individuals who are free of the 2 common HFE genotypes associated with GH, as well as of phenotypic GH (serum iron saturation lower than 50%).

The first observation was that CHO-intolerant individuals with NAFLD were not iron overloaded. This finding agrees with some,[4,5] although not all,[1–3] existing studies. In the latter reports, patients with high serum (or liver) iron indexes were studied, indicating sampling the upper ranges of the gender-specific iron storage frequency distribution. On the contrary, when patient selection relied on other criteria, such as presence or absence of NAFLD on liver biopsy, iron overload was less commonly observed.[4,5] The current investigation provides further evidence in this direction, i.e., when patients are selected from clinics where there is no special

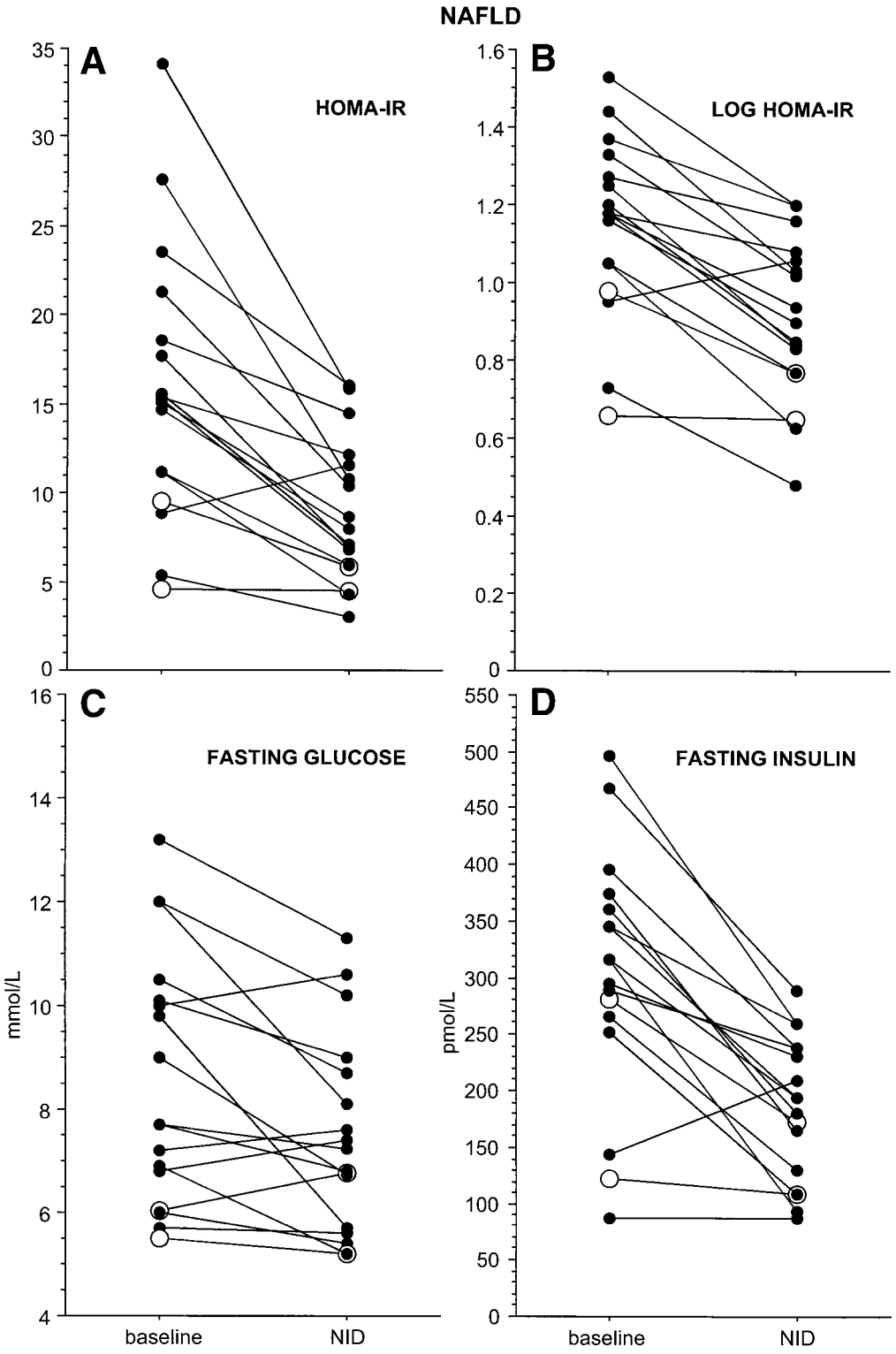

Figure 2. (*A*) Individual changes in HOMA-IR, (*B*) log HOMA-IR, (*C*) fasting glucose, (*D*) and fasting insulin between baseline and NID in individuals with NAFLD. *Open circles* represent patients with IGT/IFG. *Closed circles* represent patients with type 2 diabetes.

interest in iron storage disorders and GH is excluded, NAFLD and CHO intolerance appear commonly associated even in conditions of iron sufficiency.

A second finding of the present study was that, when compared with patients without NAFLD, individuals with NAFLD had a 250% and a 10% elevation of fasting insulin and glucose concentrations, respectively. Thus, carbohydrate-intolerant individuals with NAFLD were more hyperglycemic and hyperinsulinemic than those without NAFLD, despite comparable age, BMI, duration

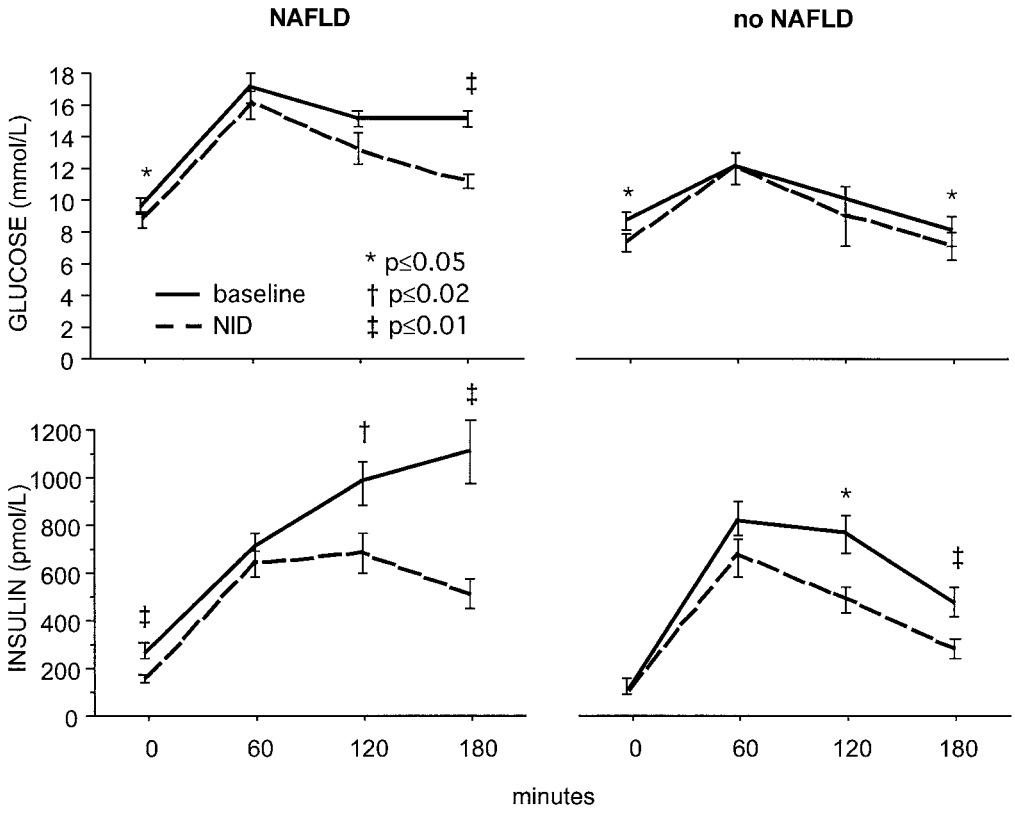

Figure 3. Plasma glucose and insulin concentrations during a 3-hour oral glucose tolerance test performed at baseline (*solid line*) and NID (*dashed line*) in patients with NAFLD and without NAFLD.

of diabetes, and glucometabolic control. This conclusion is consistent with the notion that NAFLD is closely related to insulin resistance and hyperinsulinemia[22–26] even at a preclinical stage,[22] i.e., before development of diabetes and severe obesity. A preclinical association suggests an important causal role of hyperinsulinemia in steatogenesis and several lines of evidence support this view: (1) in vitro, fatty acid oxidation is inhibited and triglyceride synthesis activated by hyperinsulinemia with consequent cytoplasmic accumulation of triacylglycerol[27–29]; (2) intraperitoneal insulin injection, in vivo, caused subcapsular steatonecrosis, a localized form of NAFLD, presumably secondary to exposure of superficial hepatocytes to high insulin concentrations[30]; (3) various insulin-sensitizing maneuvers, such as physical activity and calorie restriction,[23,31] partially reversed steatosis in humans, whereas the insulin-sensitizing drug metformin had a similar effect in rats.[32] In this context, the well-known muscle contraction-like effect of iron depletion on glucose transport in rats, calves, and normal humans[33–36] might assist with the explanation of our current findings. The insulin-sparing effect of iron depletion was linked to enhanced glucose transporter GLUT1 and 4 activity[37,38] and rather than the method used to induce iron depletion, e.g., selective dietary exclusion of the metal, bleeding, or chelation, the relevant factor consisted in reaching a depletion threshold.[33–36] Such a threshold coincided with the lowest level of storage iron sufficient to prevent anemia (e.g., NID) or with frank iron deficiency causing anemia. Thus, enhanced skeletal muscle glucose transport and metabolism[39,40] likely accounted for the reductions of plasma glucose and insulin levels noted in our patients. Although insulin resistance was not directly measured, such a conclusion appears appropriate because there is consensus on use of plasma insulin concentrations as surrogate measurements of insulin resistance in both normal and diabetic subjects,[41,42] particularly when the HOMA model is used.[18] Independent from the precise mechanism(s), it is now possible to extend the notion of an insulin-sparing effect of NID on glucose homeostasis to CHO-intolerant patients with NAFLD.

A third finding of the current study was that ALT activity halved and nearly normalized at NID. A decrease was noted in every patient with NAFLD, whereas no change occurred in those without NAFLD. As the reduction of serum ALT activity could not be attributed to methodological bias, it seems obvious it was a specific consequence of phlebotomy. There are earlier observations that phlebotomy-induced iron depletion improved ALT in small numbers of patients with NAFLD.[5,43,44] However, in those uncontrolled studies, patients were not characterized in terms of HFE mutations, glucose

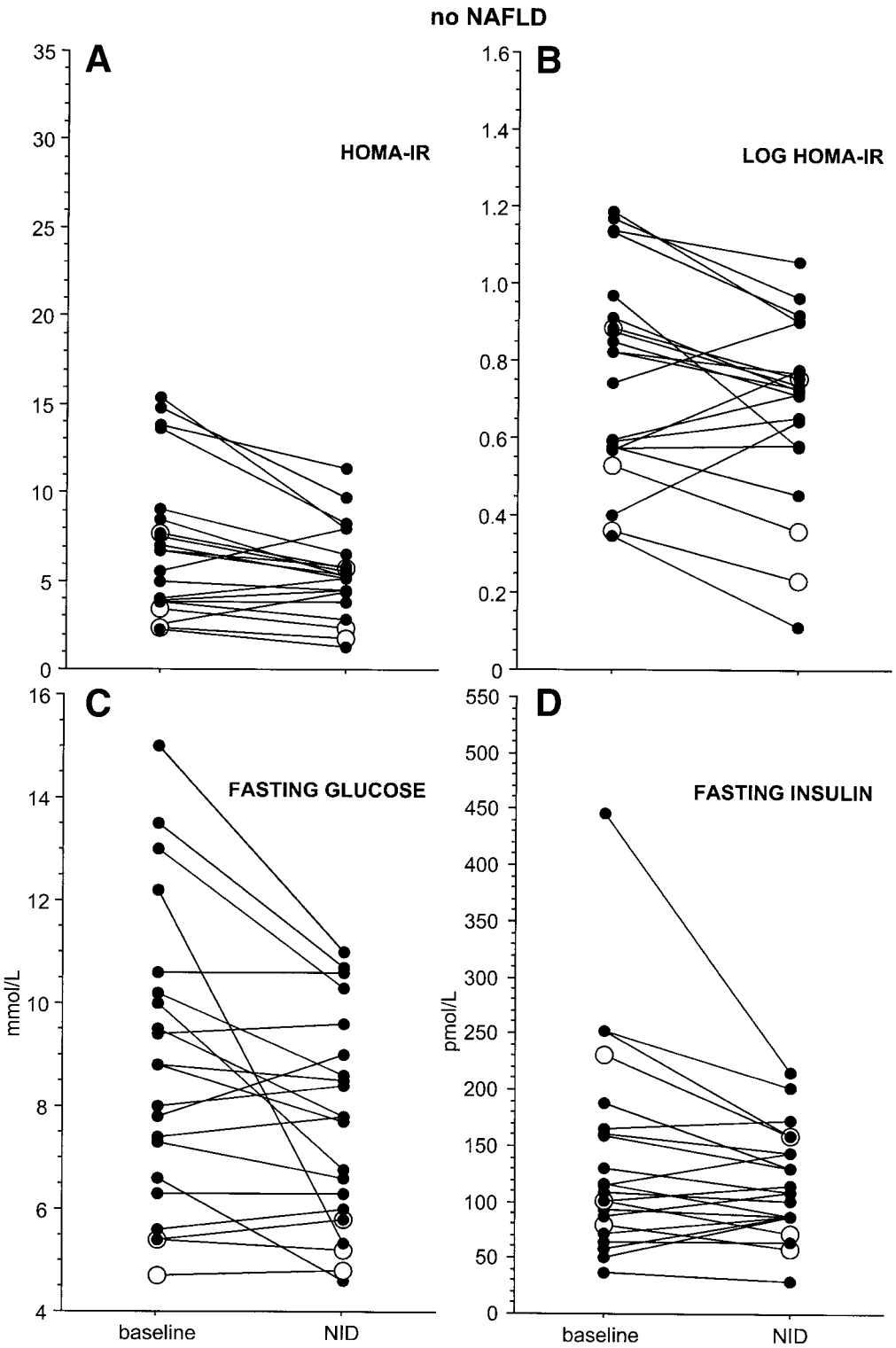

Figure 4. (*A*) Individual changes in HOMA-IR, (*B*) log HOMA-IR, (*C*) fasting glucose, (*D*) and fasting insulin between baseline and NID in individuals without NAFLD. *Open circles* represent patients with IGT/IFG. *Closed circles* represent patients with type 2 diabetes.

tolerance state, or both. In the current study, all patients were CHO-intolerant, not iron overloaded, and free of the 2 common HFE genotypes associated with GH, as well as of phenotypic GH (serum iron saturation lower than 50%). Therefore, NID seems effective in improving markers of liver inflammation and oral glucose tolerance even in patients who have no apparent iron storage disorder. We suggest that, by enhancing insulin sensitivity and reducing iron-mediated oxidative stress, NID diminished hepatocyte substrate load, lipid peroxidation,

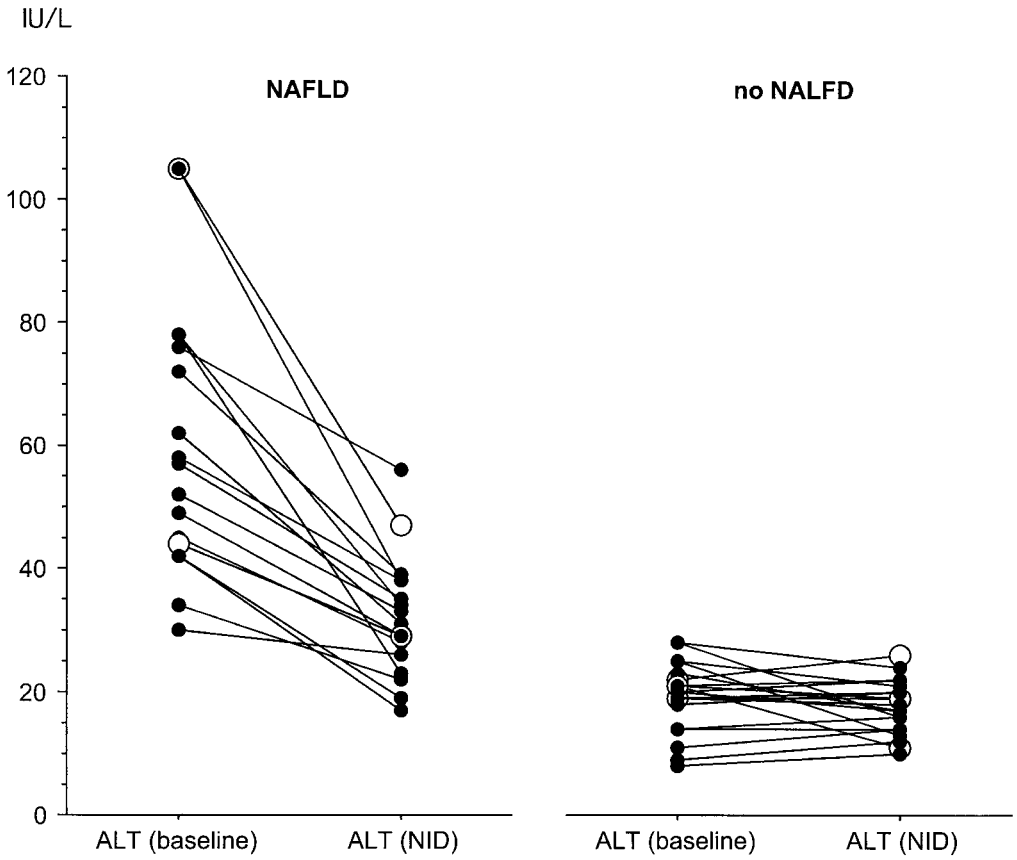

Figure 5. Individual changes of serum ALT activity from baseline to NID in patients with NAFLD and without NAFLD. *Open circles* represent patients with IGT/IFG. *Closed circles* represent patients with type 2 diabetes.

hepatocellular injury, and serum ALT activity. Albeit speculative, this hypothesis is supported by studies where oxidative stress and lipid peroxidation caused experimental NAFLD,[45] and by others, where iron chelation lowered and iron loading enhanced both oxidative stress and lipid peroxidation.[46–48]

We used clinical, not histological, criteria to diagnose NAFLD. However, we doubt other causes of liver inflammation, such as cryptic viral infection, could affect our conclusions. In fact, such an eventuality appears unlikely[49,50] and not precluding the beneficial effect of NID on markers of liver inflammation. Nonetheless, a change in ALT may not reflect the histology or progression to fibrosis, and in this study we could not assess whether or not iron depletion had favorable effects on prognostic indicators.

There is anecdotal evidence that liver fibrosis may regress after iron depletion.[44] Furthermore, hyperferritinemic individuals with insulin-resistance-associated hepatic iron overload can have symptom relief after venesection.[51] However, in our opinion iron depletion to NID should remain investigational until controlled biopsy studies will elucidate whether or not serious hepatic fibrosis will prove to be safely preventable with long-term maintenance of NID.

In summary, it was shown that CHO-intolerant individuals with NAFLD are more hyperglycemic and, especially, more hyperinsulinemic than CHO-intolerant patients without NAFLD. Despite normal body iron stores, iron depletion to NID markedly lowered insulinemia, and nearly normalized serum ALT activity indicating the detrimental effect iron sufficiency has in susceptible individuals. These results lend further credence for a central pathogenetic role of iron, insulin resistance, and hyperinsulinemia in the genesis of GH-unrelated NAFLD.

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