The Gly385(388)Arg Polymorphism of the FGFR4 Receptor Regulates Hepatic Lipogenesis Under Healthy Diet

Stefan Z. Lutz,[1,2,3] Anita M. Hennige,[2,3] Andreas Peter,[1,2,3] Marketa Kovarova,[1,2,3] Charisis Totsikas,[1] Jürgen Machann,[2,3,4] Stefan M. Kröber,[5] Bianca Sperl,[6] Erwin Schleicher,[1,2,3] Fritz Schick,[4] Martin Heni,[1,2,3] Axel Ullrich,[6] Hans-Ulrich Häring,[1,2,3] and Norbert Stefan[1,2,3]

[1]Department of Internal Medicine, Division of Endocrinology, Diabetology, Vascular Disease, Nephrology and Clinical Chemistry, University of Tübingen, 72076 Tübingen, Germany; [2]Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University of Tübingen, 72076 Tübingen, Germany; [3]German Center for Diabetes Research, 85764 Munich-Neuherberg, Germany; [4]Section on Experimental Radiology, Department of Diagnostic and Interventional Radiology, University of Tübingen, 72076 Tübingen, Germany; [5]Institute of Pathology, University of Tübingen, 72076 Tübingen, Germany; and [6]Department of Molecular Biology, Max-Planck-Institute of Biochemistry, 82152 Martinsried, Germany

ORCiD numbers: 0000-0003-4655-9937 (H.-U. Häring).

Context: The effect of a lifestyle intervention to reduce liver fat content in nonalcoholic fatty liver disease in humans is influenced by genetics. We hypothesized that the amino acid exchange in human Gly388Arg (mouse homolog: Gly385Arg) in fibroblast growth factor receptor 4 (FGFR4), which regulates bile acid, lipid, and glucose metabolism, could determine hepatic lipid accumulation and insulin sensitivity. Mechanisms of this substitution were studied in mice under normal chow and high-fat diets.

Design: In humans, the Gly388Arg polymorphism was studied for its relationship with changes in liver fat content and insulin sensitivity during 9 months of a lifestyle intervention. We also studied a knock-in mouse strain with an Arg385 allele introduced into the murine *FGFR4* gene under normal chow and high-fat diets.

Results: In humans, the *FGFR4 Arg388* allele was not associated with liver fat content or insulin sensitivity in subjects who were overweight and obese before lifestyle intervention. However, it was associated with less decrease in liver fat content and less increase in insulin sensitivity during the intervention. In mice receiving normal chow, the *FGFR4 Arg385* allele was associated with elevated hepatic triglyceride content, altered hepatic lipid composition, and increased hepatic expression of genes inducing *de novo* lipogenesis and glycolysis. Body fat mass and distribution, glucose tolerance, and insulin sensitivity were unaltered. The *FGFR4 Arg385* allele had no effect on glucose or lipid metabolism under the high-fat diet.

Conclusion: Our data indicate that the *FGFR4 Arg388(385)* allele affects hepatic lipid and glucose metabolism specifically during healthy caloric intake. (***J Clin Endocrinol Metab*** **104: 2041–2053, 2019)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA
Copyright © 2019 Endocrine Society
Received 20 July 2018. Accepted 7 December 2018.
First Published Online 12 December 2018

Abbreviations: acyl-CoA, acetyl-coenzyme A; Arg, arginine; BMI, body mass index; DGAT2, diacylglycerol acyltransferase 2; *Fasn*, fatty acid synthase; FGFR4, fibroblast growth factor receptor 4; *Gck*, glucokinase; Gly, glycine; HFD, high-fat diet; *IL-6*, interleukin-6; i.p., intraperitoneal; LI, lifestyle intervention; NAFLD, non-alcoholic fatty liver disease; NEFA, non-esterified fatty acid; OGTT, oral glucose tolerance test; *Socs3*, suppressor of cytokine signaling-3; *Srebp1*, sterol regulatory element-binding protein 1; STAT3, signal transducer and activator of transcription-3; TAG, triacylglyceride.

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Nonalcoholic fatty liver disease (NAFLD) is strongly associated with insulin resistance and is an important risk factor for the development of type 2 diabetes mellitus and cardiovascular disease (1–6). Strategies to prevent and treat NAFLD are not fully understood. Lifestyle intervention (LI) in general is an effective and safe strategy to treat NAFLD; however, not all participants benefit to the same extent (7), indicating that the success of NAFLD treatment may depend on genetic variation. In humans, we found that variations in the peroxisome proliferator-activated receptor (PPAR)-δ gene, which encodes a nuclear transcription factor controlling fatty acid oxidation and energy uncoupling, determined changes in hepatic fat content during an LI (8). Furthermore, we found that cardiorespiratory fitness was an independent predictor of reduction in liver fat content during an LI (9). In addition, presence of the -8503 A and -1927 C alleles in the gene encoding the adiponectin receptor 1 (*ADIPOR1*) predicted changes in liver fat content and insulin sensitivity during an LI (10).

Because lack of the fibroblast growth factor receptor 4 (FGFR4) in mice resulted in features of the metabolic syndrome, such as hyperlipidemia, glucose intolerance, and insulin resistance, but unexpectedly protected from hepatic steatosis (11), we hypothesized that *FGFR4* may be another candidate gene of fatty liver affecting the dynamics of liver fat accumulation in humans. FGFR4 belongs to a family of transmembrane receptor protein tyrosine kinases that includes five members: FGFR1 to FGFR5 (12, 13). FGFRs 1 to 4 are single-pass transmembrane tyrosine kinase receptors consisting of a ligand-binding extracellular domain, a single transmembrane domain, and an intracellular kinase domain (14, 15), whereas the recently identified FGFR5 lacks the intracellular tyrosine kinase domain (13). FGFRs are activated by fibroblast growth factors, which are involved in a variety of biological responses such as embryonic development, cell proliferation and migration, angiogenesis, and glucose and lipid homeostasis (16). FGFR4 plays an essential role in suppressing bile acid synthesis and promotes hepatic steatosis under a high-fat diet (HFD). This involves its ligands, the human FGF19, and its mouse ortholog FGF15, which display hormonelike effects (11, 17, 18). Most recently, an FGF19 analogue was shown to reduce liver fat content in patients with nonalcoholic steatohepatitis (19), a finding indicating that FGFR4 may also be important for the regulation of liver fat content in humans.

The polymorphism in codon 388 in the human *FGFR4* (mouse homolog *FGFR4* codon 385) results in the amino acid exchange of glycine (Gly^{388}) to arginine (Arg^{388}) in the transmembrane domain of the tyrosine kinase and is associated with cancers, such as cancer of the breast, prostate, colon, lung, and skin (20–22). The global minor allele frequency of this common variant is 0.30. However, among patients with cancer, its prevalence is increased up to 50% (20, 23). We previously found that the *FGFR4* Gly388Arg substitution induced hypersecretion of insulin in mouse islets and was associated with lower glucose levels in humans (24). Furthermore, the Gly388Arg substitution was associated with activation of the signal transducer and activator of transcription-3 (STAT3) in mice (25) which, when activated, suppresses hepatic gluconeogenic gene expression (26) and induces hepatic lipogenic gene expression (27), pointing to a gain-of-function effect of this amino acid exchange.

Therefore, in the current study, we investigated the role of the human *FGFR4* Gly388Arg (mice: Gly385Arg) polymorphism in the regulation of hepatic lipid metabolism and glycemic traits before and after an LI in humans and studied mechanisms of action of this amino acid substitution under normal chow and an HFD in mice.

Materials and Methods

Human data

Subjects

A total of 170 subjects without diabetes from Southern Germany were studied. These individuals participated in a 9-month LI study to reduce adiposity and prevent type 2 diabetes mellitus and had measurements of liver fat content before and after 9 months of the LI (3, 28). For inclusion in the study, at least one of the following criteria had to be fulfilled by the participants: a family history of type 2 diabetes mellitus, previous diagnosis of impaired glucose tolerance or gestational diabetes, or a body mass index (BMI) >27 kg/m^2. Moreover, after a standard questionnaire, physical examination, and routine laboratory tests, the participants had to be ascertained as healthy, especially without any history of liver disease.

As assessed by means of a standard questionnaire, participants did not consume more than two alcoholic drinks per day. After the baseline measurements, individuals underwent a 9-month LI composed of dietary counseling with up to 10 sessions with a dietician and at least 3 hours of aerobic endurance exercise per week, as described in detail in (28). Counseling was aimed at reducing body weight by $\geq$5%. Furthermore, participants were advised to reduce energy intake from fat to $<$30% of total energy consumed and to increase dietary fiber intake to at least 15 g/4185 kJ (1000 kcal). They were also encouraged to reduce the intake of saturated fatty acids to $<$10% of total fat. During each visit, participants presented a 3-day food diary and discussed it with the dietitians. Recommended aerobic endurance exercise was walking or swimming with an only moderate increase in heart rate. Informed written consent was also obtained from these participants, and the Ethics Committee of the University of Tübingen approved the protocol. Some of the results from these analyses was also

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presented in a doctoral thesis of the coauthor (C.T.; https://publikationen.uni-tuebingen.de/xmlui/handle/10900/45745).

Anthropometrics, liver fat content, total body fat mass, and body fat distribution

BMI was calculated as weight divided by the square of height (kg/m^2). Waist circumference was measured at the midpoint between the lateral iliac crest and lowest rib. Liver fat content was measured by localized proton magnetic resonance spectroscopy as previously described (29). Total body and visceral fat mass were measured by magnetic resonance tomography using an axial T1-weighed fast spin-echo technique.

Oral glucose tolerance test

The 170 individuals underwent a 2-hour 75-g oral glucose tolerance test (OGTT). We obtained venous plasma samples at 0, 30, 60, 90, and 120 minutes for determination of plasma glucose and insulin levels. Whole-body insulin sensitivity was calculated from glucose and insulin values obtained at five time points (0, 30, 60, 90, and 120 minutes) during a 75-g OGTT, as proposed by Matsuda and DeFronzo (30) (10,000 / $\sqrt{}$ (mean insulin × mean glucose) × (fasting insulin × fasting glucose). Furthermore, adipose tissue insulin resistance (fasting insulin × fasting free fatty acids) was calculated (31, 32).

Genotyping

For genotyping, DNA was isolated from whole blood using a commercial DNA isolation kit (NucleoSpin; Macherey & Nagel, Düren, Germany). The single-nucleotide polymorphism rs351855, a G to A exchange leading to substitution of Gly by Arg at position 388 of FGFR4, was genotyped using TaqMan assay (Applied Biosystems, Foster City, CA) (24). The overall genotyping success rate was 99.1%. Rescreening of 3.1% of the subjects gave 100% identical results.

Experimental animals

The generation of mice carrying the *FGFR4 Arg385* allele was described previously (33). In brief, to replace the Gly codon in position 385 with an Arg codon in the murine *FGFR4* gene, a *knock-in* mouse strain was generated in the genetic background of SV/129 mice. Twelve-week-old male *FGFR4 Gly/Arg385* or *FGFR4 Arg/Arg385* mice were compared with litter-matched *FGFR4 Gly/Gly385* control mice. Mice (4 weeks old) were kept on a 12-hour/12-hour light/dark cycle (lights on at 6:00 AM) and were allowed access to regular chow *ad libitum* (Diet #1310; Altromin, Lage, Germany) or to an HFD (D12451; Research Diets, New Brunswick, NJ) for 8 weeks. The HFD contained 45% kcal as fat. All animal procedures were performed in accordance with the guidelines of laboratory animal care and were approved by the local governmental commission for animal research.

Western blotting

For the analysis of insulin signal transduction, after an overnight fast, anesthetized mice were injected in the inferior vena cava with 2 units of human insulin. For Western blotting, after 7 minutes liver and skeletal muscle tissues were lysed at 4°C in lysis buffer containing 2 mM EDTA, 137 mM NaCl, 1% NP-40, 10% glycerol, 12 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, and 10 mg/μL leupeptin and aprotinin for 30 minutes and precleared by centrifugation at 12,000*g* for 20 minutes at 4°C. Western blot analysis of tissue lysates was done with antibodies against phospho-Akt [(34); Ser173; #9271; Cell Signaling Technology, Beverly, MA] and glyceraldehyde-3-phosphate dehydrogenase [(35); #2118; Cell Signaling Technology]. Signals were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

Analysis of body fat mass and liver fat content in mice

For MRI of fat mass, mice were anesthetized and measured on a 3T whole-body magnetic resonance analyzer (Magnetom Trio; Siemens Healthcare, Erlangen, Germany) applying a T1-weighted fast spin-echo technique. Mice were placed in prone position in the wrist coil of the system. Images were recorded with an in-plane spatial resolution of 0.25 mm and a slice thickness of 2 mm. Postprocessing was done as described (36). For the histological detection of liver fat content, liver tissue was fixed in 4% paraformaldehyde in PBS. Hematoxylin and eosin staining of paraffin liver sections was carried out for analysis of fat content by visual inspection of vesicles (37). To determine the liver fat content by clinical chemical methods, triacylglycerides (TAGs) were quantified in the homogenate using the ADVIA 1800 Clinical Chemistry System analyzer (Siemens Healthcare Diagnostics, Eschborn, Germany) and calculated as mg/100 mg tissue.

Analysis of fatty acid composition

For determination of the fatty acid composition of mouse hepatic TAGs, snap frozen tissue samples were homogenized in PBS containing 1% Triton X-100 with a TissueLyser (Qiagen, Hilden, Germany). The TAG fraction was isolated from the liver homogenate extracts using thin-layer chromatography. Transesterification of the fatty acids and quantification by gas chromatography with flame ionization detection were performed as previously described (37).

Analysis of mouse blood samples

Blood glucose was measured from tail bleeds using the Glucometer Elite (Bayer, Elkhart, IN). For insulin measurements, a radioimmunoassay (Linco Research, St. Charles, MO) was used. Nonesterified fatty acids (NEFAs) were analyzed in the EDTA-plasma collected after decapitation. NEFA concentrations were detected using an enzymatic method (WAKO Chemicals, Neuss, Germany) on the clinical chemistry analyzer ADVIA 1800 (Siemens Healthcare Diagnostics).

Analysis of glucose homeostasis and insulin responsiveness in mice

A glucose tolerance test was performed in mice that were fasted overnight. Mice were injected intraperitoneally (i.p.) with 2 g/kg body weight α-D-glucose. Blood was collected immediately before the application and 15, 30, 60, and 120 minutes after injection. Plasma glucose was determined as described previously. To measure glucose-stimulated insulin release, mice were injected i.p. after an overnight fast with 3 g/kg body weight α-D-glucose. Blood was collected immediately before the

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application and at 2, 5, 10, and 30 minutes after injection. To determine insulin sensitivity, an insulin tolerance test was performed, with mice receiving 1 U/kg body weight insulin i.p. (Actrapid, Novo Nordisk, Denmark). Blood was collected immediately before the application and at 15, 30 and 60 minutes after injection.

Gene expression analysis

For quantification of mRNA expression in mouse liver, tissues were frozen in liquid nitrogen. Total RNA was extracted with the AllPrep Mini Kit (Qiagen) according to the manufacturer's instructions. After treatment with RNase-free DNase I, total RNA was transcribed into cDNA using the First-Strand cDNA Kit from Roche Diagnostics (Mannheim, Germany). Quantitative PCR was performed on a LightCycler 480 (Roche Diagnostics) using Probes Master and fluorescent probes from the Universal Probe Library (Roche Diagnostics). Primers were obtained from TIB MOLBIOL (Berlin, Germany). Primer sequences can be provided upon request. Measurements were performed at least in duplicate. RNA content was normalized for the housekeeping gene *RPS13* using the $\Delta\Delta$Ct method.

Statistical analysis

Data are expressed as mean ± SEM, and the number of independent experiments or mice is indicated in the respective figure legends. Groups were compared by the Cochran-Armitage test for trend based on the linear regression model, where the significance of linear trends across the three genotypes, *Gly385(388)Gly*, *Gly385(388)Arg*, and *Arg385(388)Arg,* was tested using a dose-response effect (here, increasing numbers of the *Arg* allele), assuming a codominant and a dominant mode of inheritance or two-tailed unpaired *t* tests, as appropriate. *P* values <0.05 were considered statistically significant. The statistical software package JMP 13.0 (SAS Institute Inc, Cary, NC) was used.

Results

Humans carrying the FGFR4 Arg385 allele were more resistant to reduction of liver fat content and improvement in insulin sensitivity during the LI

The demographic and metabolic characteristics of the population at baseline and at follow-up are presented in Table 1. During a follow-up period of 9 months, there were decreases in body weight, total body fat, intramyocellular fat, and visceral fat. The largest change was found for liver fat content (−29%). Moreover, fasting as well as 2-hour glycemia and insulinemia and adipose tissue insulin resistance decreased, and whole-body insulin sensitivity increased. At baseline, the *FGFR4* Gly388Arg polymorphism was not associated with liver fat content, body fat mass, body fat distribution, or insulin sensitivity. However, during the LI, individuals carrying the minor *388Arg* allele had both less decrease in fasting and 2-hour insulinemia and less decrease in 2-hour glycemia and adipose tissue insulin resistance (Table 2) than individuals who were homozygous for the *388Gly* allele. Furthermore, subjects carrying the *388Arg* allele had a similar decrease in total body fat mass (Fig. 1A), but less reduction in liver fat content and less augmentation of insulin sensitivity compared with homozygous carriers of the *388Gly* allele (Fig. 1B and 1C).

Table 1. Subject Characteristics at Baseline and After 9 Months of Follow-Up in Caucasians

	Baseline	Follow-Up	*P* Value
Demographics and body composition			
Sex (male/female)	68/102		—
Age, y	46 ± 1	47 ± 1	—
Body weight, kg	85.4 ± 1.2	82.9 ± 1.2	<0.0001
Body mass index, kg/m^2	28.9 ± 0.3	28.1 ± 0.3	<0.0001
Body fat, kg	25.2 ± 0.7	23.0 ± 0.7	<0.0001
Visceral fat, kg	3.0 ± 0.1	2.6 ± 0.1	<0.0001
$IMCL_{tibialis\ anterior}$, arbitrary units[a]	4.0 ± 0.1	3.7 ± 0.1	<0.0001
Liver fat, %	5.1 ± 0.4	3.6 ± 0.3	<0.0001
Metabolic characteristics			
Fasting glucose, mM	5.23 ± 0.04	5.16 ± 0.04	0.01
2-h glucose, mM	6.87 ± 0.12	6.62 ± 0.12	0.02
Fasting insulin, pM	59 ± 3	51 ± 2	0.0002
2-h insulin, pM	480 ± 30	420 ± 33	0.007
Fasting FFA μM	655 ± 17	611 ± 15	0.02
2-h FFA, μM	84 ± 6	76 ± 10	0.002
HOMA-IR index	1.89 ± 0.09	1.60 ± 0.08	<0.0001
Adipose tissue IR, mmol/l·(μU/mL)	5.46 ± 3.76	4.61 ± 3.37	**0.0002**
Whole-body IS, arbitrary units	13.3 ± 0.5	15.1 ± 0.6	0.0001

Values represent mean ± SE. *P* is for paired differences after log transformation of nonnormally distributed parameters. Bolface indicates *P* values ≤ 0.05.

Abbreviations: FFA, free fatty acid; HOMA-IR, homeostasis model assessment of insulin resistance; IMCL, intramyocellular lipid; IR, insulin resistance; IS, insulin sensitivity.

[a]Available in 149 subjects.

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doi: 10.1210/jc.2018-01573

Table 2. Associations of Gly388Arg SNP in *FGFR4* With Changes in Subject Characteristics During the Lifestyle Intervention

	Gly/Gly		Gly/Arg		Arg/Arg		*P* Baseline		*P* for Change Between Genotypes	
	Baseline	Follow-Up	Baseline	Follow-Up	Baseline	Follow-Up	Additive	Dominant	Additive	Dominant
Demographics										
Sex (male/female)	29/54		31/39		8/9		**0.20**[a]	0.19[a]		
Age, y	46 ± 1	47 ± 1[b]	46 ± 1	47 ± 1[b]	45 ± 4	46 ± 0[b]	**0.53**	0.66		
Change in body composition										
Body weight, kg	85.8 ± 1.7	83.1 ± 1.6[b]	83.8 ± 1.8	81.7 ± 1.8[b]	89.6 ± 4.3	86.3 ± 4.2[b]	**0.60**	0.24	**0.74**	0.94
Body mass index, kg/m^2	29.0 ± 0.5	28.1 ± 0.5[b]	28.5 ± 0.5	27.8 ± 0.5[b]	30.1 ± 1.0	29.0 ± 1.0[b]	**0.71**	0.83	**0.83**	0.95
Body fat, kg	25.9 ± 1.1	23.7 ± 1.0[b]	23.7 ± 1.1	21.7 ± 1.1[b]	28.2 ± 2.2	24.8 ± 1.9[c]	**0.65**	0.68	**0.42**	0.35
Visceral fat, kg	3.02 ± 0.20	2.61 ± 0.19[b]	2.84 ± 0.19	2.45 ± 0.19[b]	3.26 ± 0.51	2.71 ± 0.48[b]	**0.12**	0.31	**0.61**	0.95
$IMCL_{tibialis\ anterior}$, (arbitrary units)[d]	3.91 ± 0.20	3.78 ± 0.16	4.09 ± 0.20	3.73 ± 0.21[e]	3.90 ± 0.53	3.39 ± 0.47	**0.77**	0.36	**0.30**	0.30
Liver fat, %	5.11 ± 0.67	3.06 ± 0.37[b]	5.33 ± 0.65	4.11 ± 0.51[b]	4.49 ± 0.99	4.10 ± 1.29[e]	**0.90**	0.69	**0.009**	0.007
Change in metabolic characteristics										
Fasting glucose, mM	5.24 ± 0.05	5.17 ± 0.05	5.25 ± 0.06	5.15 ± 0.06	5.11 ± 0.14	5.16 ± 0.15	**0.44**	0.83	**0.58**	0.92
2-h glucose, mM	6.95 ± 0.18	6.45 ± 0.18[b]	6.93 ± 0.18	6.89 ± 0.19	6.16 ± 0.26	6.39 ± 0.26	**0.24**	0.71	**0.02**	0.006
Fasting insulin, pM	55 ± 4	46 ± 3[b]	62 ± 5	55 ± 4[e]	67 ± 8	59 ± 5	**0.17**	0.10	**0.01**	0.01
2-h insulin, pM	465 ± 41	342 ± 34[b]	506 ± 47	487 ± 60	442 ± 105	524 ± 125	**0.74**	0.35	**0.0005**	0.06
Fasting FFA, μM	674 ± 22	615 ± 20[c]	618 ± 27	589 ± 26	708 ± 68	675 ± 31	**0.68**	0.35	**0.19**	0.56
2-h FFA, μM	92 ± 11	90 ± 19	73 ± 5	61 ± 4[e]	92 ± 17	67 ± 6[e]	**0.47**	0.30	**0.77**	0.61
HOMA-IR index	1.78 ± 0.13	1.46 ± 0.10[b]	1.97 ± 0.15[e]	1.70 ± 0.14	2.10 ± 0.28	1.88 ± 0.2	**0.25**	0.13	**0.016**	0.017
Adip. IR, mmol/L·(μU/mL)	5.41 ± 4.00	4.28 ± 3.40[b]	5.32 ± 3.59	4.70 ± 3.47	6.26 ± 3.45	5.86 ± 2.60	**0.36**	**0.44**	**0.0047**	**0.011**
$Insulin\ sensitivity_{OGTT}$, arbitrary units	14.08 ± 0.78	16.75 ± 0.94[b †]	12.52 ± 0.81	13.92 ± 0.87	12.48 ± 1.82	12.38 ± 1.53	**0.29**	0.13	**0.003**	0.003

Data represent unadjusted mean ± SE. For statistical analyses, nonnormally distributed parameters were log transformed. The genotype effect at baseline and during the intervention was tested using an additive and a dominant model. Body weight, BMI, and total body fat at baseline were adjusted for age and sex. The other parameters were additionally adjusted for total body fat. For longitudinal analyses, fold-changes in the parameters (follow-up over baseline) were adjusted for the baseline parameters. Body weight, BMI, and body fat were also adjusted for age and sex. The other parameters were additionally adjusted for body fat at baseline and at follow-up. Boldface indicates *P* values ≤ 0.05.

Abbreviations: Adip. IR, adipose tissue insulin resistance; FFA, free fatty acid; HOMA-IR, homeostasis model assessment of insulin resistance; IMCL, intramyocellular lipid; SNP, single-nucleotide polymorphism.

[a] χ^2 test.

[b] $P < 0.001$ for paired differences between baseline and follow-up.

[c] $P < 0.01$ for paired differences between baseline and follow-up.

[d] Available in 149 subjects (Gly/Gly, n = 74; Gly/Arg, n = 60; Arg/Arg, n = 15).

[e] $P < 0.05$ for paired differences between baseline and follow-up.

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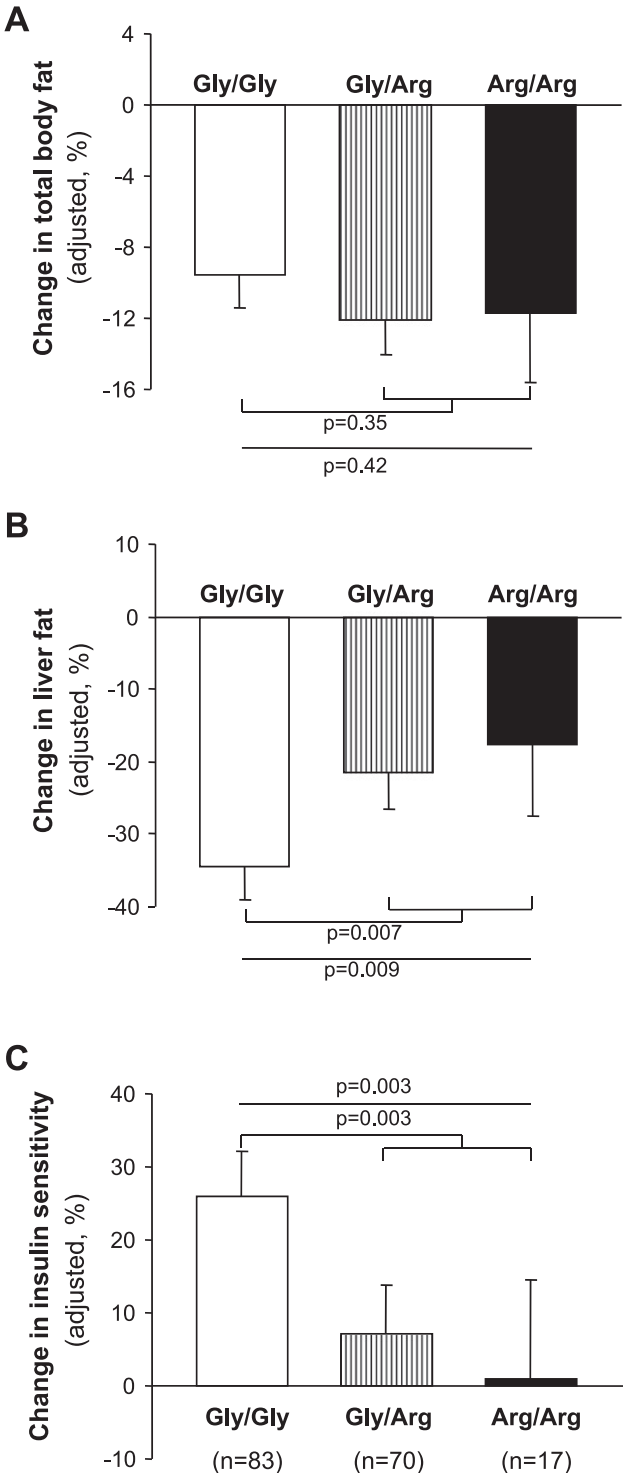

Figure 1. Percentage of change (indicated as follow-up over baseline) in (A) total body fat, (B) liver fat content, and (C) insulin sensitivity during 9 months of a lifestyle intervention in humans, depending on the respective genotype of the Gly388Arg polymorphism of FGFR4. Change in total body fat was adjusted for age, sex, and total body fat at baseline. Changes in liver fat content and insulin sensitivity were adjusted for age, sex, and body fat at baseline and at follow-up. Statistical significance is given by use of both an additive model and a dominant model for the 388Arg-encoding allele. Values represent means ± SEM.

Mice carrying the FGFR4 Arg385 allele exhibited increased liver fat content and altered hepatic lipid composition without altering body fat mass and body fat distribution under a chow diet

To investigate the role of the *FGFR4* Gly388Arg (in mice: Gly385Arg) substitution in the regulation of lipid metabolism, we used a *knock-in* mouse model in which the Gly codon in position 385 of the murine *FGFR4* gene was replaced with an Arg codon. We found no association between the *FGFR4 Arg385* allele and body weight (38), in line with total or visceral adipose tissue mass under a chow diet, as measured by MRI (Fig. 2A). Moreover, the *FGFR4 Arg385* allele was not associated with NEFA or total cholesterol levels (38). The serum TAGs tended to be higher in the *FGFR4 Arg385* mice; however, this difference was not statistically significant (*P* for trend = 0.165) (Fig. 2B).

However, the *FGFR4 Arg385* allele was associated with an elevated hepatic TAG content (*P* for trend = 0.005) (Fig. 2C). As expected, histological analyses confirmed the morphological differences in terms of increases in the number and size of lipid droplets in the liver of *FGFR4 Arg/Arg385* mice compared with that of *FGFR4 Gly/Gly385* mice (Fig. 2D).

When we analyzed the fatty acid composition of hepatic TAGs, we detected distinct alterations in the livers of *FGFR4 Arg385* mice on a chow diet (Table 3). Despite the absolute increase in liver fat content, we detected a relative reduction in saturated fatty acids of 14:0 and 18:0, as well as in polyunsaturated fatty acids of the *n*-3 series (22:6) and the *n*-6 series (20:4 and 22:6) in carriers of the *FGFR4 Arg385* allele. In contrast, the relative amount of the *n*-3 series precursor (18:3) was increased. The stearoyl-CoA-desaturase activity index of fatty acid desaturation was significantly increased for the ratio of 18:1/18:0, indicating increased fatty acid synthesis.

The FGFR4 Gly385Arg polymorphism altered glycolysis and liver *de novo* lipogenesis without affecting insulin sensitivity or glucose tolerance in mice under a chow diet

To determine whether the altered hepatic lipid metabolism was associated with changes in glucose metabolism in the *FGFR4 Arg385* mice, we examined plasma glucose and insulin levels. The *FGFR4 Arg385* allele was not associated with circulating levels of blood glucose or insulin in the fed state (38) or in the fasted state (38), although there was a trend for higher insulin levels in mutant mice in the fed state (*P* for trend = 0.09). When subjected to a glucose-stimulated insulin release test by administration of 3 g of glucose per kilogram of body

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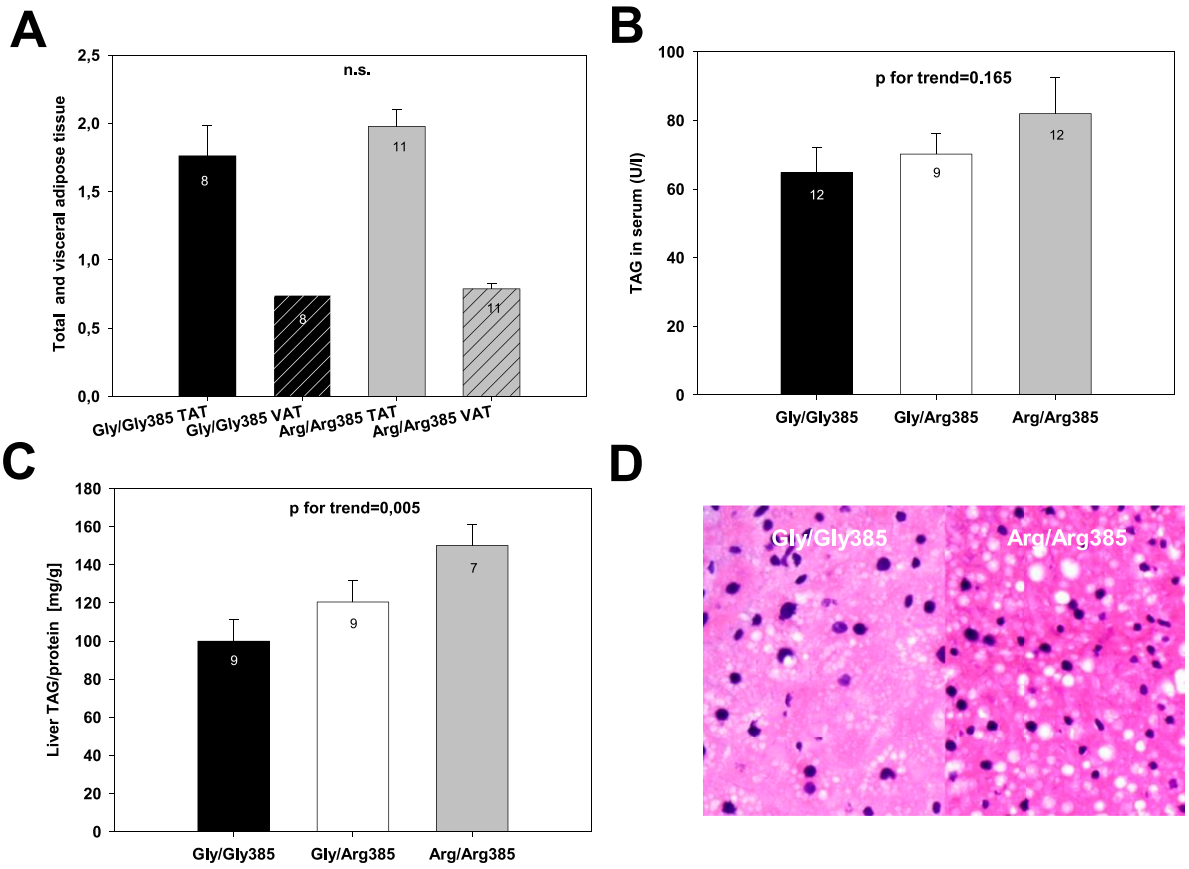

Figure 2. Fat distribution in *FGFR4 Gly/Gly385* mice (black bars), *FGFR4 Gly/Arg385* mice (white bars), and *FGFR4 Arg/Arg385* mice (gray bars) that were fed a chow diet. (A) MRI analysis of visceral and subcutaneous fat deposits (n = 3 per group). (B) Plasma triacylglycerides (n = 9 to 12). (C) Liver triacylglyceride content (n = 7 to 9). (D) Hematoxylin and eosin staining of liver sections. Images are representative of 7 to 9 stained sections per group. Data are expressed as mean ± SEM. TAT, total adipose tissue; VAT, visceral adipose tissue.

weight, *FGFR4 Arg/Arg385* mice exhibited significantly elevated insulin levels after 2 minutes compared with control *FGFR4 Gly/Gly385* mice (38).

In spite of unaltered glucose tolerance and insulin sensitivity (38), the *FGFR4 Arg385* allele was associated with higher mRNA expression of hepatic glucokinase *(Gck)*, indicating enhanced glycolysis (*P* for trend = 0.022) (Fig. 3A). Moreover, mRNA expression of the rate-limiting enzyme in lipogenesis, fatty acid synthase (*Fasn*), was also higher in carriers of the *FGFR4 Arg385* allele (*P* for trend = 0.002) (Fig. 3B), whereas the mRNA expression of acetyl-coenzyme A (acyl-CoA) carboxylase 1 (*Acc1*) and sterol regulatory element binding transcription factor 1 (*Srebp1*) were unchanged (Fig. 3C and 3D, respectively). Because the enzyme acyl-CoA: diacylglycerol acyltransferase-2 (DGAT2), which catalyzes the final step of TAG synthesis, was found to be involved in the dissociation between fatty liver and insulin resistance in mice and in humans (39, 40), we also measured *Dgat2* mRNA expression in the liver. In accordance with unchanged parameters of insulin resistance and glucose tolerance, the *FGFR4 Arg385* allele was associated with elevated *Dgat2* mRNA levels, in parallel with enhanced liver fat content in *FGFR4 Arg385* mice (*P* for trend = 0.005) (Fig, 3E).

The *FGFR4* Gly385Arg mutation was not associated with mRNA expression of the two major enzymes of hepatic gluconeogenesis, phosphoenolpyruvate-carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6Pase*) (38).

Elevated liver fat content was not associated with liver cytokine gene expression or reduced insulin signaling in liver and skeletal muscles in FGFR4 Arg385 mice under a chow diet

In parallel with findings of unaltered glucose and insulin tolerance, analysis of the transcriptional regulation of the proinflammatory genes interleukin 6 (*Il-6*) and interleukin 1 beta (*Il-1β*) revealed no association in liver tissue with the *FGFR4 Arg385* allele (38). Together with the concomitant finding that gene expression of the *Il-6* mediator suppressor of cytokine signaling 3 (*Socs3*) was unaltered independent of the presence of the *FGFR4 Arg385* allele, these results indicate that *FGFR4 Arg385* mice did not suffer from liver inflammation (38).

In addition, to exclude the effect of the mutation on cellular insulin signal transduction, we evaluated the

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Table 3. Relative Fatty Acid Composition in Percentage of the Triacylglyceride Fraction in the Liver of the Mice

	FGFR4				
	GG	GR	RR	P_{add}	$P_{adjusted\ for\ TAG}$
TAG, %	4.28 ± 0.120	4.32 ± 0.170	5.55 ± 0.305	**0.0116**	
14:0	1.12 ± 0.049	0.980 ± 0.033	0.965 ± 0.052	**0.0497**	0.2883
16:0	20.66 ± 0.636	21.68 ± 0.410	21.69 ± 0.919	0.3025	0.3399
18:0	1.55 ± 0.255	1.08 ± 0.057	1.03 ± 0.109	**0.0426**	0.278
20:0	0.058 ± 0.012	0.04 ± 0.008	0.056 ± 0.006	0.9592	0.4741
22:0	0.018 ± 0.004	0.010 ± 0.003	0.010 ± 0.002	0.1652	0.2630
24:0	0.089 ± 0.007	0.106 ± 0.011	0.111 ± 0.016	0.2217	0.0747
t-16:1 *n*-7	0.014 ± 0.0007	0.016 ± 0.002	0.016 ± 0.006	0.7934	0.2424
c-16:1 *n*-7	4.82 ± 0.719	5.97 ± 0.329	6.49 ± 0.591	0.0770	0.4495
18:1 *n*-9	22.23 ± 0.269	21.26 ± 0.567	21.65 ± 0.514	0.4296	**0.0513**
18:1 *n*-7	1.87 ± 0.107	1.78 ± 0.037	1.85 ± 0.106	0.8430	0.3243
24:1 *n*-9	0.071 ± 0.018	0.040 ± 0.011	0.022 ± 0.003	**0.0139**	0.2064
18:3 *n*-3	1.25 ± 0.107	1.69 ± 0.106	1.63 ± 0.057	**0.0421**	**0.0544**
18:4 *n*-3	1.25 ± 0.331	1.17 ± 0.068	1.15 ± 0.011	0.9736	0.8183
20:4 *n*-3	0.032 ± 0.003	0.042 ± 0.007	0.039 ± 0.003	0.2417	0.6107
20:5 *n*-3	0.220 ± 0.014	0.242 ± 0.014	0.239 ± 0.019	0.3802	0.5083
22:5 *n*-3	0.198 ± 0.029	0.239 ± 0.010	0.218 ± 0.024	0.5262	0.9345
24:5 *n*-3	0.030 ± 0.006	0.014 ± 0.004	0.015 ± 0.002	0.1317	0.0921
24:6 *n*-3	0.047 ± 0.014	0.031 ± 0.003	0.037 ± 0.005	0.6539	0.1318
22:6 *n*-3	0.838 ± 0.029	0.756 ± 0.016	0.627 ± 0.012	**0.0001**	**0.0051**
18:2 *n*-6	34.24 ± 0.567	35.17 ± 0.645	34.66 ± 0.959	0.7043	0.1292
18:3 *n*-6	1.57 ± 0.303	1.25 ± 0.027	1.19 ± 0.032	0.1390	0.2034
20:3 *n*-6	0.482 ± 0.042	0.451 ± 0.037	0.419 ± 0.039	0.2494	0.1957
20:4 *n*-6	2.58 ± 0.26	2.12 ± 0.047	1.89 ± 0.057	**0.0099**	0.0895
22:4 *n*-6	0.357 ± 0.040	0.251 ± 0.028	0.222 ± 0.012	**0.0144**	**0.0203**
24:4 *n*-6	0.013 ± 0.003	0.018 ± 0.007	0.011 ± 0.002	0.6008	0.6092
24:5 *n*-6	0.014 ± 0.003	0.012 ± 0.003	0.012 ± 0.003	0.6841	0.6846
22:5 *n*-6	0.180 ± 0.092	0.110 ± 0.001	0.093 ± 0.005	0.6145	0.5204

The genotype-specific fatty acid composition of hepatic triglyceride fraction from n = 3 mice per group a standard diet is displayed (mean ± SE). Total liver fat (triacylglyceride) content is displayed in the first lane; stearoyl-CoA-desaturase 1 activity indices as well as the *de novo* lipogenesis index are calculated and displayed in the lower lanes. *P* values are given for additive inheritance model. *P* values ≤ 0.05 were indicated as boldface text. GG, Gly/Gly; GR, Gly/Arg; RR, Arg/Arg.

insulin-stimulated serine phosphorylation of Akt in liver and skeletal muscle. We found no significant differences in basal or insulin-stimulated serine phosphorylation of Akt in liver and skeletal muscle, indicating normal insulin signaling in the *FGFR4 Arg385* mice (38).

Unaltered liver fat content as well as glucose and lipid metabolism in FGFR4 Arg385 mice under an HFD

To dissect whether the described phenotype could be aggravated with an HFD, we compared lipid and glucose metabolism of *FGFR4 Arg/Arg385*, *FGFR4 Gly/Arg385*, and *FGFR4 Gly/Gly385* mice under HFD conditions. Body weight (38), fasting blood glucose levels (38), and glucose and insulin tolerance (38) were comparable between the mice independent of the presence or absence of the *FGFR4 Arg385* allele. Interestingly, no significant differences were noted between the genotypes in regard to liver TAGs (38). Furthermore, unlike in mice under a chow diet, hepatic gene expression analysis revealed no association of the *FGFR4 Arg385* allele with the mRNA expression of *Fasn*, *Gck, or Dgat2* (38). The expression levels of *Srebp1* and *Acc1* were also comparable between the genotypes (38). Finally, an HFD resulted in elevated hepatic *Fgfr4* and *β-Klotho* mRNA expression compared with a normal chow diet (38).

Discussion

The role of FGFR4 in the regulation of lipid metabolism was first suggested on the basis of observations in FGFR4-deficient mice (17). Despite showing features of the metabolic syndrome under normal dietary conditions, these mice were protected from HFD-induced fatty liver (11). Furthermore, in another study, improved insulin resistance and glucose metabolism were found in FGFR4-deficient mice; however, consistent with the previous study, protection from HFD-induced fatty liver was observed (41). In addition, targeting of FGF19, which signals through FGFR4, is currently being discussed as a tool to treat hepatic steatosis (19, 42).

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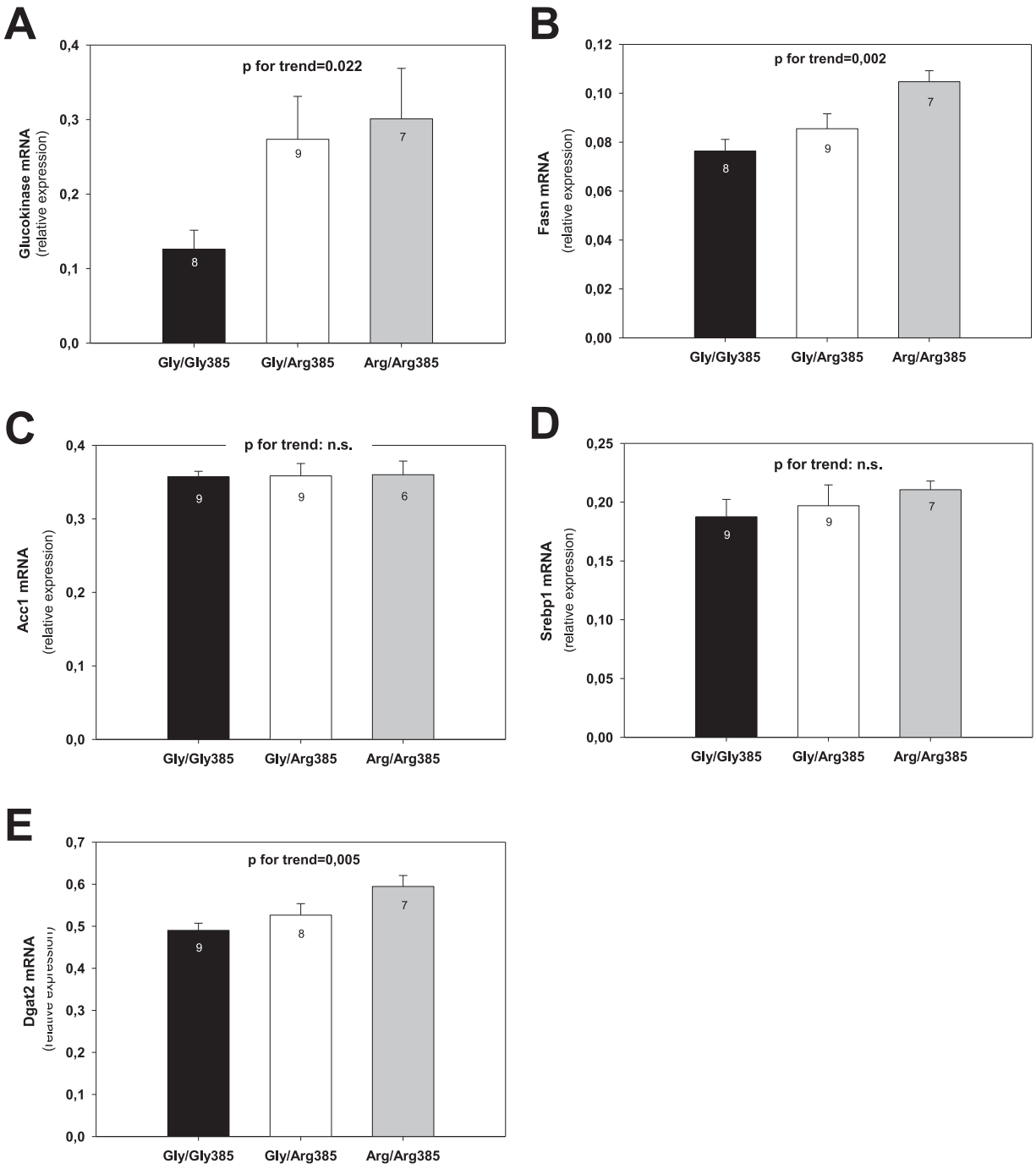

Figure 3. Gene expression analysis of *FGFR4 Gly/Gly385* mice (black bars), *FGFR4 Gly/Arg385* mice (white bars), and *FGFR4 Arg/Arg385* mice (gray bars) on a chow diet. (A) Glucokinase (n = 7 to 9). (B) Fasn (n = 7 to 9). (C) Acc1 (n = 6 to 9). (D) Srebp1 (n = 7 to 9). (E) Dgat2 (n = 7 to 9). Data are expressed as mean ± SEM.

In the current study, we focused on the human *FGFR4* Gly388Arg (mice: Gly385Arg) polymorphism, as a gain-of-function effect of the *FGFR4* Arg388 allele in humans has been demonstrated (25). The *FGFR4* Arg388 allele is associated with enhanced STAT3 tyrosine phosphorylation (25), which in turn is involved in hepatic gluconeogenesis and hepatic lipogenesis (26, 27). Here, we specifically investigated whether this amino acid exchange influences hepatic lipid and glucose metabolism.

Performing studies with this amino acid substitution in both humans and mice, we found an association of the *FGFR4 Arg388* allele (mice: *FGFR4 Arg385*) with elevated hepatic liver fat content under healthy dietary conditions. Interestingly, this mutation did not affect body fat mass or body fat distribution. Furthermore, we found that under normal dietary conditions, mice carrying the *FGFR4 Arg385* allele had (i) altered liver *de novo* lipogenesis and hepatic lipid composition, indicating increased fatty acid synthesis, and (ii) enhanced

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glycolysis and insulin release, without changes in insulin sensitivity, glucose tolerance, or insulin signaling in liver and skeletal muscle. Furthermore, no metabolic alterations were found in these mice under an HFD compared with HFD-fed controls.

The elevated liver TAG accumulation in mice carrying the *FGFR4 Arg385* allele under a chow diet is in agreement with our data in humans, where we found carriers of the minor *388Arg* allele to have a lower decrease in liver fat content during an LI. An important function of the liver is quick adaptation to changes in nutrient availability, maintaining a well-controlled balance in the fluxes of carbohydrates and lipids between the circulation and itself. Ulaganathan *et al.* (25) recently found that the substitution of the Gly 388 residue to Arg in FGFR4 resulted in an increased tyrosine phosphorylation and activation of the transcription factor STAT3, which is known to regulate both carbohydrate metabolism *in vivo* by suppressing the expression of the gluconeogenic genes *Pepck* and *G6Pase* in the liver (26) and lipid metabolism, increasing plasma levels of TAGs and total cholesterol by enhanced expression of the key hepatic lipogenic enzymes *Fasn* and *Acc* (27). Although circulating plasma lipid levels were not associated with the *FGFR4 Arg385* allele in our study, we found enhanced expression of *Fasn* in livers of these mice under normal chow conditions, demonstrating an elevated hepatic lipogenesis in carriers of the mutation. The alterations in lipogenesis were reported to be independent of the SREBP1 pathway (27), which was also the case in the current study. However, we could not detect changes in *Il-6* gene expression or in its downstream target, *Socs3*, possibly because the transcriptional regulation of *Il-6* in our mice was lower than that in previously studied mice with genetically forced expression of STAT3. Thus, together with the elevated transcription of *Gck* as a key glycolytic enzyme and unaltered fasting glucose levels and gluconeogenesis, our results indicate enhanced glucose flux toward lipogenesis in mice carrying the *FGFR4 Arg385* allele, possibly to maintain stable plasma glucose levels. Certainly, other mechanisms by which the *FGFR4 Arg385* allele may also regulate hepatic fat accumulation cannot be ruled out and remain to be clarified.

In addition, FGFR4 is known to be expressed in adult pancreatic islets (43, 44). Our data are in agreement with previous findings showing that the *FGFR4 Arg385* allele promotes pancreatic insulin secretion in both mice and humans (24). Elevated insulin levels in *FGFR4 Arg/Arg385* mice 2 minutes after the glucose load may contribute, in part, to the maintenance of normal glucose levels in these animals.

Nevertheless, in contrast to the animal data, in which no changes in insulin sensitivity were observed based on the presence or absence of the *FGFR4 Arg385* allele, humans carrying the minor *388Arg* allele displayed less improvement in whole-body and adipose tissue insulin resistance under an LI with a lower caloric load. So far, we have no explanation for this discrepancy between the human and animal data. It may well be that the *FGFR4* Gly388Arg polymorphism has a larger effect on glucose metabolism in humans than in our mouse model. Furthermore, because our measurement of insulin resistance in humans is a function of high glucose and high insulin levels during the OGTT, a *FGFR4* Gly388Arg–mediated hypersecretion of insulin may result in a higher estimate of insulin resistance in humans. In addition, it should also be noted that the previously reported changes in insulin resistance and glucose intolerance in mice (7, 31) were found in *FGFR4*-deficient animals. This may have resulted from a more severe genetic modification than in our transgenic mice.

Interestingly, although the change in body fat mass was not statistically significant among the genotypes, subjects carrying the *Gly/Gly* alleles had the lowest decrease in body fat mass. One can only hypothesize about mechanisms explaining this difference. However, because insulin resistance in adipose tissue was also most strongly decreased in carriers of this genotype, improved suppression of insulin-mediated lipolysis in carriers of the *Gly/Gly* genotype may have resulted in less decrease of fat mass in these subjects.

Under conditions of overnutrition in mice and obesity before the intervention in humans, the *FGFR4 Arg385 (388)* allele did not associate with liver fat content or insulin sensitivity. To further clarify this point, we first confirmed the finding of a previous report demonstrating marked upregulation of *Fgfr4* expression in liver under HFD conditions (45). Together with established knowledge about the effect of FGFR4 signaling on liver fat content under both chow and HFD conditions, our data indicate that the *FGFR4 Arg385* allele regulated hepatic lipogenesis predominantly under conditions of healthy nutrition. Thus, under caloric overload, the comparatively modest modulatory effect of the minor *Arg385* allele seems to be overridden by forced FGFR4 expression and a large amount of liver fat accumulation.

In our mouse model, we found a lack of hepatic inflammation in *FGFR4 Arg385* mice. The finding that transcriptional regulation of key hepatic proinflammatory genes, such as *Il-6*, *Socs3,* and *Il-1β*, was not altered in the presence of the *Arg385* allele—together with normal insulin signaling—points to a metabolically healthy fatty liver (46–48). Regarding other mechanisms of a metabolically healthy fatty liver, further evidence for a genetically determined dissociation of hepatic steatosis from insulin resistance is provided by the single-nucleotide

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polymorphism (rs738409) in *PNPLA3*. The amino acid exchange Ile148Met causes the largest known effect on hepatic steatosis in humans, without insulin resistance or dyslipidemia (49–53), but a different quality of stored hepatic TAGs, with distinct alterations in fatty acid composition (35). Of note, when we analyzed the hepatic TAG profile of *FGFR4* Arg385 mice under a chow diet, we detected somewhat similar changes, including a reduction in stearate (18:0) and an increase in linolenic acid (18:3), as well as a reduction in some major polyunsaturated fatty acids. This altered hepatic lipid composition may contribute to healthy metabolic effects of the stored hepatic lipids in *FGFR4* Arg385 mice.

Another model of a metabolically healthy fatty liver is the liver-specific *Dgat2* transgenic mouse (39). In accordance with these mouse data, we found that genetic variability in *DGAT2* is also a mediator of the dissociation between fatty liver and insulin resistance in humans (40). There is evidence that in addition to liver inflammation, the mode of storing hepatic lipids plays a key role in the determination of metabolically healthy and unhealthy fatty liver. According to this hypothesis, the accumulation of hepatic fatty acyl-CoAs is detrimental for glucose metabolism, whereas TAG storage provides protection from their deleterious effects (54, 55). DGAT2 catalyzes the final step of TAG synthesis and is predominantly expressed in the liver (56). In our study, the *FGFR4 Arg385* allele was associated with elevated *Dgat2* expression in liver tissue under a chow diet, pointing to a possible mechanistic explanation for the metabolically benign TAG accumulation in these mice. Thus, our results underline a main regulatory function of hepatic FGFR4 in lipid homeostasis, without affecting systemic glucose homeostasis.

In conclusion, we provide evidence for an association between the *FGFR4 Arg388* allele and elevated liver fat content in humans consuming a healthy diet, but not under nutritional overload. This finding was in agreement with our data in mice who carried the *FGFR4 Arg385* allele. Given that hepatic lipid accumulation was not accompanied by metabolic disorders as a result of lipotoxicity in mice carrying the *FGFR4 Arg385* allele under a healthy diet, hepatic lipid storage may lead to a metabolically healthy fatty liver, possibly because of an effective detoxification of fatty acyl-CoAs. Thus, *FGFR4* may be a candidate gene for not only progression of several cancers but also hepatic steatosis in humans.

Acknowledgments

The authors thank all study participants for their cooperation. We gratefully acknowledge the excellent assistance of Alke Guirguis, Dorothee Neuscheler, and Anja Dessecker.

Financial Support: This study was supported in part by a grant from the German Federal Ministry of Education and Research to the German Center for Diabetes Research (to H.-U.H.).

Author Contributions: S.Z.L. and N.S. designed the study, performed the analyses, and drafted the manuscript. M.K., J.M., S.M.K., and B.S. performed laboratory measurements and contributed to the discussion. C.T. was involved in the collection of the human data and contributed to the discussion. A.M.H., A.P., E.S., F.S., M.H., and A.U. contributed to the discussion. H.-U.H. and N.S. supervised the project and contributed to the discussion. All authors approved the final version of the manuscript prior to submission. H.-U.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Correspondence and Reprint Requests: Hans-Ulrich Häring, MD, Department of Internal Medicine, Division of Endocrinology, Diabetology, Vascular Disease, Nephrology and Clinical Chemistry, University of Tübingen, Otfried-Müller-Straße 10, 72076 Tübingen, Germany. E-mail: hans-ulrich.haering@med.uni-tuebingen.de.

Disclosure Summary: The authors have nothing to disclose.

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