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Methionine restriction prevents onset of type 2 diabetes in NZO mice

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ABSTRACT: Dietary methionine restriction (MR) is well known to reduce body weight by increasing energy expenditure (EE) and insulin sensitivity. An elevated concentration of circulating fibroblast growth factor 21 (FGF21) has been implicated as a potential underlying mechanism. The aims of our study were to test whether dietary MR in the context of a high-fat regimen protects against type 2 diabetes in mice and to investigate whether vegan and vegetarian diets, which have naturally low methionine levels, modulate circulating FGF21 in humans. New Zealand obese (NZO) mice, a model for polygenic obesity and type 2 diabetes, were placed on isocaloric high-fat diets (protein, 16 kcal%; carbohydrate, 52 kcal%; fat, 32 kcal%) that provided methionine at control (Con; 0.86% methionine) or low levels (0.17%) for 9 wk. Markers of glucose homeostasis and insulin sensitivity were analyzed. Among humans, low methionine intake and circulating FGF21 levels were investigated by comparing a vegan and a vegetarian diet to an omnivore diet and evaluating the effect of a short-term vegetarian diet on FGF21 induction. In comparison with the Con group, MR led to elevated plasma FGF21 levels and prevented the onset of hyperglycemia in NZO mice. MR-fed mice exhibited increased insulin sensitivity, higher plasma adiponectin levels, increased EE, and up-regulated expression of thermogenic genes in subcutaneous white adipose tissue. Food intake and fat mass did not change. Plasma FGF21 levels were markedly higher in vegan humans compared with omnivores, and circulating FGF21 levels increased significantly in omnivores after 4 d on a vegetarian diet. These data suggest that MR induces FGF21 and protects NZO mice from high-fat diet–induced glucose intolerance and type 2 diabetes. The normoglycemic phenotype in vegans and vegetarians may be caused by induced FGF21. MR akin to vegan and vegetarian diets in humans may offer metabolic benefits *via* increased circulating levels of FGF21 and merits further investigation.—Castaño-Martinez, T., Schumacher, F., Schumacher, S., Kochlik, B., Weber, D., Grune, T., Biemann, R., McCann, A., Abraham, K., Weikert, C., Kleuser, B., Schürmann, A., Laeger, T. Methionine restriction prevents onset of type 2 diabetes in NZO mice. FASEB J. 33, 7092–7102 (2019). www.fasebj.org

KEY WORDS: energy expenditure · hyperglycemia · obesity · vegan · vegetarian

It is well established that dietary restriction (energy restriction) promotes health and longevity. Additional evidence suggests that dietary protein restriction (PR) may be contributing, as recently summarized by Hill *et al.* (1). We and others have previously shown that dietary PR may reduce body weight by decreasing body fat gain, improving glucose tolerance, and increasing energy expenditure (EE), effects which are mediated by fibroblast growth factor 21 (FGF21) (2–5). Our group recently demonstrated in New Zealand obese (NZO) mice, a model for polygenic obesity and type 2 diabetes with the characteristic trait of pancreatic β-cell loss, that exogenous FGF21 treatment (6) and a dietary PR-induced increase in FGF21 plasma levels (7) prevented hyperglycemia and diabetes, despite hyperphagia.

ABBREVIATIONS: BAT, brown adipose tissue; Con, control (0.86% methionine); EE, energy expenditure; FGF21, fibroblast growth factor 21; MR, methionine restriction; NEFA, nonesterified fatty acid; NZO, New Zealand obese; OGTT, oral glucose tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; PR, protein restriction; RER, respiratory exchange ratio; sWAT, subcutaneous white adipose tissue

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Furthermore, dietary restriction of the essential amino acid methionine is known to mimic the metabolic effects of energy and PR, with FGF21 as a required mechanism (8–11). As demonstrated in rodents and humans, methionine restriction (MR) improves glucose homeostasis and insulin sensitivity and prevents weight gain (9–13). The potential health benefits of vegan and vegetarian diets may be linked to lower methionine intake. Lowering methionine intake to levels characteristic of vegan diets, which have naturally low methionine levels (14, 15), may be associated with health benefits (including lower rates of obesity and diabetes) (16) and could represent a nutritional strategy for preventing and managing type 2 diabetes in overweight and obese patients.

Circulating FGF21 produced by the liver in response to PR or MR (2, 3, 13) targets a variety of organs through the traditional FGF receptor, fibroblast growth factor receptor 1, c isoform, and the mandatory FGF21 coreceptor β-klotho (17). Pharmacological FGF21 treatment of mice has been shown to increase EE by activation of thermogenesis, improve insulin sensitivity (6, 18, 19), promote β-cell function and survival (20), and prevent pancreatic inflammation (21). The increase in EE, the induction of thermogenic genes in brown adipose tissue (BAT) and subcutaneous white adipose tissue (sWAT), and the improved insulin sensitization induced by an MR diet are lost in FGF21-deficient mice (10). In summary, this makes FGF21 a potential target for the treatment of obesity and diabetes.

FGF21 plasma concentrations correlate positively with blood glucose levels in hyperglycemic and obese NZO mice as well as in humans (6, 22, 23). This slight increase in FGF21 concentration (0.8 ng/ml in NZO mice) may represent a compensatory mechanism for deteriorating glucose homeostasis (6). In rodents, restriction of dietary protein and methionine increases circulating FGF21 levels >4 and 8 ng/ml, respectively, after 4–7 d on PR or only 6 h on MR (2, 3, 5, 7, 24). As recently demonstrated by our group, exogenous FGF21 treatment (6) and dietary PR, which increases endogenous plasma FGF21 (7), prevent hyperglycemia and diabetes in NZO mice. We concluded that the diabetes-susceptible NZO mouse model is well suited to study dietary MR-triggered FGF21-dependent outcomes and sought to investigate whether MR is associated with improved hyperglycemia and diabetes outcomes in NZO mice and humans consuming a vegan diet in the present study.

MATERIALS AND METHODS

Animals, diets, and experimental design

NZO/HIBomDife mice [Deutsches Institut für Ernährungsforschung (DIfE), Nuthetal, Germany] were single-housed in a 12-h light/dark cycle (lights on at 6:00 AM) at a temperature of 21 ± 1°C with *ad libitum* access to food and water unless otherwise noted. At the age of 3 wk, male NZO mice were placed on a high-fat diet (32 kcal%) that provided methionine at the control (Con) level (0.86% methionine, Con; S8022-E127; Ssniff Spezialdiäten, Soest, Germany; Supplemental Table S1) for 1 wk, at which point a subgroup of animals was transferred to an MR diet without cystine (0.17% methionine; S8022-E125; Ssniff Spezialdiäten), an MR diet with cystine [MR (+Cys); S8022-E124; Ssniff Spezialdiäten], or a Con diet with cystine [Con (+Cys); S8022-E126; Ssniff Spezialdiäten] for 9 wk (**Fig. 1*A***).

Six weeks after the diet switch, an oral glucose tolerance test (OGTT) was performed on the mice after a 6-h period of being unfed (glucose 2 mg/g body weight). At indicated time points, blood glucose and plasma insulin levels were measured. Body weight, food intake, body composition (quantitative magnetic resonance; 2012 Body Composition 115 Analyzer; EchoMRI, Houston, TX, USA), random blood glucose (7:00 AM; fed), and equivalent serum insulin levels were measured from tail blood weekly throughout the experiment. Eight weeks after the diet switch, EE was analyzed within metabolic chambers (PhenoMaster/LabMaster; TSE Systems, Bad Homburg, Germany). Nine weeks after the diet switch, mice were killed during midlight cycle in the 6-h unfed state using acute exposure to isoflurane, followed by blood collection. Mice were treated intraperitoneally with NaCl (0.9% w/v) or insulin (7 IU/kg body weight) 15 min before killing, and tissues (liver, sWAT, BAT, quadriceps) were collected and snap-frozen in liquid nitrogen for further analysis. Blood was centrifuged at 10,000 *g* at 4°C for 10 min. All procedures involving animals were approved by the animal welfare committees of Deutsche Institut für Ernährungsforschung (DIfE) and local authorities (Landesamt für Umwelt, Gesundheit, und Verbraucherschutz, Brandenburg, Germany).

Plasma adiponectin, FGF21, leptin, and methionine analysis in meat-restricted humans

A cross-sectional study including vegans and omnivores was conducted at the German Federal Institute for Risk Assessment (BfR) in Berlin, Germany, in 2017 (Supplemental Fig. S1, flow chart). The study was approved by the ethics committee of the Charité-Berlin University of Medicine, Germany (permission number EA4/121/16). Information on lifestyle characteristics, including physical activity level, educational level, smoking habits, and medical history, was obtained through a self-administered tablet computer–based questionnaire. Inclusion criteria were as follows: age 30–60 yr, adherence to type of diet for at least 1 yr, vegans avoid the consumption of all animal-based products, and an omnivorous diet should contain meat consumption at least 3 times per week or consumption of meat and processed meat 2 times per week, respectively. Exclusion criteria were as follows: body mass index ≥30 kg/m^2, type 2 diabetes, cardiovascular diseases, cancer, pregnancy, breastfeeding period, acute infection, and medication (cortisone and proton pump inhibitors). Detailed characteristics of the study population are provided in Supplemental Table S2. Venous blood was collected from all participants after fasting for a minimum of 8 h and fractionated into serum, plasma, and erythrocytes and stored at −80°C. Methionine was analyzed in EDTA plasma by gas chromatography–tandem mass spectrometry at Bevital (Bergen, Norway).

In a second study, biobanked human plasma samples were utilized from a previous study by Kochlik *et al.* (25) investigating the impact of dietary habits on muscle biomarkers. Ethical approval was provided by the ethics committee of the University of Potsdam, Potsdam, Germany (36/2016). Briefly, male and female participants were divided according to their nutritional habits into a vegetarian or an omnivore group (Supplemental Fig. S2, flow chart). Baseline blood samples were taken from omnivores and vegetarians. After baseline, the omnivore group followed a vegetarian diet (no meat or fish) for 4 d, and a blood sample was taken. The exclusion criterion was body fat mass ≥35% (body mass index: 18.5–25.7 kg/m^2). Plasma adiponectin, FGF21, and leptin concentrations were determined by specific ELISAs (Supplemental Table S3).

Determination of adiponectin, FGF21, insulin, and leptin in mice

Plasma adiponectin (total and high MW), FGF21, insulin, and leptin concentrations were determined by specific ELISAs (Supplemental Table S3) (2, 3, 6).

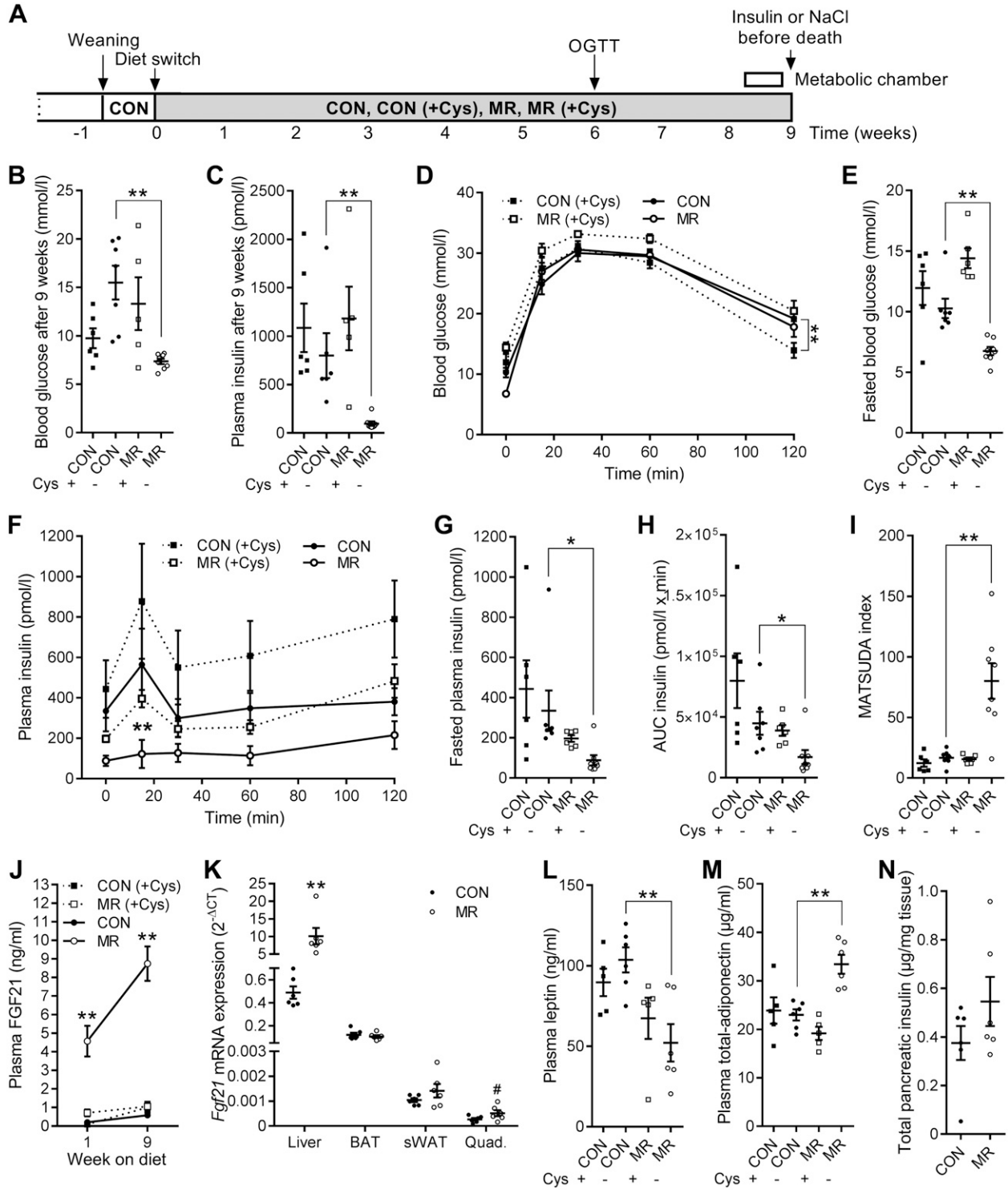

Figure 1. MR prevents hyperglycemia and improves glucose homeostasis in NZO mice. *A*) Study design. At the age of 3 wk, NZO mice were placed on a Con diet without cystine, or a Con (+Cys) diet for 1 wk, at which point a random subgroup of animals was transferred to an MR diet [MR or MR (+Cys), respectively] for 9 wk. *B, C*) Final random blood glucose (*B*) and plasma insulin concentrations (*C*). Six weeks after the dietary switch, an OGTT (glucose 2 mg/g body weight by oral gavage) was performed in mice unfed for 6 h. *D, E*) Blood glucose (*D*) and fasting blood glucose (*E*) during OGTTs. *F–H*) Plasma insulin (*F*), fasting plasma insulin (*G*), and area under the curve of insulin levels (*H*) during OGTTs. *I*) Insulin sensitivity index calculated by Matsuda. *J*) Circulating FGF21 levels. *K*) Final gene expression of *Fgf21* in indicated tissues. *L, M*) Final circulating leptin (*L*) and total adiponectin levels (*M*) assessed by ELISA. *N*) Final total pancreatic insulin. Quad., quadriceps. Data are presented as means ± SEM ($n = 6$–8/group). Differences *vs.* the Con group were calculated by 1-way ANOVA [with Bonferonni *post hoc* analyses (*B, C, E, G–I, L, M*)], 2-way ANOVA [with Bonferonni *post hoc* analyses (*D, F, J*)], and a 2-tailed Student's *t* test (*K, N*). $^{\#}P \leq 0.1$ and $^{\#}P < 0.05$; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

Pancreatic insulin content

For detection of pancreatic insulin content, whole pancreas was homogenized in ice-cold acidic ethanol (0.1 M HCl in 70% v/v ethanol) and incubated for 24 h at 4°C. After centrifugation (16,000 *g*, 10 min), insulin was detected by ELISA (Supplemental Table S3).

Hematoxylin and eosin staining

Formalin-fixed and paraffin-embedded liver sections (2 μm) were stained with hematoxylin and eosin (MilliporeSigma, Burlington, MA, USA). Microscopic images were captured with the Keyence BZ-9000 microscope (Keyence, Osaka, Japan).

Western blot analysis

Western blot analysis was performed as previously described (7, 26) using 20 μg protein per sample solution. A detailed list of antibodies is provided in Supplemental Table S3.

Detection of liver triacylglycerol and glycogen concentrations

Hepatic triacylglycerol content was measured using the commercial TR-210 kit (Randox, Crumlin, United Kingdom). Quantification of hepatic glycogen content was performed as previously described (6).

Detection of plasma triacylglycerol and nonsterified fatty acid

Plasma triacylglycerol content was measured using the commercial TR0100 kit (MilliporeSigma). Plasma nonsterified fatty acid (NEFA) levels were enzymatically analyzed using the NEFA-Homologous Recombination (2) Assay (Wako Pure Chemicals, Tokyo, Japan).

Determination of methionine plasma concentration by isotope-dilution liquid chromatography–tandem mass spectrometry

In total, 5 μl of plasma was added to 45 μl methanol containing 135 pmol L-methionine-d_3 (C/D/N Isotopes, Pointe-Claire, Canada) as an internal standard. Samples were thoroughly vortexed, ultrasonicated, and centrifuged at 16,000 *g*. Supernatants were evaporated to dryness under reduced pressure using a Savant SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Methionine and its stable isotope-labeled analog were derivatized to butyl esters by addition of 100 μl 3 M HCl in 1-butanol (MilliporeSigma). Samples were heated to 65°C for 15 min followed by evaporation to dryness under reduced pressure. Dried residues of the derivatized samples were reconstituted in 500 μl methanol, thoroughly vortexed, ultrasonicated, and centrifuged at 16,000 *g*. Supernatants were subjected to liquid chromatography–tandem mass spectrometry analysis conducted with an Agilent 1260 Infinity LC system coupled to an Agilent 6490 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). Chromatographic separation was achieved on a YMC Triart C18 Plus Column (YMC, Kyoto, Japan) (3.0 × 100 mm, 3 μm) connected to a guard column (3.0 × 10 mm, 3 μm) of the same material. Water and acetonitrile, both acidified with 0.1% formic acid, were used as eluents. Ionization of eluted analytes occurred within an electrospray ion source operating in the positive ion mode. Quantification of derivatized methionine in relation to its deuterated analog was carried out using the multiple reaction monitoring approach. The mass transitions *m/z* 206.1 → 103.9 for methionine and *m/z* 209.1 → 107.0 for methionine-d_3 were used for quantification. Two further mass transitions per compound were recorded as qualifiers.

Real-time PCR

RNA extraction from liver, quadriceps, sWAT, and BAT and real-time PCR were conducted as previously described (6). Target gene expression was normalized to β-actin (liver, sWAT, quadriceps) or TATA box binding protein (BAT) as endogenous control.

Statistical analysis

Data were analyzed using the Prism 6 software (GraphPad Software, La Jolla, CA, USA) applying 1- or 2-way ANOVA (with Bonferonni *post hoc* analyses) and unpaired or paired, 2-tailed Student's *t* test. All data are expressed as means ± SEM, with values of $P \leq 0.05$ considered statistically significant. Samples were randomized, and no data were omitted. The experimenters were not blind to group assignment.

RESULTS

MR prevents hyperglycemia and improves glucose homeostasis in NZO mice

To test whether MR protects against high-fat diet–induced glucose intolerance and diabetes, 3-wk-old male NZO mice were placed on a Con diet for 1 wk. At that point, a subgroup of mice was transferred to an MR (0.17% methionine) diet for 9 wk (Fig. 1*A*). Nine weeks after diet switch, Con mice showed increased blood glucose levels (15.5 ± 1.7 mM), whereas MR mice exhibited normal blood glucose levels (7.4 ± 0.3 mM) at the end of the intervention (Fig. 1*B*). Final plasma insulin levels (Fig. 1*C*) were significantly increased in Con (799.3 ± 232.3 pM) mice compared with MR mice (96.9 ± 22.7 pM).

After 6 wk of dietary intervention, an OGTT was performed (Fig. 1*D*). Fasting blood glucose levels were lower in MR mice (6.8 ± 0.3 mM; Fig. 1*E*) compared with Con mice (10.3 ± 0.8 mM). The area under the curve did not differ between the 2 groups (unpublished results), indicating no improvement in glucose clearance. However, insulin levels during OGTTs (Fig. 1*F*) were significantly decreased in MR mice compared with Con mice. Fasting plasma insulin levels in Con mice (335.3 ± 100.8 pM; Fig. 1*G*) were higher than in MR mice (88.1 ± 25.4 pM). The area under the insulin curve (Fig. 1*H*) was decreased in MR mice (16,976.4 ± 5765.0 pM × min) compared with Con mice (44,819.8 ± 9401.0 pM × min). Insulin sensitivity, determined using the Matsuda index (27), was improved in MR mice (80.1 ± 14.6; Fig. 1*I*) in comparison with Con mice (16.8 ± 2.4).

Plasma FGF21 levels (Fig. 1*J*) were significantly increased in MR mice after 1 and 9 wk of dietary intervention. To examine which organ was responsible for the increase in FGF21, we measured *Fgf21* mRNA expression in liver, BAT, sWAT, and quadriceps (Fig. 1*K*)

after 9 wk of dietary intervention, demonstrating MR is a stimulator of hepatic FGF21. In contrast, there was no effect from diet on BAT, sWAT, and quadricep *Fgf21* mRNA expression levels (Fig. 1*K*). Plasma leptin concentrations (Fig. 1*L*) were decreased in MR mice (52.1 ± 11.6 ng/ml) in comparison with Con mice (103.6 ± 7.8 ng/ml). Adiponectin mediates metabolic effects of FGF21 on insulin sensitivity (28), and as shown in Fig. 1*M*, total plasma adiponectin was increased in MR mice (33.4 ± 2.0 μg/ml) compared with Con mice (23.0 ± 1.1 μg/ml). The high MW form of adiponectin measured by ELISA and Western blot was also increased in MR mice compared with Con mice (Supplemental Fig. S3). As a consequence, the leptin-adiponectin ratio, a measure of systemic insulin resistance (29), was significantly elevated in Con mice (leptin-adiponectin ratio: Con = $4.5 \times 10^{-3} \pm 2.5 \times 10^{-4}$; MR = $1.7 \times 10^{-3} \pm 4.3 \times 10^{-4}$). Finally, in order to confirm that MR mice are capable of synthesizing insulin and are protected from β-cell loss, we measured the total pancreatic insulin content (Fig. 1*N*), showing an increase in MR-fed mice by number compared with the Con mice.

Dietary cystine reverses the effects of MR in NZO mice

Wanders *et al.* (24) demonstrated that dietary cysteine supplementation blunts the MR-mediated FGF21 induction. In order to test if the beneficial effects of MR are dependent on FGF21, we treated a subgroup of NZO mice with an MR diet containing 0.3% cystine. All parameters described above were analyzed. First of all, similar to Wanders *et al.* (24), MR (+Cys)–fed mice did not show an increase in FGF21 plasma concentration as detected in the MR group without cystine (Fig. 1*J*), and total plasma adiponectin was not increased in MR (+Cys) mice compared with Con (+Cys) mice (Fig. 1*M*). Plasma leptin concentrations were not decreased in MR (+Cys) mice in comparison with Con (+Cys) mice (Fig. 1*L*). Final blood glucose levels and plasma insulin concentrations were not different between Con (+Cys) and MR (+Cys) groups (Fig. 1*B, C*). In addition, the insulin sensitivity (Matsuda index) during the OGTTs was not improved (Fig. 1*I*) because of higher blood glucose levels and unaffected plasma insulin concentrations (Fig. 1*D–H*) during the OGTTs in MR (+Cys) mice. Taken together, our observations suggest MR has only positive effects on glucose homeostasis and insulin sensitivity when FGF21 levels are elevated.

Switch to vegetarian diet increases FGF21 in humans

Given that vegan and vegetarian diets exhibit a lower methionine intake than an omnivore diet (14, 15), we asked whether FGF21 levels can be elevated by switching from an omnivore to a vegetarian diet in humans. First, the FGF21 plasma and methionine concentrations were analyzed in NZO mice. Plasma FGF21 levels correlated inversely ($P = 0.025$) with plasma methionine levels in NZO mice (**Fig. 2*A***). Similar to the observation made in NZO mice on an MR diet, plasma FGF21 levels were robustly increased in vegan (Fig. 2*B*) and vegetarian subjects (Fig. 2*C*) compared with omnivore subjects. Furthermore, plasma FGF21 concentrations increased significantly in omnivore humans given a vegetarian diet for 4 d (Fig. 2*D*). Expressed as percentage change from baseline, FGF21 concentrations increased by 232% in omnivore humans consuming a vegetarian diet for 4 d. Plasma FGF21 levels did not correlate with plasma methionine levels in humans (unpublished results). Interestingly, neither leptin nor adiponectin concentrations were different between the omnivore and vegetarian humans (Supplemental Fig. S4), but the leptin-to-adiponectin ratio was numerically lower in vegan compared with omnivore subjects (Supplemental Table S2). These data may suggest increased insulin sensitivity in vegan human subjects.

MR lowers weight gain and delays growth

The evaluation of body weight, body composition, and size demonstrated that MR mice were lighter than Con mice after 9 wk of dietary intervention (**Fig. 3*A***). Relative to body weight, fat and lean mass percentage did not differ between Con and MR mice at the end of the study (Fig. 3*B, C*). Additionally, MR mice were shorter than Con mice at the end of the study (Fig. 3*D*). These data suggest

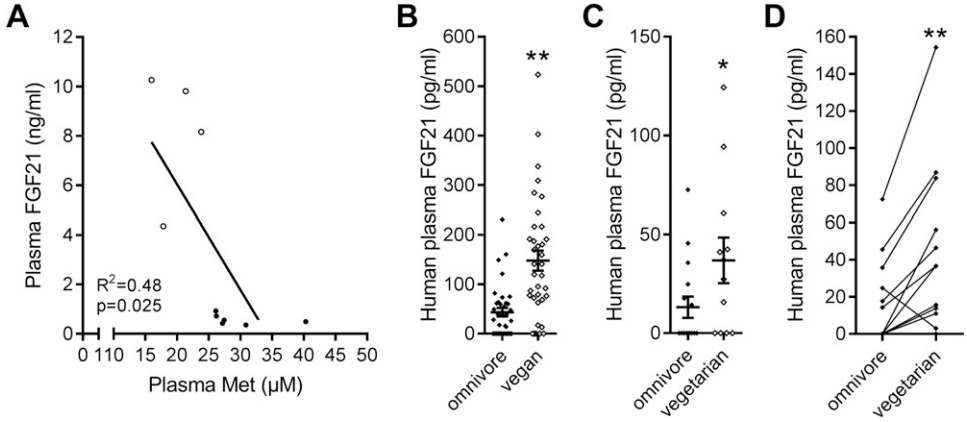

Figure 2. Switching to a vegetarian diet increases FGF21 in humans. *A*) Correlations between plasma FGF21 and methionine levels of NZO mice. *B–D*) Plasma FGF21 concentrations of omnivore ($n = 36$) or vegan ($n = 36$) humans (*B*), of omnivore ($n = 16$) or vegetarian ($n = 12$) humans at baseline (*C*), and omnivore human subjects ($n = 16$) given a vegetarian diet for 4 d (*D*). Black circles, Con; white circles, MR. Data are presented as means ± SEM. Pearson correlation (*A*); 2-tailed Student's *t* test for difference between groups (*B, C*); and paired, 2-tailed Student's *t* test (*D*). $^*P \leq 0.05$, $^{**}P \leq 0.01$.

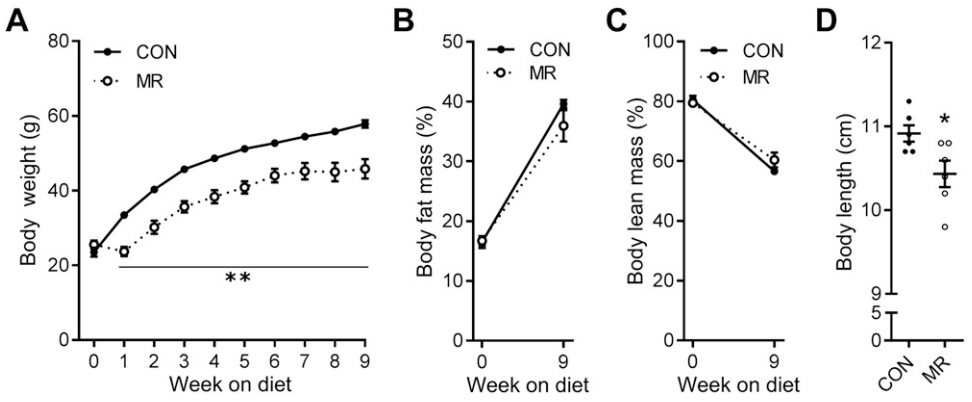

Figure 3. MR lowers weight gain and delays growth in NZO mice. Mice were treated as described in Fig. 1. *A*) Weekly body weight. *B*, *C*) Percentage body fat (*B*) and percentage body lean (*C*) at the beginning and end of the study. *D*) Final body length. Data are presented as means ± SEM (n = 6–8/group). Differences *vs.* the Con group were calculated by 2-way ANOVA with Bonferonni *post hoc* analyses (*A*–*C*) and 2-tailed Student's t test (*D*). *$P \leq 0.05$, **$P \leq 0.01$.

promotion of euglycemia by MR is not driven by reduction in body fat.

MR increases EE and improves metabolic flexibility

Recent studies have shown that FGF21 is required for MR-induced effects on EE (10) and that pharmacological FGF21 treatment increases EE in NZO mice (6). As expected, MR mice showed an increase in EE during the dark and light periods 8 wk after the dietary intervention (**Fig. 4*A***). This effect was evident irrespective of whether EE was expressed on a per-animal basis (Supplemental Fig. S5*A*), normalized to body weight (Fig. 4*B*) or lean mass (Fig. 4*C*), and not caused by an increase in locomotor activity (Fig. 4*D*). The respiratory exchange ratio (RER) showed a higher flexibility in MR mice because it strongly decreased during the light phase (Fig. 4*E, F*), reflecting increased fatty acid oxidation. Food intake did not differ between both groups (Fig. 4*G*), but water intake (Fig. 4*H*) was increased in Con mice during the dark phase, which may reflect increased blood glucose and subsequent polyuria.

Dietary cystine reversed the increased EE of MR mice. EE was not increased in MR (+Cys) compared with Con (+Cys) mice, irrespective of whether EE was expressed on a per-animal basis, normalized to body weight or lean mass (Supplemental Fig. S5*B*). Furthermore, the RER showed no difference between these 2 groups (Supplemental Fig. S5*C*).

MR decreases hepatic fat storage and elevates expression of browning markers in sWAT

Final liver weight was significantly lower in the MR than in Con mice (**Fig. 5*A***), possibly related to decreased triacylglycerol (Fig. 5*B*) and glycogen (Fig. 5*C, D*) concentrations. The former might explain the reduced triacylglycerol concentrations in the plasma of MR mice (Fig. 5*E*). Histologic assessment revealed morphologic changes that are consistent with triacylglycerol accumulation in Con mice (Fig. 5*F*). Surprisingly, no differences were observed between the groups in gonadal white adipose tissue and BAT mass. sWAT mass was lower in MR mice (Fig. 5*A*) compared with Con mice, which may be related to lower leptin concentrations in MR mice (Fig. 1*L*). Weights of quadriceps, brain, and pancreas mass showed no differences between the groups, whereas heart and kidney mass were decreased in MR mice (Fig. 5*A*). The increase in kidney mass in the Con mice may reflect the diabetic nephropathy, whereas MR may prevent renal hypertrophy (30, 31). In relation to body weight, heart mass did not differ between the groups (unpublished results).

Pharmacological FGF21 treatment is known to induce thermogenic and lipogenic genes in BAT and sWAT, which is prone to browning (6). Therefore, we tested whether the increase in EE caused by MR was associated with changes in thermogenic and lipogenic markers in BAT and sWAT. Even though we could not observe an increase in sWAT mass in MR mice, the sWAT thermogenic genes uncoupling protein 1 and cell death–inducing DFFA-like effector A were robustly increased, and PR/SET domain 16 mRNA expression tended to be higher in MR mice (Supplemental Fig. S6*A*), which provides strong evidence for sWAT browning. Feeding the MR diet increased the mRNA expression of genes associated with lipogenesis and glucose uptake within the sWAT (stearoyl-CoA desaturase, fatty acid synthase, and glucose transporter 1 by trend; Supplemental Fig. S6*B*). At the same time, BAT thermogenic gene cell death–inducing DFFA-like effector A was robustly increased, and uncoupling protein 1 tended (P = 0.11) to be higher in MR mice (Supplemental Fig. S6*C*). Lipogenic genes (stearoyl-CoA desaturase, fatty acid synthase) were greatly elevated, and acetyl-CoA carboxylase 1 and glucose transporter 4 tended to be higher in response to MR feeding (Supplemental Fig. S6*C, D*). These data indicate that MR-induced FGF21 might induce browning and thermogenic effects in NZO mice, accounting for the increase in EE.

Dietary MR improves hepatic insulin sensitivity

In order to test whether MR improves insulin sensitivity and might explain the improvement in blood glucose levels, all mice were treated with NaCl or insulin before killing, and stimulation of Akt phosphorylation as read

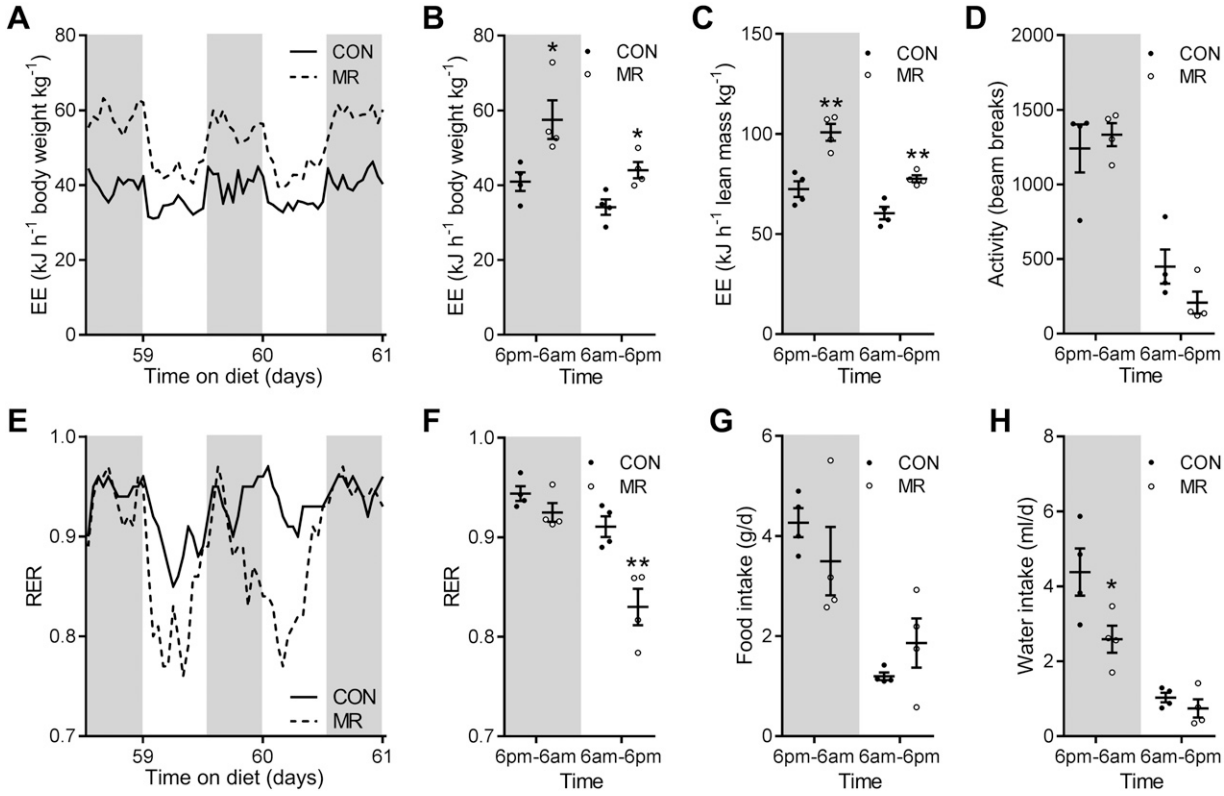

Figure 4. MR increases EE in NZO mice. Mice were treated as described in Fig. 1. *A*) EE normalized to body weight in NZO mice consuming Con or MR diets for 8 wk. *B–D*) Mean EE normalized to body weight (*B*) or lean mass (*C*), and mean activity (*D*) for indicated periods throughout the 8 wk after diet switch. *E–H*) RER (*E*), mean RER for indicated periods (*F*), mean food intake (*G*), and water intake (*H*) for indicated periods throughout the eighth week after diet switch. Data are presented as means ± SEM ($n = 4$/group). Differences *vs.* the Con group were calculated by a 2-tailed Student's *t* test (*B–D*, *F–H*). $^*P \leq 0.05$, $^{**}P \leq 0.01$.

out from insulin signaling pathway was measured. Insulin response (phosphorylation of Akt) in the liver, quadriceps, and sWAT was increased in both Con and MR mice, but it was improved to a much higher extent in the MR group (Fig. 5*G* and Supplemental Fig. S7*A*, *C*). In the BAT, insulin sensitivity was significantly improved only in MR mice (Supplemental Fig. S7*B*).

Hepatic glycogen content was reduced in MR mice (Fig. 5*C*). In order to test whether a reduced gluconeogenesis rate might protect against hyperglycemia, as hypothesized in our previous study (7), we measured hepatic phosphoenolpyruvate carboxykinase (PEPCK). Surprisingly, as shown in Fig. 5*H*, the protein expression of PEPCK is increased in MR mice, pointing to an increased gluconeogenesis rate in the normoglycemic MR mice.

DISCUSSION

In general, improvement in metabolic health can be achieved by restriction of energy intake (32, 33), protein, (7) or single amino acids (34–36). As shown in rodent models, restrictions of branched-chain amino acids (leucine, isoleucine, and valine) and methionine elicit positive metabolic effects, such as improving whole-body glucose metabolism (34–36). Compelling literature indicates in nondiabetic rodent models that MR improves insulin sensitivity, decreases body and fat mass, and increases EE, with FGF21 as the underlying mechanism (10, 11, 13, 36). As recently demonstrated by our group, FGF21 treatment (6) and dietary PR-induced FGF21 (7) prevented hyperglycemia and diabetes in NZO mice. Here, we demonstrate for the first time that MR prevents the onset of hyperglycemia in obese, diabetes-susceptible NZO mice, presumably *via* increased plasma adiponectin, independent of food intake and adiposity. The beneficial effects were mediated by FGF21 because, in the presence of cystine, MR did not increase FGF21 and did not improve glucose homeostasis. Dipeptide cystine can be reduced to cysteine, which was shown to abolish the effects of MR on improved glucose tolerance and insulin sensitivity because it reverses the MR-mediated reduction in hepatic glutathione, thereupon blunting the increase in circulating FGF21 (13, 24). Consequently, the MR-mediated increase in EE, reduction in fasting insulin, and increase in adiponectin levels were reversed by cysteine supplementation (24, 37). This is precisely what we observed in our study. Adding cystine to the diet abolished the MR-induced rise in circulating FGF21, prevented the increase in EE, and reversed the reduced plasma insulin levels and the increased adiponectin levels. Thus, FGF21 appears to be the major trigger responsible for the beneficial effects of MR.

FGF21 has been shown to enhance both the expression and secretion of adiponectin in adipocytes (38). As expected,

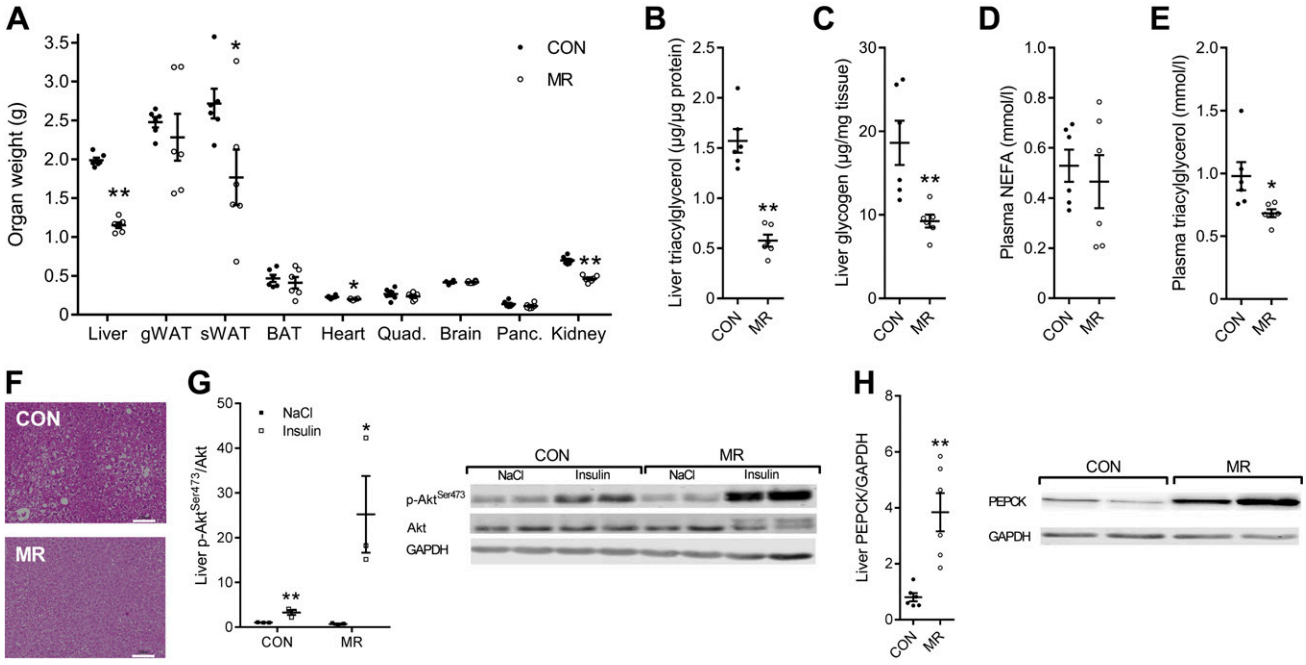

Figure 5. Dietary MR decreases hepatic fat storage and improves hepatic insulin sensitivity in NZO mice. Mice were treated as described in Fig. 1. Nine weeks after the diet switch, mice unfed for 6 h were treated intraperitoneally with NaCl or insulin (7 IU/kg body weight) 15 min before killing. *A*) Final weight of indicated organs. *B*, *C*) Final liver triacylglycerol (*B*) and glycogen content (*C*). *D*, *E*) Final plasma NEFA (*D*) and triacylglycerol (*E*). *F*) Hematoxylin and eosin staining of liver revealed morphologic changes consistent with triacylglycerol accumulation in Con mice. Scale bars, 100 µm. *G*, *H*) Western blots of total and phosphorylated Akt (*G*) and total PEPCK (*H*) in liver. Detecting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. gWAT, gonadal white adipose tissue; Panc., pancreas; Quad., quadriceps. Data are presented as means ± SEM (n = 3–6/group). Differences between the groups were calculated by a 2-tailed Student's t test. *$P \leq 0.05$, **$P \leq 0.01$.

NZO mice with increased circulating FGF21 exhibit increased adiponectin levels. Many studies have clearly shown that adiponectin has insulin-sensitizing effects (39, 40). Therefore, the improvement of peripheral insulin sensitivity observed by Matsuda (Fig. 1*I*) might be explained by the activated FGF21-adiponectin axis in these mice. However, it was shown by Stone *et al.* (41) that adiponectin is not necessary to improve the insulin sensitivity by MR. Thus, the question of how important the activated FGF21-adiponectin axis is for promoting euglycemia in NZO mice through a MR diet remains to be answered.

In addition to hepatic glutathione being critical to *Fgf21* transactivation (24), hepatic glutathione has been shown to activate the phosphatase and tensin homolog, which degrades phosphatidylinositol (3–5)-trisphosphate and therefore decreases Akt activation (13). In contrast, lower glutathione by MR diets (due to decreased cystine) amplifies hepatic insulin-dependent signaling, based on reduced degradation of phosphatidylinositol (3–5)-trisphosphate, which might explain the increased hepatic Akt phosphorylation (in addition to FGF21) in this study. Taken together, MR is not preventing hyperglycemia *per se* but only in the context of decreased cystine availability, which then robustly activates the FGF21-adiponectin axis in NZO mice.

Protein and MRs are known to increase whole-body EE, which is mediated by FGF21 and associated with activation of BAT and browning of sWAT (2, 3, 5, 24). Recently shown in NZO mice, exogenous FGF21 treatment increases EE and induces thermogenic markers in sWAT (6). Consistent with this study, increased FGF21 levels, in response to MR, induced browning of sWAT in NZO mice. This might explain the increase in EE, given that the BAT mass did not differ between the groups. Interestingly, protein-restricted NZO mice did not show browning of sWAT, despite increased FGF21 levels, but exhibited an elevated BAT mass, which explained higher EE (7). It can be speculated that the higher magnitude of FGF21 induction on MR diet (similar to exogenous FGF21 treatment) might be the reason for this difference. Whereas plasma FGF21 levels reached ~4 ng/ml under PR (7), MR generated plasma FGF21 levels around 9 ng/ml (Fig. 1*J*). This is mirrored by an ~10-fold increase in hepatic *Fgf21* mRNA expression on PR relative to control-fed mice (7) and an ~20-fold increase in *Fgf21* mRNA expression in the liver on MR relative to Con mice. Additionally, the increase in EE with no changes in energy intake between the groups might cause the lower body and liver weight in MR mice. The latter can be explained by a reduction in triacylglycerols and glycogen, which serve as a substrate for nonshivering thermogenesis. FGF21 is known to inhibit growth hormone signaling and thereby growth in general (42), effects that were also visible in this study.

Besides increased EE, improved metabolic flexibility might contribute to the improved insulin sensitivity, a beneficial antidiabetic effect of MR. Whereas MR-fed mice show a pronounced rhythm in substrate utilization in response to light-dark cycles (Fig. 4*E*, *F*), Con-fed mice are

not able to oxidize fatty acids like the MR-fed mice during the light cycle. This is in accordance with rats fed an MR diet switching between glucose (RER ranging around 1.0) and fatty acid (RER toward 0.7) utilization during light and dark cycles, respectively (43). A similar effect of an improved metabolic flexibility in NZO mice was observed by intermittent food withdrawal, which enhanced lipid metabolism and prevented diabetes (33). The high rate of energy metabolism due to browning of sWAT and the capacity to switch between substrate utilization might therefore account for the suppression of diabetes.

Moreover, dysregulated gluconeogenesis and glycogenolysis induces hyperglycemia (44, 45). Interestingly, humans with type 2 diabetes and NZO mice (prone to develop diabetes) show an increased gluconeogenesis rate (46–48). We concluded in a recent study with NZO mice (7) that the prevention of diabetes through PR required reduced gluconeogenesis. As demonstrated by Stone *et al.* (13) using hyperinsulinemic-euglycemic clamps, the suppression of hepatic glucose production by insulin was greater in MR-fed mice compared with controls. In this study, MR-fed mice showed the lowest glycogen levels in the liver. The hepatic PEPCK protein, however, which catalyzes a rate-limiting step in hepatic gluconeogenesis, was increased. Insulin is known to have an inhibitory effect on *Pepck* mRNA expression. Therefore, with the lowest plasma insulin levels in mice fed an MR diet (Fig. 1C), the inhibitory effect of insulin on *Pepck* gene expression might be reduced, causing an increase in PEPCK expression.

Several studies suggest that plant-based diets have protective effects for human health because of protective phytochemicals and reduced fat and protein contents (16, 49). In addition, vegan food has the lowest amount of methionine per total protein content compared with beef, dairy, and eggs, which may be associated with health benefits (including lower rates of diabetes) (15, 16). Given that the considerably lower intake of methionine in vegans (15) does not elicit a significant decrease of plasma methionine levels, it is not surprising that no correlation between FGF21 and methionine could be observed in humans in this study. Greater insight into potential associations between plasma methionine and FGF21 in humans may be achieved by analyzing postprandial samples, but not after fasting for 8 h, when homeostatic regulation of blood amino acid levels takes place as a result of a decreased liver and muscle amino acid metabolism to buffer against dramatic decreases in circulation (50, 51). However, based on the increased circulating FGF21 levels in vegan and vegetarian humans and the observed FGF21 induction by the switch to a vegetarian diet in omnivore humans, it is tempting to speculate that the normoglycemic phenotype in vegans and vegetarians is caused by induced FGF21. In this study, the FGF21 induction was lower in humans on a vegetarian diet compared with a vegan diet, likely reflecting the consumption of dairy products and eggs in the vegetarian group. Therefore, a strict MR diet (*e.g.*, a vegan diet) may be a beneficial nutritional strategy in type 2 diabetes patients and should be further investigated.

CONCLUSIONS

In summary, we demonstrate for the first time superior efficacy of dietary MR in preventing the onset of diet-induced diabetes in male NZO mice. This protective effect is not caused by hypophagia or loss of body and fat mass but rather by an increase in plasma adiponectin levels and EE, presumably due to browning of sWAT. The protection from hyperglycemia and diabetes is dependent on FGF21 and not on MR *per se*. MR-mediated increase in FGF21 might contribute to the normoglycemic phenotype in vegans. Finally, patients at high risk of developing type 2 diabetes may benefit from dietary changes in favor of MR without protein and calorie restriction. FJ

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AUTHOR CONTRIBUTIONS

T. Castaño-Martinez, F. Schumacher, S. Schumacher, R. Biemann, A. McCann, K. Abraham, C. Weikert, and T. Laeger made substantial contributions to the acquisition of data; T. Castaño-Martinez and T. Laeger drafted the manuscript; T. Castaño-Martinez, F. Schumacher, S. Schumacher, B. Kochlik, D. Weber, T. Grune, R. Biemann, A. McCann, K. Abraham, C. Weikert, B. Kleuser, A. Schürmann, and T. Laeger made substantial contributions to the analysis and interpretation of data and critically revised the manuscript for important intellectual content; T. Castaño-Martinez and T. Laeger made substantial contributions to the conception and design; T. Laeger is the guarantor of this work; and all authors gave final approval of the version of the manuscript to be published.

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