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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 functionally active amino acid exchange in humans Gly388Arg (mouse homologue: Gly385Arg) in the fibroblast growth factor receptor 4 (FGFR4), which regulates bile acid, lipid and glucose metabolism, may determine the dynamics of hepatic lipid accumulation and insulin sensitivity in humans. Mechanisms of this substitution were studied in mice under normal chow and high-fat diet.

Design-In humans the Gly388Arg polymorphism was studied for its relationship with the change of liver fat content and insulin sensitivity during 9 month 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 diet.

Results-In humans, the *FGFR4Arg388* allele did not associate with liver fat content or insulin sensitivity in overweight and obese subjects before the lifestyle intervention. However, it associated with less decrease of liver fat content and less increase of insulin sensitivity during the intervention. In mice, under normal chow, the *FGFR4Arg385* allele 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. No effects of the *FGFR4Arg385* allele on glucose or lipid metabolism were found under high-fat diet.

Conclusion-Our data indicate that the *FGFR4Arg388(385)* allele affects hepatic lipid and glucose metabolism specifically during a healthy caloric intake.

We assessed the role of the amino acid exchange Gly388(385)Arg in the fibroblast growth factor receptor 4 in hepatic lipid accumulation and its implications for glucose metabolism. .

Abbreviations:

ACC, acetyl-CoA-carboxylase; DGAT2, acyl-CoA:diacylglycerol acyltransferase 2; FASN, fatty acid synthase; FGFR4, fibroblast growth factor receptor 4; G6Pase, glucose-6-phosphatase; Gck, glucokinase; HFD, high-fat diet; IL-1β, interleukin-1β; IL-6, interleukin-6; i.p. intraperitoneal; IR, insulin receptor; i.v., intravenous; LI, lifestyle intervention; NEFA, non-esterified fatty acid; NAFLD, non-alcoholic fatty liver disease; PEPCK, phosphoenolpyruvate carboxykinase; SNP, single nucleotide polymorphism; SREBP1, sterol regulatory element-binding protein 1; SOCS-3, suppressor of cytokine signaling-3; TAG, triacylglyceride.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is strongly associated with insulin resistance and is an important risk factor for the development of type 2 diabetes 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. We found in humans that variations in the peroxisome proliferator-activated receptor (PPAR)-δ gene, which encodes a nuclear transcription factor controlling fatty acid oxidation and energy uncoupling, determine the change of hepatic fat content during a LI [8]. Furthermore, we found that cardiorespiratory fitness was an independent predictor of the reduction of liver fat content during a LI [9]. In addition, carriers of the -8503 A and -1927 C alleles in the gene encoding the adiponectin receptor 1 (*ADIPOR1*) predicted the change of liver fat content and insulin sensitivity during a LI [10].

Since 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, that may affect the dynamics of liver fat accumulation in humans. FGFR4 belongs to a family of transmembrane receptor protein tyrosine kinases including 5 members (FGFR1-FGFR5) [12, 13]. FGFRs 1-4 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], while the recently identified FGFR5 lacks the intracellular tyrosine kinase domain [13]. FGFRs are activated by fibroblast growth factors (FGFs), which are involved in a variety of biological responses like 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. This involves its ligands, the human FGF19 and its mouse orthologue FGF15, which display hormone-like effects [11, 17, 18]. Most recently, an FGF19 analogue was shown to reduce liver fat content in patients with non-alcoholic 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 homologue *FGFR4* codon 385) results in the amino acid exchange glycine (Gly^{388}) to arginine (Arg^{388}) in the transmembrane domain of the tyrosine kinase, and is associated with cancer diseases, 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% [23, 24]. We previously found that the *FGFR4* Gly388Arg substitution induced hypersecretion of insulin in mouse islets and associated with lower glucose levels in humans [25]. Furthermore, the Gly388Arg substitution was found to be associated with an activation of the signal transducer and activator of transcription-3 (STAT3) in mice [26]

which, if activated, suppresses hepatic gluconeogenic [27] and induces hepatic lipogenic gene expression [28], pointing to a gain-of-function effect of this amino acid exchange.

Therefore, in the present 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 a LI in humans, and studied mechanisms of action of this amino acid substitution under normal chow and a high-fat diet in mice.

Materials and Methods

Human data

Subjects

A total of 170 subjects from Southern Germany without diabetes were studied. These individuals participated in a 9 month lifestyle intervention study to reduce adiposity and to prevent type 2 diabetes and had measurements of liver fat content before and after 9 months of the lifestyle intervention [3, 29]. For inclusion in the study, at least one of the following criteria has to be fulfilled by the participants: a family history of type 2 diabetes, previous diagnosis of impaired glucose tolerance or gestational diabetes, or a BMI > 27 $kg \cdot m^{-2}$. Moreover, after a standard questionnaire, physical examination and routine laboratory tests the participants have to be ascertained healthy, especially without any history of liver disease.

As assessed by means of a standard questionnaire, they did not consume more than 2 alcoholic drinks per day. After the baseline measurements, individuals underwent 9- month lifestyle intervention comprising of dietary counseling with up to 10 sessions with a dietician and at least 3 h of aerobic endurance exercise per week as described in detail in [29]. Counselling aimed to reduce body weight by ≥ 5%. Furthermore, participants were recommended to reduce the energy intake from fat to < 30% of total energy consumed, and to increase dietary fibre 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 the heart rate. Part of the results from these analyses was also presented in a doctoral thesis of the co-author CT (https://publikationen.uni-tuebingen.de/xmlui/handle/10900/45745). Informed written consent was also obtained from these participants and the Ethics Committee of the University of Tübingen had approved the protocol.

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

Body mass index was calculated as weight divided by the square of height ($kg \cdot m^{-2}$). 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 (^{1}H-MRS) as previously described [30]. 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 2hr 75g 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 5 time points (0, 30, 60, 90, 120 min) during a 75g OGTT, as proposed by Matsuda and DeFronzo (10 000 / √(mean insulin x mean glucose) x (fasting insulin x fasting glucose) [31]. Furthermore, adipose tissue insulin resistance (fasting insulin x fasting free fatty acids) was calculated [32, 33].

Genotyping

For genotyping DNA was isolated from whole blood using a commercial DNA isolation kit (NucleoSpin; Macherey & Nagel, Düren, Germany). The single nucleotide polymorphism (SNP) rs351855, a G to A exchange leading to substitution of glycine (Gly) by arginine (Arg) at position 388 of FGFR4, was genotyped using TaqMan assay (Applied Biosystems, Foster City, CA, USA) [25]. The overall genotyping success rate was 99.1%. Re-screening of 3.1% of the subjects gave 100% identical results.

Experimental animals

The generation of mice carrying the *FGFR4Arg385* allele has been described previously [34]. 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 *FGFR4Gly/Arg385* or *FGFR4Arg/Arg385* mice were compared with litter-matched *FGFR4Gly/Gly385* control mice. Mice (4 week old) were kept on a 12h/12h light/dark cycle (lights on at 6:00 am) and were allowed to access to a regular chow ad libitum (Diet#1310, Altromin, Lage, Germany) or to high-fat diet (HFD) (D12451, Research Diets, New Brunswick, NJ) for 8 weeks. The HFD contained 45 % kcal as fat. All animal procedures were performed in accordance to 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, anaesthetized mice were injected into the inferior vena cava with 2 units of human insulin. For Western Blotting, after 7 min 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 PMSF, 10 mg/µl leupeptin and aprotinin for 30 min and precleared by centrifugation at 12000xg for 20 min at 4°C. Western blot analysis of tissue lysates was done with antibodies against phospho-Akt (Ser473) and GAPDH (#9271 and #2118, respectively, Cell Signaling Technology, Beverly, MA). Signals were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

Analysis of body fat mass and liver fat content in mice

For magnetic resonance imaging (MRI) of fat mass, mice were anaesthetized and measured on a 3 T 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. Post-processing was done as described [35]. For the histological detection of liver fat content, liver tissue was fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Hematoxylin and eosin staining of paraffin liver sections was carried out for analysis of fat content by visual inspection of vesicles [36]. To determine the liver fat content by clinical chemical methods, triglycerides (TAGs) were quantified in the homogenate using the ADVIA 1800 clinical chemistry analyzer (Siemens Healthcare Diagnostics, Eschborn, Germany) and calculated as mg/100mg tissue.

Analysis of fatty acid composition

For the 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. Trans-esterification of the fatty acids and quantification by gas chromatography with flame ionization detection was performed as previously described [36].

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. Non-esterified fatty acids (NEFA) 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, Eschborn, Germany).

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 min after injection. Plasma glucose was determined as described above. 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 application and 2, 5, 10 and 30 min after injection. To determine insulin sensitivity, an insulin tolerance test was performed with mice receiving 1 units/kg body weight insulin i.p. (Actrapid, Novo Nordisk, Denmark). Blood was collected immediately before the application and 15, 30 and 60 min after injection.

Gene expression analysis

For quantification of mRNA expression in mouse liver, tissues were frozen in liquid nitrogen. Total RNA was extracted with AllPrep Mini Kit (QIAGEN, Hilden, Germany) 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 duplicates. RNA content was normalized for the housekeeping gene *RPS13* using the ΔΔCt method.

Statistical analysis

Data are expressed as mean ± SEM and the number of independent experiments or mice are 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 FGFR4Arg385 allele are more resistant to the reduction of liver fat content and the improvement of insulin sensitivity during a lifestyle intervention

The demographic and metabolic characteristics of the population at baseline and at follow-up are presented in the table 1. During a follow-up period of 9 months there was a decrease of 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 2h glycemia and insulinemia and adipose tissue insulin resistance also 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, the minor *388Arg* allele associated both with less decrease of fasting and 2h insulinemia, and in addition, with less decrease of 2h glycemia and of adipose tissue insulin resistance (table 2) compared to individuals who were homozygous for the *388Gly* allele. Furthermore,

subjects carrying the *388Arg* allele had a similar amount of decrease of total body fat mass (figure 1, panel A), but less reduction of liver fat content and less augmentation of insulin sensitivity, in comparison with homozygous carriers of the *388Gly* allele (figure 1, panels B and C).

Mice carrying the FGFR4Arg385 allele exhibit increased liver fat content and altered hepatic lipid composition without altering body fat mass and body fat distribution under 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 in position 385 of the murine *FGFR4* gene the Gly codon was replaced with an Arg codon. We found no association of the *FGFR4Arg385* allele with body weight (supplemental figure 1A [37]), and in line, as measured by MRI, with total or visceral adipose tissue mass under chow diet (figure 2A). Moreover, the *FGFR4Arg385* allele was not associated with NEFA or total cholesterol levels (supplemental figure 1B and 1C, respectively [37]). The serum TAGs tended to be higher in the *FGFR4Arg385* mice, however, this difference was not statistically significant (figure 2B, p for trend=0.165).

However, the *FGFR4Arg385* allele associated with an elevated hepatic TAG content (figure 2C, p for trend=0.005). As expected, histological analyses confirmed the morphological differences in terms of an increase in the number and size of lipid droplets in the liver of *FGFR4Arg/Arg385* mice compared to *FGFR4Gly/Gly385* mice (figure 2D).

When we analyzed the fatty acid composition of hepatic TAGs, we detected distinct alterations in livers of *FGFR4Arg385* mice on chow diet (table 3). Despite the absolute increase of liver fat content, we detected a relative reduction of saturated fatty acids 14:0 and 18:0, as well as of polyunsaturated fatty acids of the n-3 series (22:6), as well as the n-6 series (20:4 and 22:6), in carriers of the *FGFR4Arg385* allele. In contrast, the relative amount of the n-3 series precursor (18:3) was increased. The SCD 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 alters glycolysis and liver de novo lipogenesis without affecting insulin sensitivity or glucose tolerance in mice under chow diet

To determine whether the altered hepatic lipid metabolism associated with changes in glucose metabolism in the *FGFR4Arg385* mice, we examined plasma glucose and insulin levels. The *FGFR4Arg385* allele was not associated with circulating levels of blood glucose or insulin, neither in the fed (supplemental figure 1D and 1E, respectively [37]) nor in the fasted state (supplemental figure 1F and 1G, respectively [37]), 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 3g of glucose/kg body weight, *FGFR4Arg/Arg385* mice exhibited significantly elevated insulin levels after 2 min compared to control *FGFR4Gly/Gly385* mice (supplemental figure 1H [37]).

In spite of unaltered glucose tolerance and insulin sensitivity (supplemental figure 2A and 2B [37]), the *FGFR4Arg385* allele associated with higher mRNA expression of the hepatic glucokinase *(Gck)*, indicating enhanced glycolysis (figure 3A, p for trend=0.022). Moreover, the mRNA expression of the rate limiting enzyme in lipogenesis, the fatty acid synthase (*Fasn*), was also higher in carriers of the *FGFR4Arg385* allele (figure 3B, p for trend=0.002), while the mRNA expression of acetyl-Coenzyme A carboxylase 1 (*Acc1*) and sterol regulatory element binding transcription factor 1 (*Srebp1*) were unchanged (figure 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 [38, 39], we also measured *Dgat2* mRNA expression in the liver. In accordance with unchanged parameters of insulin resistance or glucose tolerance, the *FGFR4Arg385* allele associated with elevated *Dgat2*

mRNA levels, in parallel to enhanced liver fat content in *FGFR4Arg385* mice (figure 3E, p for trend=0.005).

The *FGFR4* Gly385Arg mutation did not associate with the mRNA expression of the two major enzymes of hepatic gluconeogenesis, phosphoenolpyruvate-carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6Pase*) (supplemental figure 2C and 2D, respectively [37]).

Elevated liver fat content is not associated with liver cytokine gene expression or reduced insulin signaling in liver and skeletal muscle in FGFR4Arg385 mice under chow diet

In parallel to unaltered glucose and insulin tolerance, analysis of the transcriptional regulation of the pro-inflammatory genes interleukin 6 (*Il-6*) and interleukin 1 beta (*Il1b*) revealed no association in liver tissue with the *FGFR4Arg385* allele (supplemental figure 2E and 2F, respectively [37]). Together with the concomitant finding that gene expression analysis of the *Il-6* mediator suppressor of cytokine signaling 3 (*Socs3*) was unaltered, independent of the presence of the *FGFR4Arg385* allele, these results indicate that *FGFR4Arg385* mice did not suffer from liver inflammation (supplemental figure 2G [37]).

In addition, to exclude an effect of the mutation on cellular insulin signal transduction we evaluated the 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 *FGFR4Arg385* mice (supplemental figure 2H and 2I, respectively [37]).

Unaltered liver fat content, as well as glucose and lipid metabolism in FGFR4Arg385 mice under high-fat diet

To dissect whether the described phenotype can be aggravated upon a high-fat diet (HFD), we compared lipid and glucose metabolism of *FGFR4Arg/Arg385*, *FGFR4Gly/Arg385*, and *FGFR4Gly/Gly385* mice under HFD conditions. Body weight (supplemental figure 3A [37]), fasting blood glucose levels (supplemental figure 3B [37]), and glucose and insulin tolerance (supplemental figure 3B and 3C, respectively [37]) were comparable between the mice, independent of the presence or absence of the *FGFR4Arg385* allele. Interestingly, no significant differences were noted between the genotypes in regard to liver TAGs (supplemental figure 3D [37]). Furthermore, in contrast to mice under a chow diet, hepatic gene expression analysis revealed no association of the *FGFR4Arg385* allele with the mRNA expression of *Fasn, Gck, or Dgat2* (supplemental figure 3E, 3F, and 3I respectively [37]). The expression levels of *Srebp1* and *Acc1* were also comparable between the genotypes (supplemental figure 3G and 3H, respectively [37]). Finally, HFD resulted in elevated hepatic *Fgfr4* and *β-Klotho* mRNA expression compared to normal chow (supplemental figure 4 [37]).

Discussion

A role of FGFR4 in the regulation of lipid metabolism was first suggested based on observations in FGFR4-deficient mice [17]. Despite showing features of the metabolic syndrome under normal dietary conditions, these mice were protected from high-fat diet (HFD)-induced fatty liver [11]. Furthermore, in another study, improved insulin resistance and glucose metabolism was found in FGFR4-deficient mice, but consistent with the previous study, a protection from HFD-induced fatty liver was observed [40]. In addition, targeting FGF19, which signals through FGFR4, is currently being discussed as a tool to treat hepatic steatosis [19, 41].

In the present 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 [26]. The *FGFR4* Arg388 allele associates with enhanced signal transducer and activator of transcription-3 (STAT3) tyrosine phosphorylation [26], which in turn is involved

in hepatic gluconeogenesis and hepatic lipogenesis [27, 28]. Here, we specifically investigated whether this amino acid exchange influences hepatic lipid and glucose metabolism.

Performing studies with this amino acid substitution, both, in humans and in mice, we found an association of the *FGFR4Arg388* allele (mice: *FGFR4Arg385*) 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 *FGFR4Arg385* allele had i) altered liver *de novo* lipogenenesis and hepatic lipid composition, indicating increased fatty acid synthesis and ii) enhanced 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 HFD, when compared to HFD-fed controls.

The elevated liver TAG accumulation in mice carrying the *FGFR4Arg385* allele under chow diet is in agreement with our data in humans, where we found carriers of the minor *388Arg* allele to have a lower decrease of liver fat content during a LI. An important function of the liver is a 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.* recently found that the substitution of the glycine 388 residue to arginine 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 [27], and lipid metabolism, increasing plasma levels of TAGs and total cholesterol by enhanced expression of the key hepatic lipogenic enzymes *Fasn* und *Acc* [28]. While in our study circulating plasma lipid levels were not associated with the *FGFR4Arg385* allele, 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 [28], which was also the case in the present 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 in the previously studied mice with genetically forced expression of STAT3. Thus, together with the elevated transcription of the Glucokinase (*Gck)* as a key glycolytic enzyme, beside unaltered fasting glucose levels and gluconeogenesis, our results indicate an enhanced glucose flux towards lipogenesis in mice carrying the *FGFR4Arg385* allele, possibly to maintain stable plasma glucose levels. Certainly, other mechanisms by which the *FGFR4Arg385* 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 [42, 43]. Our data are in agreement with previous findings showing that the *FGFR4Arg385* allele promotes pancreatic insulin secretion, both, in mice and humans [25]. The elevated insulin levels in *FGFR4Arg/Arg385* mice two minutes after the glucose load may in part contribute to the maintenance of normal glucose levels in these animals.

Though, in contrast to the animal data, where no changes in insulin sensitivity was observed based on the presence or absence of the *FGFR4Arg385* allele, humans carrying the minor *388Arg* allele displayed a lower improvement of whole-body and adipose tissue insulin resistance under a LI with a lower caloric load. So far we have no explanation for this discrepancy between the human and the animal data. It may well be that in humans the *FGFR4* Gly388Arg polymorphism may have a larger impact on glucose metabolism than in our mouse model. Furthermore, as our measurement of insulin resistance in humans is a function of high glucose and high insulin levels during the oral glucose tolerance test, a *FGFR4* Gly388Arg-mediated hypersecretion of insulin may result in a higher estimate of

insulin resistance in humans. In addition, it should also be taken into account, that the previously reported changes in insulin resistance and glucose intolerance in mice (7, 32) were found in *FGFR4*-deficient animals. This may have resulted from a more severe genetic modification, compared to our transgenic mice.

Interestingly, although the change of body fat mass was not statistically significant among the genotypes, subjects carrying the *Gly/Gly* alleles had the lowest decrease of body fat mass. One can only hypothesize about mechanisms possibly explaining this difference. However, as adipose tissue insulin resistance 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 a lower decrease of fat mass in these subjects.

Under conditions of overnutrition in mice and obesity prior to the intervention in humans, the *FGFR4Arg385(388)* allele did not associate with liver fat content or insulin sensitivity. To further clarify this point, we first could confirm the finding of a previous report demonstrating marked up-regulation of *Fgfr4* expression in liver under HFD conditions [44]. Together with the established knowledge about the impact of FGFR4 signaling on liver fat content, both, under chow and HFD conditions, our data indicate that the *FGFR4Arg385* allele regulates hepatic lipogenesis predominantly under conditions of a healthy nutrition. Thus, under a 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. As transcriptional regulation of key hepatic pro-inflammatory genes, such as *Il-6*, *Socs3* and *Il1b* was not altered in the presence of the *Arg385* allele, together with normal insulin signaling, this points to a metabolically healthy fatty liver [45-47]. In respect to other mechanisms of a metabolically healthy fatty liver, further evidence for a genetically determined dissociation of hepatic steatosis from insulin resistance is being provided by the single nucleotide polymorphism (rs738409) in *PNPLA3*. The amino acid exchange Ile148Met causes the largest known effect on hepatic steatosis in humans, without insulin resistance or dyslipidemia [48-52], but different quality of stored hepatic TAGs with distinct alterations in fatty acid composition [36]. Of note, when we analyzed the hepatic TAG profile of *FGFR4* Arg385 mice under chow diet, we detected somewhat similar changes, including a reduction of stearate (18:0) and an increase of linolenic acid (18:3), as well as a reduction of 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 acyl:CoA:diacylglycerol acyltransferase 2 (*Dgat2*) transgenic mouse [38]. 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 [39]. There is evidence that, besides 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 are detrimental for glucose metabolism while TAG storage protects from their deleterious effects [53, 54]. DGAT2 catalyzes the final step of TAG synthesis and is predominantly expressed in the liver [55]. In our study, the *FGFR4Arg385* allele was associated with elevated *Dgat2* expression in liver tissue under 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 affection of systemic glucose homeostasis.

In conclusion, we provide novel evidence for an association of the *FGFR4Arg388* allele with elevated liver fat content in humans under a healthy diet, but not under nutritional overload. This finding was in agreement with our data in mice carrying the *FGFR4Arg385*

allele. Given that hepatic lipid accumulation was not accompanied by metabolic disorders in consequence of lipotoxicity in mice carrying the *FGFR4Arg385* allele under a healthy diet, the hepatic lipid storage may lead to a metabolically healthy fatty liver, possibly due to an effective detoxification of fatty acyl-CoAs. Thus, *FGFR4* may be a candidate gene, not only for progression of several cancers, but also for hepatic steatosis in humans.

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Contribution and disclosure statements:
SZL and NS designed the study, performed analyses, and drafted the manuscript. MK, JM, SMK, BS performed laboratory measurements and contributed to discussion. CT was involved in the collection of the human data and contributed to the discussion. AMH, AP, SE, SF, MH, AU contributed to discussion. HUH and NS supervised the project and contributed to discussion. All authors approved the final version of the manuscript prior to submission. HUH 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. The authors declare that there are no conflicts of interest.

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Fig.1. Percent of change (indicated as follow-up over baseline) is presented in total body fat (A), liver fat content (B) and insulin sensitivity (C) during 9 month of a lifestyle intervention in humans, dependent on the respective genotype of the Gly388Arg polymorphism of FGFR4. Change in total body fat was adjusted for age, gender and total body fat at baseline. Changes in liver fat content and insulin sensitivity were adjusted for age, gender, and body fat at baseline and at follow-up. Statistical significance is given by using both an additive and a dominant model for the 388Arg encoding allele.

Fig.2. Fat distribution in FGFR4Gly/Gly385 (black bars), FGFR4Gly/Arg385 (white bars) and FGFR4Arg/Arg385 (gray bars) mice that were fed a chow diet. (A) MRI analysis of visceral and subcutaneous fat deposits (n=3 per group). (B) Plasma triacylglycerides (n=9-12). (C) Liver triacylglyceride content (n=7-9). (D) Hematoxylin and eosin staining of liver sections. Images are representative of 7-9 stained sections per group.

Fig.3. Gene expression analysis of FGFR4 Gly/Gly385 (black bars), FGFR4 Gly/Arg385 (white bars) and FGFR4Arg/Arg385 (gray bars) mice on a chow diet. (A) Glucokinase (n=7-9) (B) Fasn (n=7-9) (C) Acc1 (n=6-9) (D) Srebp1 (n=7-9) (E) Dgat2 (n=7-9).

Table 1. Subject characteristics at baseline and after 9 months of follow-up in Caucasians. Values represent mean ± SE; p for paired differences after log transformation of non-normally distributed parameters. *available in 149 subjects. FFA, free fatty acids. IR, insulin resistance; IS, insulin sensitivity.

Table 1. Subject characteristics at baseline and after 9 months of follow-up in Caucasians.

	Baseline	Follow-up	p value
Demographics and body composition			
Gender (Males/Females)	68/102		-
Age (years)	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
$IMCL_{tibialis\ anterior}$(arb.units.)*	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 (arb. units)	13.3 ± 0.5	15.1 ± 0.6	0.0001

Table 2. Associations of the Gly388Arg SNP in *FGFR4* with changes in subject characteristics during the lifestyle intervention. Data represent unadjusted mean ± SE. For statistical analyses, non-normally distributed parameters were log transformed. +p<0.05, ‡p<0.01 and †p<0.001 for paired differences between baseline and follow-up. #χ2-test *available in 149 subjects (Gly/Gly n=74; Gly/Arg n=60; Arg/Arg n=15). 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 gender. 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 gender. The other parameters were additionally adjusted for body fat at baseline and at follow-up. FFA, free fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance; IMCL, intramyocellular lipids; Adip. IR, adipose tissue insulin resistance; SNP, single nucleotide polymorphism; OGTT, oral glucose tolerance test.

Table 2. Associations of the 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										
Gender (males/females)	29/54		31/39		8/9		**0.20**$^{\#}$	0.19$^{\#}$		
Age (years)	46 ± 1	47 ± 1†	46 ± 1	47 ± 1†	45 ± 4	46 ± 0†	**0.53**	0.66		
Change in body composition										
Body weight (kg)	85.8 ± 1.7	83.1 ± 1.6†	83.8 ± 1.8	81.7 ± 1.8†	89.6 ± 4.3	86.3 ± 4.2†	**0.60**	0.24	**0.74**	0.94
Body mass index (kg/m^2)	29.0 ± 0.5	28.1 ± 0.5†	28.5 ± 0.5	27.8 ± 0.5†	30.1 ± 1.0	29.0 ± 1.0†	**0.71**	0.83	**0.83**	0.95
Body fat (kg)	25.9 ± 1.1	23.7 ± 1.0†	23.7 ± 1.1	21.7 ± 1.1†	28.2 ± 2.2	24.8 ± 1.9‡	**0.65**	0.68	**0.42**	0.35
Visceral fat (kg)	3.02 ± 0.20	2.61 ± 0.19†	2.84 ± 0.19	2.45 ± 0.19†	3.26 ± 0.51	2.71 ± 0.48†	**0.12**	0.31	**0.61**	0.95
$IMCL_{tibialis\ anterior}$(arb.units.)*	3.91 ± 0.20	3.78 ± 0.16	4.09± 0.20	3.73± 0.21^{+}	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†	5.33 ± 0.65	4.11 ± 0.51†	4.49 ± 0.99	4.10 ± 1.29^{+}	**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†	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†	62 ± 5	55 ± 4^{+}	67 ± 8	59 ± 5	**0.17**	0.10	**0.01**	0.01
2 h insulin (pM)	465 ± 41	342 ± 34†	506 ± 47	487 ± 60	442 ± 105	524 ± 125	**0.74**	0.35	**0.0005**	0.06
Fasting FFA (µM)	674 ± 22	615 ±	618 ± 27	589 ±	708 ± 68	675 ±	**0.68**	0.35	**0.19**	0.56

		20[‡]		26		31				
2 h FFA (µM)	92 ± 11	90 ± 19	73 ± 5	61 ± 4[+]	92 ± 17	67 ± 6[+]	**0.47**	0.30	**0.77**	0.61
HOMA-IR index	1.78 ± 0.13	1.46 ± 0.10[†]	1.97 ± 0.15[+]	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[†]**	**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$_{OGTT}$ (arb. units)	14.08 ± 0.78	16.75 ± 0.94[†]	12.52 ± 0.81	13.92 ± 0.87	12.48 ± 1.82	12.38 ± 1.53	**0.29**	0.13	**0.003**	0.003

Table 3. Relative fatty acid composition in percent of the triacylglyceride fraction in the liver of the mice. The genotype specific relative 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; SCD1 activity indices as well as the *de novo* lipogenesis (DNL) index are calculated and displayed in the lower lanes. P values are given for additive inheritance model. TAG, triacylglycerides.

Table 3. Relative fatty acid composition in percent 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

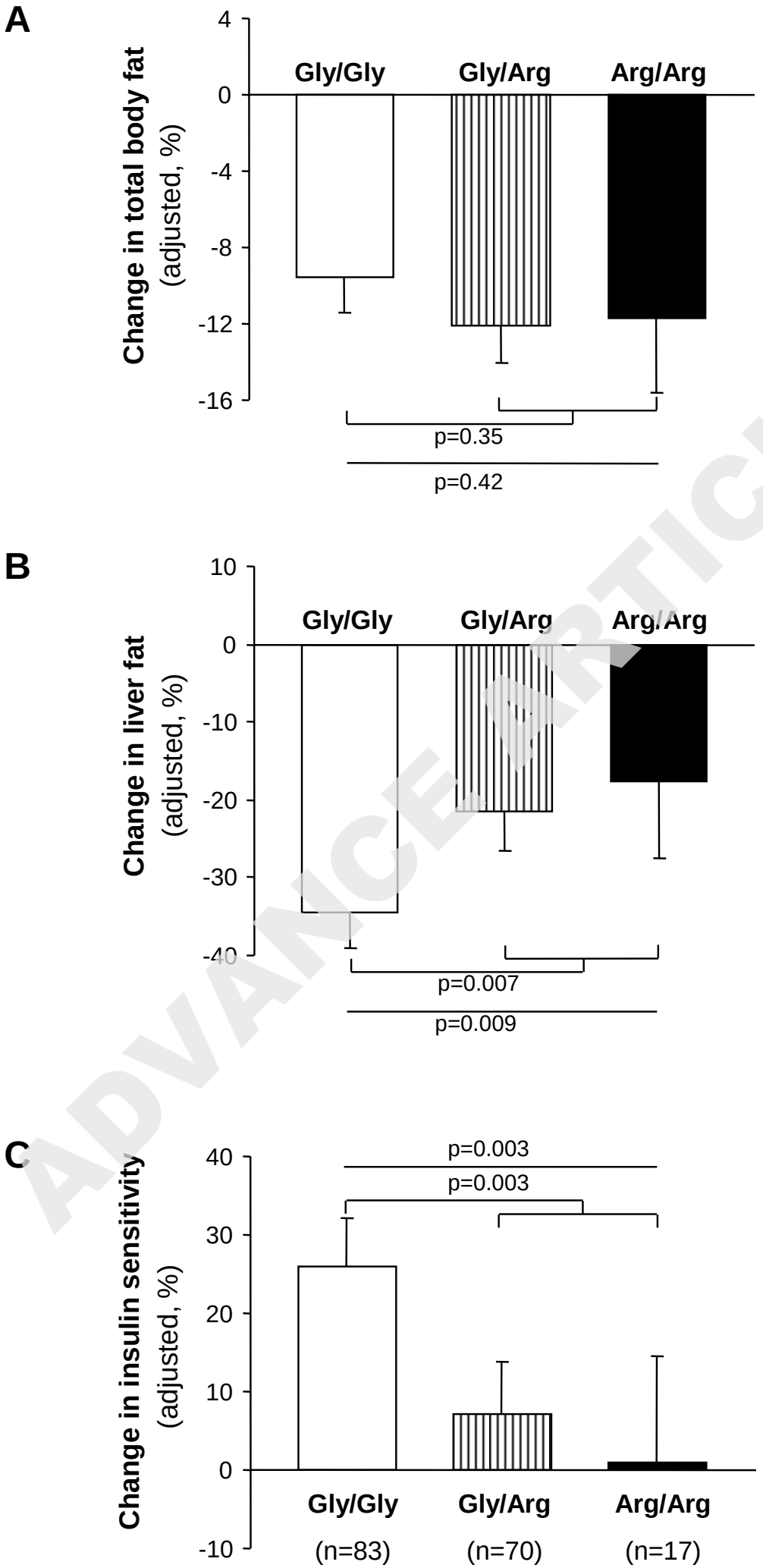

Figure 2

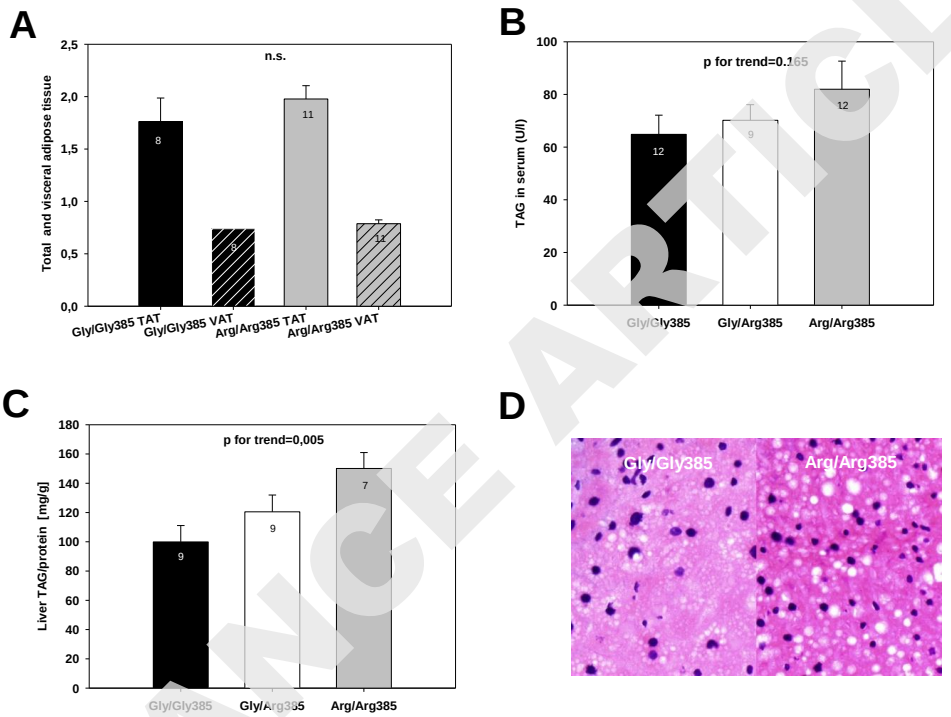

Figure 3