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Context: There is an ongoing debate about whether and how fructose is involved in the pathogenesis of nonalcoholic fatty liver disease (NAFLD). A recent experimental study showed an increased intrahepatic triglyceride (IHTG) content in mice deficient for aldolase B (aldo $B^{-/-}$), the enzyme that converts fructose-1-phosphate to triose phosphates.

Objective: To translate these experimental findings to the human situation.

Design: Case-control study.

Setting: Outpatient clinic for inborn errors of metabolism.

Patients or Other Participants: Patients with hereditary fructose intolerance, a rare inborn error of metabolism caused by a defect in aldolase B (n = 15), and healthy persons matched for age, sex, and body mass index (BMI) (n =15).

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Abbreviations: aldo $B^{-/-}$, aldolase B deficient; AUC, area under the curve; BMI, body mass index; DNL, *de novo* lipogenesis; F1P, fructose-1-phoshpate; HIRI, hepatic insulin resistance index; HOMA2, Homeostasis Model Assessment 2; iAUC, incremental area under the curve; IHTG, intrahepatic triglyceride; NAFLD, nonalcoholic fatty liver disease; OGTT, oral glucose tolerance test.

Main Outcome Measure: IHTG content, assessed by proton magnetic resonance spectroscopy.

Results: IHTG content was higher in aldo $B^{-/-}$ patients than controls (2.5% vs 0.6%; $P = 0.001$) on a background of lean body mass (median BMI, 20.4 and 21.8 kg/m^2, respectively). Glucose excursions during an oral glucose load were higher in aldo $B^{-/-}$ patients ($P = 0.043$). Hypoglycosylated transferrin, a surrogate marker for hepatic fructose-1-phosphate concentrations, was more abundant in aldo $B^{-/-}$ patients than in controls ($P < 0.001$). Finally, plasma β-hydroxybutyrate, a biomarker of hepatic β-oxidation, was lower in aldo $B^{-/-}$ patients than controls ($P = 0.009$).

Conclusions: This study extends previous experimental findings by demonstrating that aldolase B deficiency also results in IHTG accumulation in humans. It suggests that the accumulation of fructose-1-phosphate and impairment of β-oxidation are involved in the pathogenesis. (***J Clin Endocrinol Metab*** **104: 5056–5064, 2019)**

In parallel with the current obesity epidemic, nonalcoholic fatty liver disease (NAFLD) has emerged as a threat to global health (1). NAFLD can lead to end-stage liver failure and hepatocellular carcinoma, and patients are also at risk for extrahepatic complications, such as dyslipidemia, type 2 diabetes, chronic kidney disease, and cardiovascular disease (2).

The pathophysiology of NAFLD is complex and involves genetic, inflammatory, (gut) microbiotic, metabolic, nutritional, and lifestyle factors (3). A recent dietary intervention study showed that overfeeding with saturated fat or carbohydrates increased intrahepatic triglyceride (IHTG) content, albeit by different mechanisms: the former by stimulating fatty acid flux from adipose tissue toward the liver and the latter by increasing hepatic *de novo* lipogenesis (DNL) (4).

There is an ongoing discussion on whether the type of carbohydrate (*i.e.*, glucose or fructose) matters. Although fructose is preferentially metabolized in the liver (5), several epidemiological studies have failed to demonstrate that increased fructose intake is associated with increased IHTG content (6, 7). Furthermore, intervention studies comparing added glucose vs added fructose, under isocaloric or hypercaloric conditions, showed no difference in IHTG content (8, 9). On the other hand, other experimental studies have shown that added fructose, not glucose, specifically increases DNL and plasma triglycerides (10, 11).

The catabolism of fructose in the liver involves a two-step process: (i) phosphorylation of fructose by ketohexokinase and (ii) aldolase B–mediated conversion of fructose-1-phoshpate (F1P) to triose phosphates, which subsequently enter the glycolytic/gluconeogenic pathway. Lanaspa *et al.* (12) recently elucidated a potential mechanism by which fructose can cause IHTG accumulation. They showed that aldolase B knockout (aldo $B^{-/-}$) mice displayed a (paradoxical) increase in IHTG content, which could be prevented by blocking ketohexokinase. These observations ascribe a central role for intrahepatic F1P to the pathogenesis of fructose-induced IHTG accumulation.

The aim of the current study was to translate these experimental findings to the human situation. For this, we studied 15 aldo $B^{-/-}$ patients (*i.e.*, patients with hereditary fructose intolerance, Online Mendelian Influence in Man # 229600) in comparison with 15 age-, sex-, and body mass index (BMI)–matched controls.

Materials and Methods

Participants

In this case-control study, adult aldo $B^{-/-}$ patients were recruited from multiple outpatient metabolic clinics in the Netherlands and Belgium and compared with healthy control participants matched for age, sex, and BMI. Diagnosis of hereditary fructose intolerance was confirmed by a fructose tolerance test (n = 1), measurement of aldolase activity in liver biopsy tissue (n = 2), or DNA analysis (n = 12). Exclusion criteria for this study were contraindications for MRI or inability to give informed consent.

Participants visited our metabolic ward after an overnight fast of at least 8 hours. All completed a health questionnaire regarding (among others) habitual alcohol consumption and medical history. Height was determined with the participants standing upright against a stadiometer, and weight was measured by using electronic scales while the participants wore underwear only. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was ascertained with the participants in the standing position by using a measuring tape at the level of the umbilicus.

All participants gave written informed consent before inclusion in the study. The study was performed according to the Declaration of Helsinki (13) and approved by the medical ethical committee of Maastricht University Medical Center.

Dietary intake

Because aldo $B^{-/-}$ patients do not tolerate fructose, they follow a fructose-restricted diet. As a consequence, macro- and micronutrient composition of their diet may differ from that in controls, which, in turn, may affect the primary outcome of interest. Dietary intake was therefore assessed by using a 3-day food journal along with a personal interview by the clinical researcher (N.S.). Dietary macro- and micronutrient

composition was determined based on the Dutch food composition table (14). Daily fructose intake was calculated with a recently developed, extensive sugar composition table (15), which consists of data from the national Dutch food composition table and internationally available food composition databases (*i.e.,* McCance and Widdowson's composition of foods integrated data set, United Kingdom; the Danish food composition database; and the Finnish food composition database, Fineli). If the fructose content of a specific product was not available, either values of a comparable food product were used or sucrose content was estimated according to total sugar content of the specific product. Plasma fatty acid composition was measured as a biomarker of saturated fat intake.

Laboratory measurements

Glucose tolerance was determined with a standard 75-g oral glucose tolerance test (OGTT). Participants were instructed to ingest 82.5 g dextrose monohydrate dissolved in 250 mL water over the course of 5 minutes, according to the World Health Organization (16). Whole blood glucose levels were determined every 15 minutes by using the YSI2300 STAT Plus Glucose Lactate Analyzer (YSI, Yellow Springs, OH). Plasma insulin levels were measured at baseline, 30 minutes, 60 minutes, and 120 minutes by using the Human Insulin Kit (Meso Scale Discovery, Rockville, MD). Subsequently, the area under the curve (AUC) and incremental area under the curve (iAUC) were calculated for blood glucose and plasma insulin excursions. The degree of insulin resistance was estimated with the Homeostasis Model Assessment 2 (HOMA2) calculator (http://www.dtu.ox.ac.uk). The hepatic insulin resistance index (HIRI) was calculated as the square root of the product of the glucose (mmol/L) and insulin (pg/mL) AUC during the first 30 minutes of the OGTT (17), and expressed as arbitrary units.

At 5 minutes before the ingestion of dextrose monohydrate, blood was drawn for determination of serum uric acid, total cholesterol, high-density lipoprotein cholesterol, and triglycerides (enzymatic colorimetric assay, Cobas 8000 instrument, Roche Diagnostics, Mannheim, Germany). Low-density lipoprotein cholesterol was calculated by using the Friedewald formula. EDTA-treated blood samples were used for the determination of fatty acid composition and β-hydroxybutyrate, as described in detail elsewhere (18, 19).

Glycoprofiling of transferrin was carried out by using high-resolution mass spectrometry (20). In brief, 2 μL of eluted plasma was analyzed on a microfluidic 6540 high-performance liquid chromatography/chip quadrupole time-of-flight instrument (Agilent Technologies, Santa Clara, CA). Data analysis was performed by using Agilent Mass Hunter Qualitative Analysis software B.04.00. Agilent BioConfirm software was used to deconvolute the charge distribution raw data to reconstructed mass data. If no monoglycosylated transferrin was present in the samples, abundances were estimated at the average reconstructed masses of 77,351 Da. These abundances were used to calculate the ratio between monoglycosylated transferrin and diglycosylated transferrin.

Visceral adipose tissue area, IHTG, and liver stiffness

Visceral adipose tissue area was measured through two-dimensional T1-weighted turbo spin echo MRI using a 3.0-T Achieva whole body MRI scanner (Philips Healthcare, Best, the Netherlands) with a 16-element torso coil (XL Torso Coil, Philips Healthcare). Ten 5-mm-thick transverse slices with a slice gap of 10 mm centered at the top of the L5 vertebral body were acquired. Scan parameters were as follows: field of view, 400 × 322 mm; acquired voxel size, 1.30 × 1.96 × 5.00 mm; reconstructed voxel size, 0.78 × 0.78 × 5.00 mm; repetition time/echo time, 400/10 ms; number of signal averages, 1; turbo spin echo factor, 4. Visceral adipose tissue area was subsequently calculated offline with the ITK-SNAP software application (http://www.itksnap.org) (21).

IHTG content was measured with proton magnetic resonance spectroscopy using the same MRI scanner as for the visceral adipose tissue measurement. Spectra were obtained from a 20 × 20 × 20-mm voxel placed centrally in the lower right lobe of the liver, avoiding large blood vessels and the subcutaneous fat layer. Second-order FASTMAP-based shimming was performed. The following acquisition parameters were used: repetition time/echo time, 4000/32.5 ms; data points, 2048. A total of 32 spectra were acquired, with two signal averages per spectrum. In these spectra, the water signal was suppressed by using frequency-selective prepulses. The unsuppressed water signal was acquired as internal reference (eight spectra with two signal averages per spectrum). The participants were instructed to breathe exactly at the 4s-rhythm of the sequence to keep the repetition time constant. Spectra were analyzed as described elsewhere (22). Briefly, the spectra were fitted by using a home-written script in MATLAB R2014b (Mathworks, Natick, MA). The CH_2 peak and the unsuppressed water peak were corrected for T2 relaxation. Intrahepatic triglyceride content was expressed as the area percentage of the CH_2 peak compared with the water peak.

The degree of liver stiffness, as a proxy for fibrosis (23), was measured with the participant in the supine position by using transient elastography (FibroScan, Echosens, Paris, France). The standard M probe was placed in the intercostal region on the lateral side of the right upper abdominal quadrant. A low-frequency elastic shear wave was passed through the liver, after which the velocity (which differs according to different components of the liver) was measured. Subsequently, the shear wave velocity was converted into liver stiffness, expressed in kilopascals (kPa). The median value of 10 valid measurements (*i.e.,* interquartile range/median ratio ≤ 30%) was used for analysis. All measurements were performed by the clinical researcher (N.S.).

Statistical analyses

Data are expressed as median (interquartile range) and were analyzed with a Mann-Whitney *U* test. Linear regression analyses were conducted to adjust for differences in dietary intake. The relationships between IHTG content, glucose AUC, and hypoglycosylated transferrin were analyzed with a Spearman rank correlation. Results were considered statistically significant at $P < 0.05$. All analyses were carried out with the SPSS software, version 23 for Windows (IBM, Chicago, IL).

Results

General characteristics and dietary intake

General characteristics of the 15 aldo $B^{-/-}$ patients and age-, sex-, and BMI-matched control participants are displayed in Table 1. Aldo $B^{-/-}$ patients were lean

Table 1. Characteristics of the Study Population

Characteristic	Healthy Controls	Aldo $B^{-/-}$ Patients
Men/women, n/n	11/4	11/4
Age, y	28 (25–52)	31 (24–44)
BMI, kg/m^2	21.8 (21.0–23.3)	20.4 (19.3–24.8)
Waist circumference, cm	87 (84.3–91.3)	77.5 (73.8–93.5)
VAT, cm^3	264 (143–331)	328 (119–407)
Alcohol intake, U/d	0.6 (0.1–1.7)	0.1 (0.0–0.3)[a]
HOMA2-insulin resistance	0.5 (0.3–0.6)	0.5 (0.4–0.7)
Total cholesterol, mmol/L	4.1 (3.9–5.2)	5.0 (4.3–6.2)
HDL cholesterol, mmol/L	1.4 (1.1–1.8)	1.6 (1.4–2.3)
LDL cholesterol, mmol/L	2.4 (1.8–3.3)	3.1 (2.0–4.2)
Triglycerides, mmol/L	0.9 (0.5–1.2)	0.8 (0.7–1.3)
Uric acid, mmol/L	0.33 (0.28–0.38)	0.30 (0.23–0.34)

Unless otherwise noted, data are expressed as median (interquartile range).

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VAT, visceral adipose tissue.

[a] $P < 0.05$ vs controls, analyzed with a Mann-Whitney *U* test. For technical reasons, visceral adipose tissue area estimates could be obtained only for 14 aldo $B^{-/-}$ patients and 10 controls.

(median BMI, 20.4 kg/m^2) and consumed significantly less alcohol than controls ($P = 0.04$) (Table 1). Visceral adipose tissue area, HOMA2-IR, uric acid, and plasma lipids did not differ between groups (Table 1).

As expected, dietary fructose intake was very low in aldo $B^{-/-}$ patients ($P < 0.0001$ vs controls) [Fig. 1(a)]. Total caloric intake nevertheless did not differ between aldo $B^{-/-}$ patients and controls ($P = 0.93$) [Fig. 1(b)]. Of all macronutrients, aldo $B^{-/-}$ patients consumed significantly more protein ($P = 0.005$) [Fig. 1(c)] and saturated fat ($P = 0.026$) [Fig. 1(d)]. Fatty acid composition measurement of plasma triglycerides, cholesteryl esters, and phospholipids confirmed the higher saturated fat intake, in particular dairy products, as noted by a higher proportion of C14:0 in all lipid fractions and a higher C15:0 in the cholesteryl ester and phospholipid fractions [Fig. 2(a)–2(c)]. Total fat and carbohydrate intake did not significantly differ between groups ($P = 0.15$ [Fig. 1(e)] and $P = 0.11$ [Fig. 1(f)], respectively).

IHTG and glucose tolerance

IHTG content was higher in aldo $B^{-/-}$ patients than controls ($P = 0.001$) [Fig. 3(a)]. The prevalence of hepatic steatosis in aldo $B^{-/-}$ was 3 of 15 (20%) and 5 of 15 (33%), depending on the population-based and pathology-based cutoff values of 5.6% and 3.0%, respectively (24, 25). Only one control individual had an

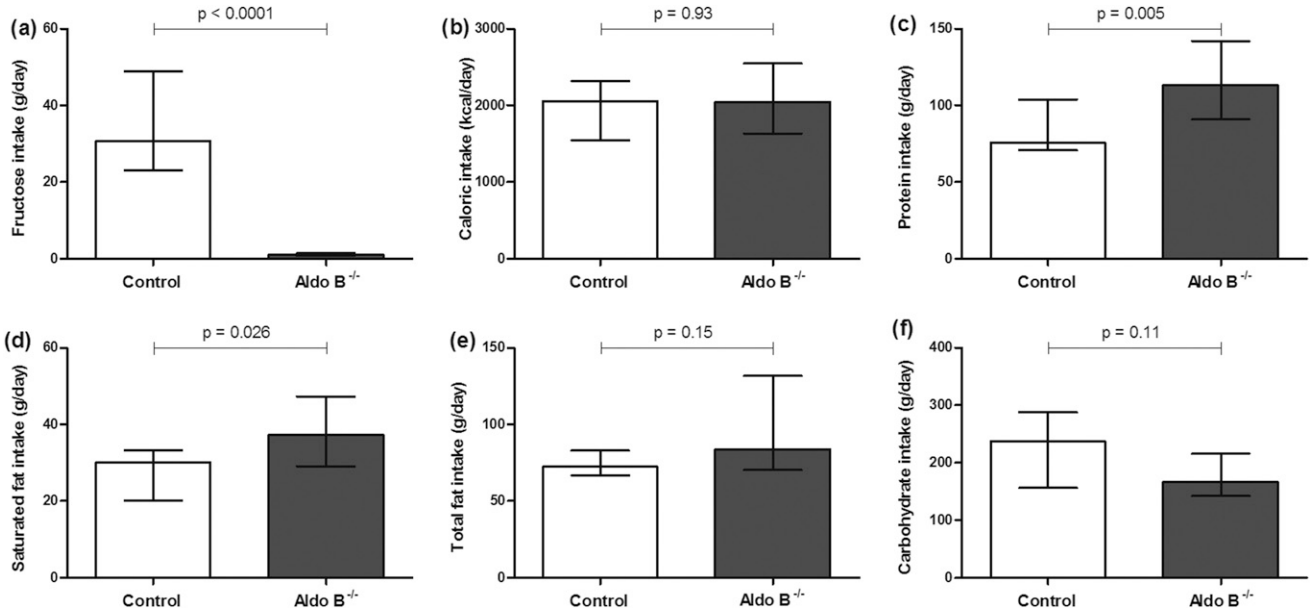

Figure 1. Dietary intake. Daily intake of (a) fructose, (b) total calories, (c) protein, (d) saturated fat, (e) total fat, and (f) carbohydrates in aldo $B^{-/-}$ patients (gray bars) and controls (white bars). Data are expressed as median (interquartile range). Analyzed with a Mann-Whitney *U* test.

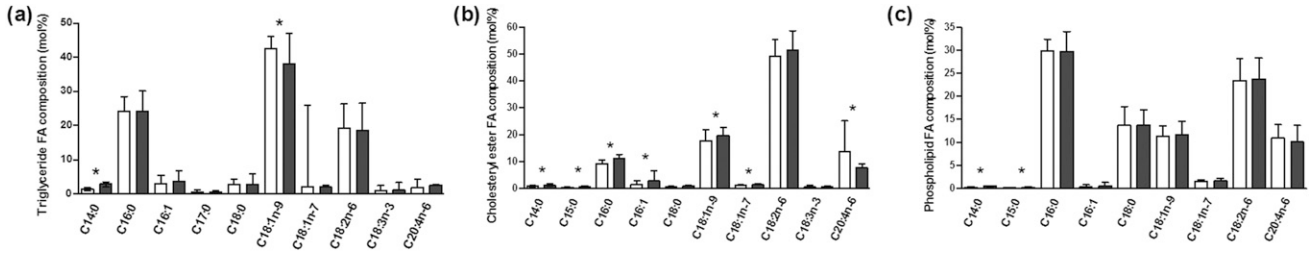

Figure 2. Plasma fatty acid composition as a biomarker of saturated fat intake. Fatty acid composition of (a) plasma triglycerides, (b) cholesteryl esters, and (c) phospholipids in aldo $B^{-/-}$ patients (gray bars) and controls (white bars). *$P < 0.05$ vs controls. Analyzed with a Mann-Whitney *U* test.

ITHG content that was >3.0%. Statistical adjustment for saturated fat or protein intakes did not affect the difference in IHTG content between aldo $B^{-/-}$ patients and controls ($P = 0.005$ and $P = 0.03$, respectively, for the adjusted difference in IHTG content). Liver stiffness did not differ between aldo $B^{-/-}$ patients and controls ($P = 0.098$) [Fig. 3(b)]. One of 15 aldo $B^{-/-}$ patients (7%) had a high liver stiffness measurement (9.0 kPa), corresponding with fibrosis stage ≥3 (26).

Plasma glucose excursions were higher in aldo $B^{-/-}$ patients during a standard 75-g OGTT [Fig. 3(c)]. Consequently, both the AUC and iAUC were greater in aldo $B^{-/-}$ patients ($P = 0.043$ and $P = 0.034$, respectively). IHTG content correlated significantly with glucose AUC and iAUC in the overall population: *i.e.*, aldo $B^{-/-}$ and controls combined ($r_s = 0.42$, $P = 0.025$; $r_s = 0.45$, $P = 0.021$, respectively). Plasma insulin excursions appeared somewhat higher at all time points in aldo $B^{-/-}$ patients [Fig. 3(d)], but were not statistically different ($P = 0.49$ and $P = 0.65$ for the AUC and iAUC, respectively). The HIRI did not differ between both groups [920 (interquartile range, 753 to 1320) in aldo $B^{-/-}$ patients vs 700 (interquartile range, 582 to 1330) in controls; $P = 0.29$].

Hypoglycosylated transferrin and β-hydroxybutyrate levels

To gain more insight into the pathogenesis of the increased IHTG content in aldo $B^{-/-}$ patients, we measured glycosylation of transferrin as a measure of intrahepatic F1P concentrations. Previous studies have shown that untreated aldo $B^{-/-}$ patients have an abnormal transferrin glycosylation pattern (27, 28) as a consequence of F1P-mediated competitive inhibition of mannose phosphate isomerase (MPI) [Fig. 4(a)] (29). Hypoglycosylated transferrin (expressed as the ratio of monoglycosylated transferrin divided by the intact glycoprotein, *i.e.*, diglycosylated transferrin) was also more abundant in aldo $B^{-/-}$ patients on a fructose-restricted diet ($P < 0.001$ vs controls) [Fig. 4(b)]. Hypoglycosylated transferrin levels correlated with IHTG content, not with glucose AUC, in the overall population ($r_s = 0.56$, $P = 0.002$; $r_s = 0.31$, $P = 0.11$, respectively).

We subsequently measured plasma β-hydroxybutyrate levels, a biomarker of hepatic fatty acid oxidation, and observed significantly lower levels in aldo $B^{-/-}$ patients than in controls [$P = 0.009$; Fig. 4(c)].

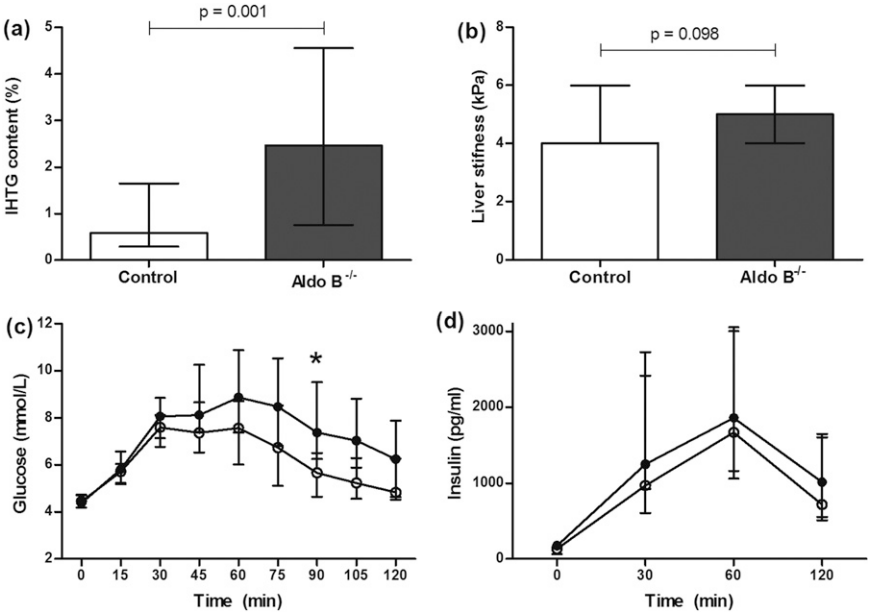

Figure 3. IHTG content, liver stiffness, and oral glucose tolerance test results. (a) IHTG content, (b) liver stiffness, and (c and d) glucose and insulin excursions during an oral glucose tolerance test (c and d) in aldo $B^{-/-}$ patients (gray bars/solid circles) and controls (white bars/open circles). Data are expressed as median (interquartile range). *$P < 0.05$ vs controls. Analyzed with a Mann-Whitney *U* test.

Discussion

The contribution of dietary fructose to the development and progression of NAFLD is still under debate, mainly because the exact mechanism by which

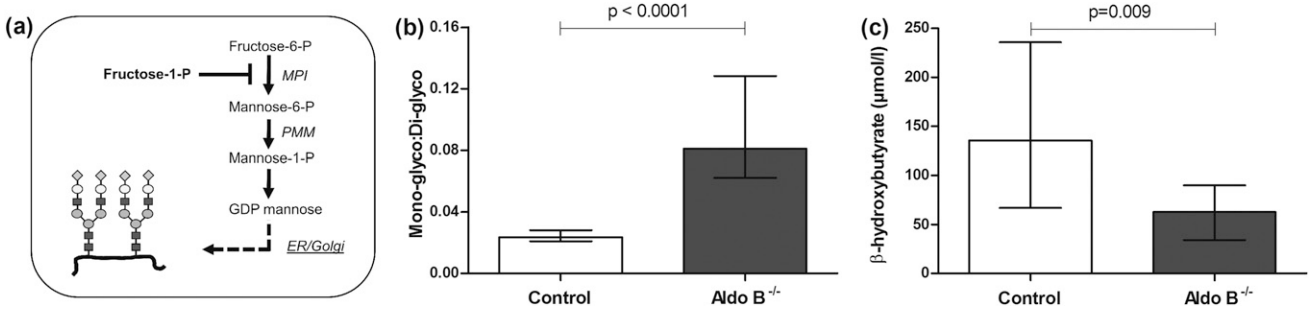

Figure 4. Hypoglycosylated transferrin and β-hydroxybutyrate levels. Normal transferrin consists of two sialylated, biantennary complexes attached to the glycosylation sites of the protein. (a) Suppression of transferrin glycosylation via the competitive inhibition of mannose phosphate isomerase (MPI) by fructose-1-phosphate (fructose-1-P), results in the loss of one or both antennary structures (*i.e.*, more monoglycosylated transferrin). (b) Monoglycosylated transferrin/diglycosylated transferrin, and (c) β-hydroxybutyrate in aldo $B^{-/-}$ patients (gray bars) and controls (white bars). Data are expressed as median (interquartile range). Analyzed with a Mann-Whitney *U* test. ER, endoplasmic reticulum; GDP, guanosine diphosphate; mannose-6-P, mannose-6-phosphate; monoglyco:di-glyco, monoglycosylated transferrin/diglycosylated transferrin; PMM, phosphomannomutase.

fructose leads to liver fat accumulation has not been fully elucidated. In the current study we showed that aldo $B^{-/-}$ patients, who are characterized by an impaired breakdown of F1P, displayed a higher IHTG content compared with controls. This was accompanied by a decrease in glucose tolerance.

In the current study, aldo $B^{-/-}$ patients were matched to healthy controls with respect to age, sex, and BMI, the latter being one of the principal determinants of IHTG content (30). Because aldo $B^{-/-}$ patients do not tolerate fructose, it was inevitable that dietary intake would differ between the groups. Previous studies have shown that short-term changes in dietary intake can affect IHTG content (22, 31). Three-day food journals completed before the measurements revealed that aldo $B^{-/-}$ patients consumed fewer alcohol-containing beverages and more saturated fat and protein. A recently published intervention study reported that overfeeding obese individuals with saturated fat increased IHTG to a greater extent than did overfeeding unsaturated fat or simple sugars (4). In contrast, a 6-week high-protein diet (30% of total energy) that was rich in meat and dairy foods significantly decreased IHTG content in patients with type 2 diabetes (32). Interestingly, Bartolotti *et al.* (33) showed that the effects of a high-saturated-fat diet on IHTG content was blunted when this diet was combined with a high-protein diet. In the current study, a few arguments corroborate the suggestion that dietary differences do not entirely account for the observed differences in IHTG content between aldo $B^{-/-}$ patients and controls. First, alcohol intake was low in both groups (median intake, 0.1 U/d in aldo $B^{-/-}$ patients and vs 0.6 U/d in controls), and its potential effect on IHTG content, if any, would minimize the difference in IHTG content between both groups. Second, statistical adjustment for saturated fat or protein intake did not affect the outcomes.

The results of the current study in aldo $B^{-/-}$ patients chronically treated with a fructose-restricted diet (containing only small amounts of fructose) agree with the outcomes of a recent experimental study in aldo $B^{-/-}$ mice chronically exposed to small amounts of fructose in the chow (~0.3%). Analogous to our proton magnetic resonance spectroscopy findings and the increased proportion of hypoglycosylated transferrin (used as plasma biomarker of intrahepatic F1P) in aldo $B^{-/-}$ patients, aldo $B^{-/-}$ mice were also characterized by a greater IHTG content and increased intrahepatic F1P levels (12). Subsequent detailed examination of the potential pathways involved in the pathogenesis of IHTG accumulation revealed upregulation of DNL genes and increased cytosolic expression of glucokinase in aldo $B^{-/-}$ mouse livers (12). Interestingly, intrahepatic F1P is a potent disruptor of the nuclear glucokinase-glucokinase regulatory protein complex, which causes migration of glucokinase toward the cytosolic space, where it facilitates the conversion of glucose to glucose-6-phosphate, the first step in glycolysis and subsequent DNL (34). Although the current study was not specifically designed to elucidate the exact mechanism by which aldo $B^{-/-}$ causes hepatic fat accumulation in humans, we observed reduced serum β-hydroxybutyrate levels in aldo $B^{-/-}$ patients, used as a biomarker of hepatic fatty acid β-oxidation (and indirectly of DNL, which is inversely linked to β-oxidation) (22, 23).

In the current study, aldo $B^{-/-}$ patients were more glucose intolerant than age-, sex-, and BMI-matched controls, which is generally explained by β-cell insufficiency or insulin resistance. Although Gerst *et al.* recently demonstrated that aldolase B is also involved in pancreatic islet function (35), their results suggested that higher aldolase B activity is associated with reduced insulin secretion, which appears to be in contrast to our findings. Previous studies have shown that IHTG content

is positively associated with endogenous glucose production (36, 37), which is line with the positive association between IHTG content and the iAUC for glucose in the current study. However, indices of hepatic insulin resistance, such as HOMA2–insulin resistance and HIRI (17), did not significantly differ between aldo $B^{-/-}$ patients and controls. Whether this is the consequence of a lack of statistical power or truly indicates a dissociation between IHTG and insulin resistance, as observed in other genetically determined conditions (38, 39), remains to be elucidated.

Our study has several strengths and limitations. First, although hereditary fructose intolerance is a rare inborn error of metabolism [estimated prevalence between 1 in 23,000 to 31,000 people (40–42)], we were able to include a unique sample of carefully phenotyped aldo $B^{-/-}$ patients. Second, as already mentioned, this study was not specifically designed to unravel the underlying mechanism by which aldolase B deficiency causes IHTG accumulation in humans. For this, future studies using stable isotopes to assess DNL are required. Finally, dietary intake was assessed with a 3-day food journal and personal interview by the clinical researcher, which could be subjective and susceptible to underreporting (43). However, we used an objective biomarker for some aspects of dietary intake [*i.e.,* fatty acid composition (44)] that confirmed the results from food journals.

In summary, the current study extends previous observations in aldo $B^{-/-}$ mice by showing that aldo $B^{-/-}$ patients are also characterized by an increased IHTG content. Our findings suggest that the accumulation of intermediates of fructolysis (*i.e.*, F1P) can cause IHTG accumulation via impaired β-oxidation.

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