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Effects of TM6SF2 rs58542926 polymorphism on hepatocellular lipids and insulin resistance in early type 2 diabetes

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Abstract *Background and aims:* Increased hepatocellular lipid content (HCL) is linked to insulin resistance, risk of type 2 diabetes and related complications. Conversely, a single-nucleotide polymorphism (*TM6SF2^{EK};* rs58542926) in the *transmembrane 6 superfamily member 2*-gene has been associated with nonalcoholic fatty liver disease (NAFLD), but lower cardiovascular risk. This case-control study tested the role of this polymorphism for tissue-specific insulin sensitivity during early course of diabetes.
Methods and results: Males with recent-onset type 2 diabetes with (TM6SF2^{EK}: n = 16) or without (TM6SF2^{EE}: n = 16) the heterozygous *TM6SF2*-polymorphism of similar age and body mass index, underwent Botnia-clamps with [6,6-$^{2}H_2$]glucose to measure whole-body-, hepatic-

Abbreviations: Alanine aminotransferase, ALT; aspartate aminotransferase, AST; AST to platelet ratio index, APRI; body mass index, BMI; estimated glomerular filtration rate, eGFR; endogenous glucose production, EGP; difference between fasting and insulin-suppressed endogenous glucose production, ΔEGP; fasting β-cell function, HOMA-B; fasting insulin resistance, HOMA-IR; high-sensitivity C-reactive protein, hsCRP; fibrosis-4, FIB4; free fatty acid, FFA; glucokinase regulatory protein, GCKR; German Diabetes Study, GDS; gamma-glutamyl transferase, GGT; hemoglobin A1c, HbA_{1c}; hepatocellular lipids, HCL; high-density lipoprotein, HDL; low density lipoprotein, LDL; mild age-related diabetes, MARD; membrane-bound O-acyltransferase domain-containing 7, MBOAT7; mild obesity-related diabetes, MOD; magnetic resonance spectroscopy, MRS; nonalcoholic fatty liver disease, NAFLD; patatin-like phospholipase domain-containing 3, PNPLA3; rates of glucose disappearance, R_d; energy expenditure, REE; standard deviation, SD; standard error of the mean, SEM; severe insulin-deficient diabetes, SIDD; severe insulin-resistant diabetes, SIRD; single-nucleotide polymorphisms, SNP; triacylglycerol, TAG; transmembrane 6 superfamily member 2, TM6SF2; TM6SF2 polymorphism rs58542926 carriers (TM6SF2^{EK}) and non-carriers, TM6SF2^{EE}; carbon dioxide output, VCO_2; very-low-density lipoprotein, VLDL; oxygen uptak, VO_2.

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Type 2 diabetes;
Insulin sensitivity

and adipose tissue-insulin sensitivity. HCL was assessed with ^{1}H-magnetic-resonance-spectroscopy. A subset of both groups (n = 24) was re-evaluated after 5 years. Despite doubled HCL, TM6SF2^{EK} had similar hepatic- and adipose tissue-insulin sensitivity and 27% higher whole-body-insulin sensitivity than TM6SF2^{EE}. After 5 years, whole-body-insulin sensitivity, HCL were similar between groups, while adipose tissue-insulin sensitivity decreased by 87% and 55% within both groups and circulating triacylglycerol increased in TM6SF2^{EE} only.
Conclusions: The *TM6SF2*-polymorphism *rs58542926* dissociates HCL from insulin resistance in recent-onset type 2 diabetes, which is attenuated by disease duration. This suggests that diabetes-related metabolic alterations dominate over effects of the *TM6SF2*-polymorphism during early course of diabetes and NAFLD.

1. Introduction

Elevated hepatocellular lipid (HCL) content is the key feature of nonalcoholic fatty liver disease (NAFLD), which associates with obesity, insulin resistance, type 2 diabetes, and other diabetes-related comorbidities [1]. Aside from environment and lifestyle, inherited factors can also increase the risk of NAFLD, its progression, and comorbidities, as shown for variations in *patatin-like phospholipase domain-containing 3 (PNPLA3)*, *transmembrane 6 superfamily member 2 (TM6SF2)*, *glucokinase regulatory protein (GCKR)* and *membrane-bound O-acyltransferase domain-containing 7 (MBOAT7)* [2,3].

Interestingly, some single-nucleotide polymorphisms (SNP) such as the *rs738409(G)* allele in *PNPLA3* may lead to progressive NAFLD, but not necessarily to insulin resistance or higher risk of type 2 diabetes [4]. However, persons featuring the severe insulin-resistant diabetes (SIRD) endotype are more frequently carriers of the *PNPLA3* variant and have a higher prevalence of NAFLD and markers of hepatic fibrosis [4].

The *rs58542926* SNP in the *TM6SF2* gene also associates with NAFLD including fibrosis and cirrhosis [5,6], but with lower plasma triacylglycerol (TAG) and very-low-density lipoprotein (VLDL) cholesterol concentrations [5,7,8]. The *TM6SF2* gene encodes a protein, predominantly expressed in the liver and localized to the endoplasmic reticulum where TAG-rich particles are assembled [5,9]. Experimental modification of *TM6SF2* expression confirmed its role in regulating lipid storage and secretion [9–12], e. g. by showing increased HCL and decreased secretion of TAG-rich apolipoproteins by *TM6SF2* inhibition in human hepatocytes [9]. Contradictory data exist for the role of the *TM6SF2* polymorphism in development of type 2 diabetes. While some studies reported a relationship between this polymorphism and insulin resistance or type 2 diabetes [5,13–19] others did not find these associations [20–22]. These conflicting results may be at least partly due to differences in the duration of diabetes and/or in the degree of chronic glucolipotoxicity and low-grade inflammation. A possible relationship between insulin sensitivity and diabetes-related comorbidities in *TM6SF2* carriers during the early course of diabetes has not been reported yet. Likewise, it is unclear whether diabetes endotypes exhibit differences in the prevalence of the *TM6SF2* polymorphism. Growing evidence exists that diabetes shows a broad spectrum of the phenotypic heterogeneity by genetic variation modifying main features of the disease progression and onset of comorbidities and complication [23]. However, this pathophysiological heterogeneity is not captured by current position statements and guidelines for diagnosis and treatment of diabetes [23,24].

This study aimed at determining whether the *TM6SF2* polymorphism: (i) is related to whole-body and tissue-specific insulin sensitivity in recent-onset type 2 diabetes, and (ii) affects the metabolic changes during the early course of diabetes and therefore *TM6SF2* carriers require different diabetes management than non-carriers during the early course of type 2 diabetes.

2. Methods

2.1. Participants

From the German Diabetes Study (GDS), 16 male persons with recently diagnosed type 2 diabetes (known duration <1 year) carrying the *TM6SF2* polymorphism *rs58542926* (TM6SF2^{EK}) and 16 male non-carriers (TM6SF2^{EE}) matched for age, body mass index (BMI) and known diabetes duration were included. This study identified only 5 female carriers of the *TM6SF2* polymorphism, which does not allow for separate statistical analysis (power of 6% only). Also, a pooled analysis of both sexes combined would not be adequate due to the unbalanced sample size between male and female participants and the known sex-specific differences in glucose tolerance between *TM6SF2* polymorphisms in mice and humans [21]. Data of the follow-up examinations after 5 years were available in subgroups. The comparison of both groups during follow-up represents only an exploratory outcome of this study, so that the data availability from all participants for both baseline and follow-up was not mandatory for inclusion. Indeed, 6 participants with TM6SF2^{EK} (n = 3: loss of contact, n = 1: uncontrolled hypertension, n = 1: suspected myopathy, n = 1: personal reasons) and 2 with TM6SF2^{EE} (n = 2: personal reasons)

were lost during follow-up. Further, magnetic resonance spectroscopy (MRS) could not be performed in 2 participants with TM6SF2^{EK} (n = 1: technical reasons, n = 1: personal reasons) and in 2 with TM6SF2^{EE} (n = 1: technical reasons, n = 1: safety consideration because of metal implant). We applied the sex-specific classification rules to assign all participants to the predefined clusters such as mild age-related diabetes (MARD), mild obesity-related diabetes (MOD), severe insulin-deficient diabetes (SIDD) or SIRD using the nearest centroid approach as previously described [25]. The GDS is a prospective observational study investigating the natural course of diabetes and its comorbidities (ClinicalTrials.gov registration no: NCT01055093). Its study design and cohort profile have been described elsewhere in detail [26]. Briefly, specific exclusion criteria were history of acute or chronic diseases including cancer, medication affecting the immune system, hemoglobin A1c (HbA_{1c}) > 9.0% (75 mmol/mol), and other diabetes types. The GDS excludes participants with known liver disease and other relevant diseases at baseline and re-evaluates comorbidities annually in telephone interviews. Participants withdrew their oral glucose-lowering medication for at least 3 days, long-acting incretins for at least 1 week and insulin for 12 h before all measurements [26]. Of note, none of the participants received thiazolidinediones. All participants gave written informed consent before study inclusion, which was performed according to the latest version of the Declaration of Helsinki and approved by the local ethics board of the Medical Faculty of Heinrich Heine University in Düsseldorf, Germany.

2.2. Hyperinsulinemic-euglycemic clamp test

The Botnia clamp comprises an intravenous glucose tolerance test followed by a hyperinsulinemic-euglycemic clamp test as described in detail before [26]. A primed continuous D-[6,6-$^2H_2$]glucose infusion is used to assess insulin sensitivity during fasting and clamp conditions [26]. The clamp was started with a priming dose of 10 mU*body weight [kg]$^{-1}$ *min^{-1} for 10 min, followed by a continuous infusion of short-acting human insulin (Insuman® Rapid, Sanofi-Aventis, Frankfurt am Main, Germany) (1.5 mU*body weight [kg]$^{-1}$ *min^{-1}) for 3 h to assess whole-body insulin sensitivity from insulin-stimulated rates of glucose disappearance (R_d) during clamp steady-state [26]. Hepatic insulin sensitivity was assessed from the difference between fasting and insulin-suppressed endogenous glucose production (ΔEGP) [27]. Adipose tissue insulin sensitivity was assessed from insulin-mediated free fatty acid (FFA) suppression and calculated as 1 - (average FFA during clamp steady-state/baseline FFA) [27].

2.3. Whole-body substrate metabolism

Before and during clamp stead-state, open-circuit indirect calorimetry (Vmax Encore 29n, CareFusion, Höchberg, Germany) was employed to measured oxygen uptake (VO_2) and carbon dioxide output (VCO_2) and to assess resting lipid, carbohydrate oxidation and energy expenditure (REE) [26].

^{1}H-MRS. HCL was measured using a 3-T whole-body MR scanner (Achieva X-series, Philips Healthcare, Best, The Netherlands) as described in detail before [26].

2.4. Genotyping

Genomic DNA was extracted from whole blood and genotyping was performed by real-time polymerase chain reaction-based allelic discrimination with probe-based genotyping assay for the *rs58542926* SNP in *TM6SF2* (Thermofisher, Darmstadt, Germany) (Supplementary CTAT Table). The genotype concordance of >99.8% was determined using the TaqMan Genotyper software v.1.3 (Thermofisher) [4].

2.5. Laboratory analyses

Fasting plasma glucose, insulin, FFA, HbA_{1c}, high-density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, total cholesterol, TAG, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and high-sensitivity C-reactive protein (hsCRP) were measured as described before [26]. The estimated glomerular filtration rate (eGFR) was calculated based on creatinine and cystatin C [28] and urinary albumin was measured by a routine lab method and levels of >20 mg/l were considered to indicate nephropathy [26].

2.6. Calculations

Homeostasis model assessment of β-cell function was used to assess fasting β-cell function (HOMA-B) and fasting insulin resistance (HOMA-IR) from fasting glucose and insulin concentrations [29]. The fibrosis-4 (FIB4), AST to platelet ratio index (APRI) and AST/ALT index were used as noninvasive surrogate tests for hepatic fibrosis and calculated from routine lab parameters [30].

2.7. Statistical analyses

Results are given as median [1st and 3rd quartiles] or mean ± standard error of the mean (SEM) as appropriate. Variables were compared using Wilcoxon matched-pairs signed rank test, unpaired two-tailed Students t-test and two-tailed Mann-Whitney *U* test as indicated and presented to determine differences over time and between groups. Nominal variables were compared by Chi-square and Fisher's exact test as appropriate. Relationships between variables were investigated using Spearman rank correlation analyses. The power calculation was based on the primary outcome, i. e. the difference of whole-body (skeletal muscle) insulin sensitivity between carriers and non-carriers of the *TM6SF2* polymorphism in the cross-sectional comparison at baseline. The standardized mean difference (Cohen's d), a generally accepted measure of the effect size (d) [31], was used for power

Table 1 Participants' characteristics at baseline.

	$TM6SF2^{EK}$	$TM6SF2^{EE}$	p-value
Male	16	16	
Known diabetes duration [days]	186 [93; 299]	142 [97; 176]	0.147
Age [years]	49 [44; 56]	50 [46; 58]	0.546
BMI [kg/m]	32 [27; 38]	32 [26; 37]	0.792
Waist-hip ratio [a.u.]	0.96 [0.94; 1.00]	1.00 [0.91; 1.03]	0.688
Body fat mass [kg]	34 [24; 40]	34 [23; 41]	0.940
Lean body weight [kg]	70 [65; 78]	69 [60; 79]	0.763
Diastolic BP [mmHg]	93 [79; 99]	83 [77; 95]	0.309
Systolic BP [mmHg]	144 [127; 159]	134 [127; 155]	0.692
Fasting plasma glucose [mmol/l]	6.6 [5.9; 8.2]	6.1 [5.4; 6.6]	0.235
HbA_{1c} [%]	6.2 [5.6; 6.6]	6.0 [5.3; 6.6]	0.375
(HbA_{1c} [mmol/mol])	(44 [37; 48])	(42 [34; 49])	
Fasting plasma insulin [mU/l]	15 [10; 28]	18 [11; 25]	0.821
Fasting plasma C-peptide [ng/dl)]	3.0 [2.4; 4.1]	3.6 [2.0; 4.4]	0.845
Fasting plasma FFA [μmol/l]	581 [462; 718]	490 [440; 607]	0.780
Fasting plasma triglycerides [mg/dl]	116 [98; 174]	111 [78; 178]	0.665
Total cholesterol [mg/dl]	183 [169; 216]	183 [159; 206]	0.451
HDL-cholesterol [mg/dl]	43 [36; 50]	45 [40; 48]	0.821
LDL-cholesterol [mg/dl]	122 [106; 149]	113 [94; 131]	0.152
GGT [U/l]	26 [23; 29]	30 [25; 57]	0.131
ALT [U/l]	32 [23; 55]	33 [22; 50]	0.140
AST [U/l]	26 [20; 33]	20 [17; 32]	0.865
hsCRP [mg/dl]	0.2 [0.1; 0.4]	0.2 [0.1; 0.5]	0.850
eGFR [ml/min per 1.73 m^2]	90 [73; 100]	93 [87; 103]	0.184
Microalbuminuria [mg/l]	3.5 [2.3; 14]	4.7 [1.4; 13.5]	0.948
REEbasal [kcal/day]	2040 [1670; 2190]	2018 [1818; 2282]	0.780
REEclamp [kcal/day]	2068 [1839; 2276][$]	2055 [1873; 2233]	0.953
HOMA-B [a.u.]	142 [56; 188][§]	173 [87; 184]	0.525

Data are shown as absolute numbers, as median [1st; 3rd quartile], as applicable. p-values based on two-tailed Mann-Whitney *U* test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BL, baseline; eGFR, estimated glomerular filtration rate; FFA, free fatty acids; FU, follow-up; GGT, gamma-glutamyl transferase; HbA_{1c}, glycated hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; REEbasal and REEclamp, resting energy expenditure in fasting conditions and during clamp; $TM6SF2^{EK}$, carriers and $TM6SF2^{EE}$, non-carriers of the *TM6SF2* gene variant with type 2 diabetes. HOMA-B, Homeostasis model assessment of β-cell function assessed from fasting glucose and insulin concentrations. $ Measurements of n = 1 missing. § 2 participants that received insulin treatment were excluded from analyses.

analyses using G*Power (G*Power Version 3.1.9.7, Kiel, Germany). Cohen's d for equally sized groups is calculated from known mean values (m1, m2) and known standard deviations (SD1, SD2) using the formula (m1 - m2)/σ, where the pooled standard deviation (σ) is defined by the formula $\sqrt{((SD1^2 + SD2^2)/2)}$ [31]. In the absence of estimates for m and SD of clamp-derived whole-body insulin sensitivity in carriers of the polymorphism in the literature, the power calculation of the present study used previously published values for whole-body insulin sensitivity from persons with well-controlled type 2 diabetes and healthy humans (5.6 ± 2.0 vs. 9.0 ± 2.0 $mg*kg^1 * min^1$) [27]. Assuming a similar SD and half of the mean difference between persons with and without type 2 diabetes for whole-body insulin sensitivity in carriers, we calculated the effect size of 1.2 using G*Power between carriers and non-carriers of the *TM6SF2* polymorphism with recent-onset type 2 diabetes.

Based on the 2-sample-2-sided *t*-test, this power calculation using G*Power revealed that a standardized mean difference of 1.2 (for a large effect size) can be detected in a sample size of n = 16 per group with a power of 90%. As our experiments showed later, the effect size for whole-body insulin sensitivity (Cohen's d: 1.3) was even larger. All statistical tests were two-sided and a p-value less than 5% was accepted to indicate significant differences. All statistical analyses were performed using SPSS for Windows 23.0 (SPSS Inc., Chicago, IL, USA) and all graphs were generated using GraphPad Prism, Version 8.3.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Participants' characteristics at baseline

Carriers ($TM6SF2^{EK}$) and non-carriers ($TM6SF2^{EE}$) had similar basic anthropometric and metabolic parameters (Table 1). Known diabetes duration (Table 1), distribution of diabetes endotypes (p = 0.779) [25] (Table S1) and of glucose-lowering medication were comparable between both groups (p = 0.481) (Table S2, Figure S1A). Of note, there was no difference between statin, acetylsalicylic acid, β1-receptor blocker, angiotensin-converting-enzyme or proton-pump inhibitor treatment between groups (Table

S2). None of the participants of the present study was carrier of the SNP rs738409(G) allele in PNPLA3.

3.2. Tissue-specific insulin sensitivity at baseline

TM6SF2^{EK} and TM6SF2^{EE} had comparable fasting endogenous glucose production (EGP) (1.7 [1,6,2,1] vs. 1,9 [1,6,2,1], p = 0.926). They also featured similar HOMA-IR (Fig. 1E). During the clamp, whole-body insulin sensitivity was 27% higher in carriers of TM6SF2^{EK} (Fig. 1F), whereas hepatic and adipose tissue insulin sensitivity were similar in both groups (Fig. 1G and H).

3.3. HCL and liver fibrosis risk at baseline

As expected, TM6SF2^{EK} exhibited doubled HCL than TM6SF2^{EE} carriers (Fig. 1A). Of note, 56% of TM6SF2^{EK} and 38% of TM6SF2^{EE} had diagnosis of NAFLD (p = 0.370), as

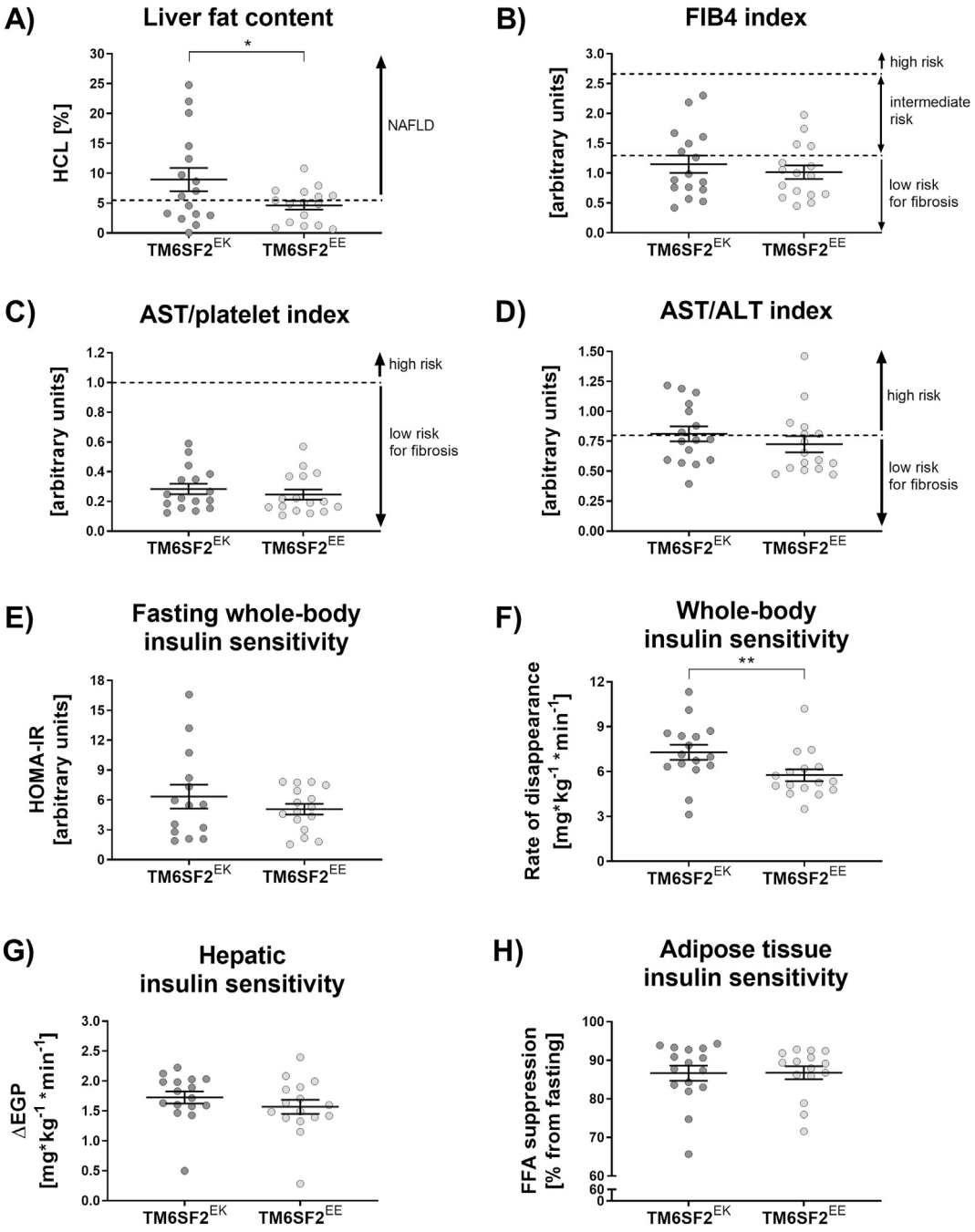

Figure 1 Hepatocellular lipid content, fibrosis risk and tissue-specific insulin sensitivity in recent-onset type 2 diabetes. **(A)** Hepatocellular lipid content (HCL), **(B)** fibrosis-4 (FIB4), **(C)** AST/platelet (APRI) and **(D)** AST/ALT index were used for hepatic fibrosis. **(E)** Homeostasis model assessment of insulin resistance (HOMA-IR) was used to assess fasting whole-body (hepatic) insulin resistance. **(F)** Whole-body (skeletal muscle), **(G)** hepatic and **(H)** adipose tissue insulin sensitivity were measured during clamp. Data are shown as individual values and mean ± SEM. $*p < 0.05$, $**p < 0.01$, p-values based on Students t-test and Mann-Whitney *U* test.

defined by HCL >5.56% [32]. Fasting circulating lipids, transaminases (Table 1) and non-invasive fibrosis tests (Fig. 1B, C, D) were not different between groups. According to FIB4, none of the participants was at high risk of hepatic fibrosis, as defined by the cutoff >2.67 [30], while 38% of TM6SF2^{EK} and 25% of TM6SF2^{EE} were at intermediate risk at the cutoff ≥1.3 (p = 0.704) [30]. According to APRI, no participant was at high risk of hepatic fibrosis, as defined by the cutoff >1 [33], whereas 44% of TM6SF2^{EK} and 38% of TM6SF2^{EE} were at risk (p = 0.999), according to the cutoff ≥0.8 for the AST/ALT ratio [30].

3.4. Participants' characteristics during follow-up

Comparing both groups at 5 years revealed no differences regarding the glucose-lowering medication, in statin and acetylsalicylic acid, β1-receptor blocker, angiotensin-converting-enzyme or proton-pump inhibitor treatment, main anthropometric and metabolic variables between TM6SF2^{EK} and TM6SF2^{EE} carriers (Table S2, 3, Figure S1A). Comparing the changes within each group, we found that TM6SF2^{EK} neither changed their body weight nor lean body mass (Table S4, Fig. 2B, Figure S1B), whereas TM6SF2^{EE} carriers had slightly increased their body mass and body fat content (Table S4, Fig. 2A and B). Of note, only TM6SF2^{EE} carriers developed increased fasting blood glucose and HbA_{1c} by 25% and 14%, respectively, and higher serum insulin concentrations when compared to baseline (Fig. 2C–F, Table S4, Figure S1C). Fasting lipid concentrations, whole-body substrate metabolism remained unchanged over 5 years in both groups (Table S3, S4). Kidney function and urinary albumin levels (Table S4) remained unchanged. Medication (glucose-lowering, acetylsalicylic acid, β1-receptor blocker, angiotensin-converting-enzyme inhibitor or proton-pump inhibitor) did not differ within each group over time (Table S2, Figure S1A). Use of statins slightly increased among TM6SF2^{EE} but not TM6SF2^{EK} during the following 5 years (Table S2).

3.5. Tissue-specific insulin sensitivity at follow-up

Comparison between both groups at 5 years of diabetes, showed similar fasting EGP (data not shown) and a trend towards lower HOMA-IR in TM6SF2^{EK} compared to TM6SF2^{EE} (p = 0.057) (Table S3). At 5 years of type 2 diabetes, previously existing differences in whole-body insulin sensitivity had disappeared between carriers of TM6SF2^{EK} and TM6SF2^{EE} (Fig. 3E and F). Hepatic insulin sensitivity was similar between both groups at 5 years (follow up in TM6SF2^{EK}: 1.77 [1.58; 2.01] vs. TM6SF2^{EE}: 1.55 [1.38; 1.89], p = 0.667). Adipose tissue insulin sensitivity was comparable between TM6SF2^{EK} and TM6SF2^{EE} at 5 years (Fig. 3G and H). Comparing the changes within each group, fasting EGP (data not shown) did not change after 5 years of diabetes. HOMA-IR remained unchanged in persons with TM6SF2^{EK} during 5 years of diabetes progression (Fig. 3C, Table S3), but tended to increase vs. baseline in TM6SF2^{EE} (Fig. 3D, Table S3). Whole-body insulin sensitivity was similar in both groups compared to baseline (Fig. 3E and F). Hepatic insulin sensitivity remained unchanged in both TM6SF2^{EK} (p = 0.193) and TM6SF2^{EE} (p = 0.463) during 5 years of diabetes progression in both groups. Adipose tissue insulin sensitivity decreased by 87% in TM6SF2^{EK} (p < 0.01) and 55% in TM6SF2^{EE} (p < 0.001) compared to baseline (Fig. 3G and H).

3.6. HCL and liver fibrosis risk at follow-up

Comparisons between TM6SF2^{EK} and TM6SF2^{EE} at 5 years of diabetes showed that initially existing differences in HCL disappeared after 5 years (Fig. 3A and B). Of note, even after exclusion of data with loss of follow-ups the detected differences at baseline remained significant (p < 0.05). NAFLD was present in 6 of 8 (75%) of TM6SF2^{EK} and all 12 TM6SF2^{EE} carriers with available MRS data (p = 0.147). Both groups had similar circulating TAG, total, HDL and LDL cholesterol as well as AST and ALT, but GGT was slightly higher in TM6SF2^{EE} (Table S3). At 5 years, TM6SF2^{EK} and TM6SF2^{EE} showed similar FIB4 (p = 0.625), APRI (p = 0.341) and AST/ALT ratio (p = 0.437). Comparing changes within each group, HCL had increased by 32% in TM6SF2^{EK} (Fig. 3A) and by 71% in TM6SF2^{EE} (Fig. 3B) at 5 years of diabetes. While circulating TAG increased in TM6SF2^{EE} only (Fig. 2G and H), total, HDL and LDL cholesterols and transaminases did not change in both groups after 5 years of type 2 diabetes when compared to baseline (Table S4). FIB4 was comparable in both groups vs. baseline (follow up in TM6SF2^{EK}: 1.1 [0.7; 1.4], p = 0.084 and TM6SF2^{EE}: 0.9 [0.7; 1.2], p = 0.761). The APRI (follow up in TM6SF2^{EK}: 0.2 [0.2; 0.3], p = 0.232 and TM6SF2^{EE}: 0.2 [0.2; 0.3], p = 0.715) and AST/ALT ratio were also unchanged (follow up in TM6SF2^{EK}: 0.7 [0.6; 0.8], p = 0.322 and TM6SF2^{EE}: 0.6 [0.6; 0.8], p = 0.855) compared to baseline.

3.7. Correlation analyses

In TM6SF2^{EK}, HCL associated positively with body fat mass (r = 0.43, p < 0.05) and hsCRP (r = 0.53, p < 0.01), while changes in HCL correlated negatively with whole-body insulin sensitivity (r = −0.86, p < 0.05). Changes in whole-body insulin sensitivity associated negatively with changes in BMI (r = −0.68, p < 0.05). Adipose tissue insulin sensitivity associated negatively with fasting blood glucose (r = −0.43, p < 0.05), HbA_{1c} (r = −0.41, p < 0.05) and fasting FFA (r = −0.43, p < 0.05) and negatively with changes in BMI (r = −0.75, p < 0.05). Changes in adipose tissue insulin sensitivity associated negatively with fasting TAG (r = −0.77, p < 0.05).

In TM6SF2^{EE}, HCL associated negatively with adipose tissue insulin sensitivity (r = −0.66, p < 0.001) and correlate positively with HOMA-IR (r = 0.64, p < 0.001). Changes of HCL correlated positively with fasting TAG (r = 0.62, p < 0.05) and positively with changes in BMI (r = 0.59, p < 0.05), fasting blood glucose (r = 0.66, p < 0.05), HbA_{1c} (r = 0.89, p < 0.001), fasting insulin (r = 0.64, p < 0.05), hsCRP (r = 0.64, p < 0.05), HOMA-IR

($r = 0.73$, $p < 0.05$) and negatively with HOMA-B ($r = -0.67$, $p < 0.05$). Adipose tissue insulin sensitivity associated negatively with fasting blood glucose ($r = -0.51$, $p < 0.01$), HbA_{1c} ($r = -0.42$, $p < 0.05$) and tended to correlate negatively with TAG levels ($r = -0.35$, $p = 0.056$). Furthermore, changes in adipose tissue insulin sensitivity associated negatively with BMI ($r = -0.75$, $p < 0.05$) and body fat mass ($r = -0.75$, $p < 0.05$).

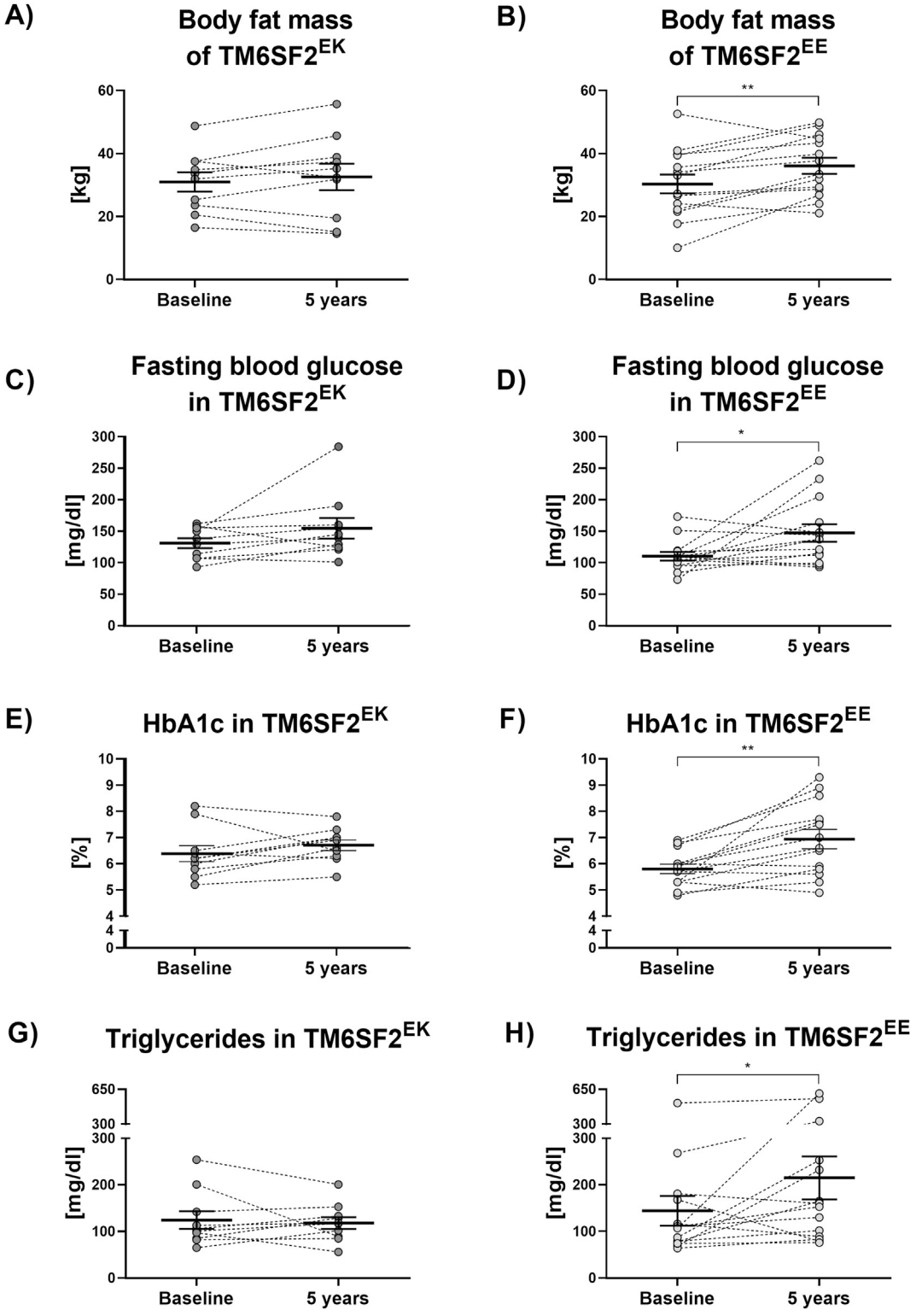

Figure 2 Changes in participants' characteristics at 5 years of type 2 diabetes diagnosis. **(A, B)** Individual data showing progression patterns in body fat mass, **(C, D)** fasting blood glucose, **(E, F)** HbA_{1c} and **(G, H)** triglycerides between baseline and follow-up. Data are shown as individual values and mean ± SEM. $*p < 0.05$, $**p < 0.01$, p-values based on Wilcoxon matched-pairs signed rank and Mann-Whitney *U* test.

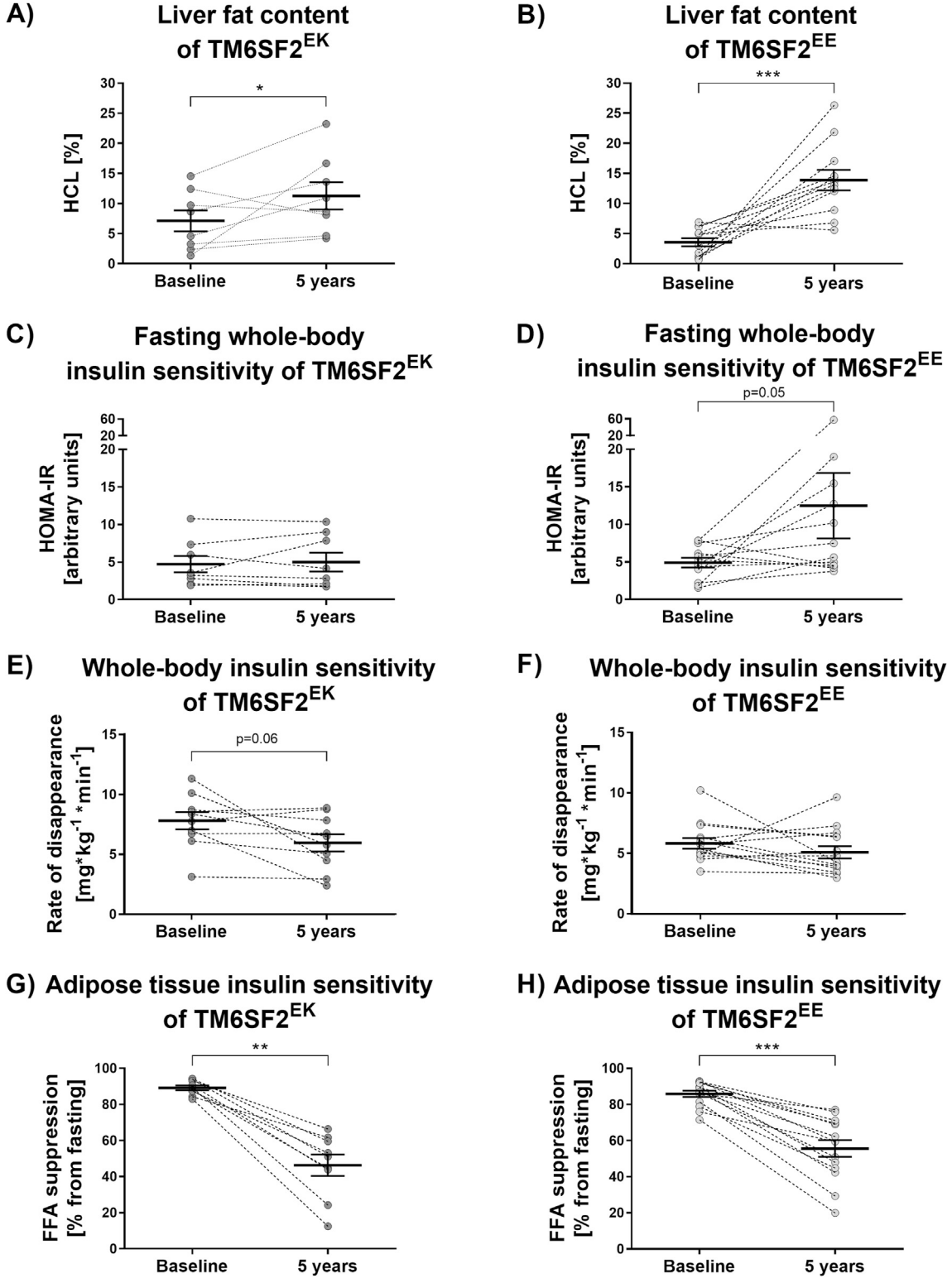

Figure 3 Hepatocellular lipid content and tissue-specific insulin sensitivity at 5 years of type 2 diabetes diagnosis. (A, B) Data showing progression patterns in hepatocellular lipid content (HCL), **(C, D)** fasting whole-body (hepatic) from HOMA-IR, **(E, F)** whole-body (skeletal muscle), and **(G, H)** adipose tissue insulin sensitivity during clamp between baseline and follow-up. Data are shown as individual values and mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, p-values based on Wilcoxon matched-pairs signed rank and Mann-Whitney *U* test.

4. Discussion

This study shows that individuals with recent-onset type 2 diabetes carrying the *TM6SF2* variant (TM6SF2^{EK}) feature almost doubled liver fat content, but even higher whole-body (skeletal muscle) insulin sensitivity and comparable hepatic and adipose tissue insulin sensitivity when compared to non-carriers (TM6SF2^{EE}). Importantly, increasing adipose tissue insulin resistance in both groups and metabolic changes during the early course of diabetes in non-carriers such as rising glycemia, lipidemia, and fasting insulin resistance likely account for the disappearance of initial differences in HCL between TM6SF2^{EK} and TM6SF2^{EE} carriers.

The present study extends the observation of greater HCL contents in TM6SF2^{EK} carriers [13] for a cohort with recent-onset type 2 diabetes, but did not confirm previously found lower plasma TAG and lipoproteins in TM6SF2^{EK} carriers [5,34]. The present report cannot support the reported higher risk of advanced hepatic fibrosis [6], in TM6SF2^{EK}, which however is likely due to short disease duration, excellent metabolic control, and the overall low initial fibrosis stage in the present cohort.

Interestingly, TM6SF2^{EK} carriers with recent-onset type 2 diabetes featured higher whole-body insulin sensitivity than non-carriers. Whereas genome-wide association studies described a higher risk for type 2 diabetes in the carriers [20,21], previous studies did not detect differences in insulin resistance between carriers and non-carriers in mixed cohorts with and without type 2 diabetes. These studies did not consider different degrees of glycemic control, known duration of diabetes and/or sex-specific analyses [5,13–17]. Of note, previous studies used the HOMA-IR for assessing insulin sensitivity, which is an accepted surrogate parameter but rather reflects hepatic insulin resistance in the fasted state [35]. Indeed, HOMA-IR was also not different between both groups in the present study, as was hepatic insulin sensitivity as measured under clamp conditions.

While TM6SF2^{EK} carriers showed no difference in hepatic insulin sensitivity, whole-body insulin sensitivity was markedly higher despite almost doubled HCL when compared to TM6SF2^{EE}. Whole-body insulin sensitivity was assessed from insulin-stimulated R_d, which mainly reflects skeletal muscle insulin sensitivity [36]. Interestingly, this study found a correlation of HCL with HOMA-IR in non-carriers, but not in carriers of the *TM6SF2* polymorphism, suggesting that this polymorphism dissociates ectopic lipid storage from insulin resistance in the liver in type 2 diabetes. Although HCL and NAFLD generally correlate negatively with hepatic and skeletal muscle insulin sensitivity [1,35], the present findings underline the concept that intracellular lipotoxic metabolites - rather than ectopic storage of neutral lipids, i. e. HCL are driving insulin resistance [2,37,38]. Of note, molecular mechanisms underlying the association between HCL and hepatic insulin resistance may involve inadequate mitochondrial adaptation to higher lipid flux [39] and subsequent intracellular accumulation of lipid metabolites such as diacylglycerols, which inhibit proximal insulin signaling [1], or ceramides, which may also stimulate inflammatory pathways [40]. Studies in human hepatocytes with reduced TM6SF2 expression [9–12] and one stable-isotope tracer study in *TM6SF2* carriers [34] found reduced hepatic lipid efflux due to disturbed secretion of TAG-rich apolipoproteins in carriers of the *TM6SF2* variant. Decreased secretion of TAG-rich lipids could protect peripheral tissues such as skeletal muscle from lipotoxic metabolites and thereby explain the higher whole-body insulin sensitivity in the risk allele carriers. Of note, the present study found no increase in fasted plasma TAG and cholesterols, in agreement with a previous report [41], which may be due to the fact that individual plasma TAG concentrations result from different mechanisms involving not only the liver.

Within hepatocytes, redistribution of lipotoxic metabolites, e. g. directing diacylglycerols into certain intracellular compartments would protect from activation of novel protein kinase isoforms and thereby from lipotoxic hepatic insulin resistance [1]. Indeed, one study reported higher hepatic and adipose tissue insulin sensitivity and a lack of hypertriglyceridemia despite 34% higher HCL in carriers of the *TM6SF2* polymorphism in a mixed cohort of humans with and without diabetes [13]. Thus, the absence of insulin resistance at the level of the adipose tissue in that and the present study argues against a key role of the adipose tissue for ectopic fat deposition in this specific genotype, which has been postulated for common type 2 diabetes [1,42]. In humans without diabetes, carriers of the *TM6SF2* polymorphism had higher hepatic and adipose insulin resistance, and higher muscle insulin sensitivity as assessed by oral glucose tolerance test-derived indexes of glucose homeostasis [19]. Thus, besides higher muscle insulin sensitivity, additional effects on insulin sensitivity of the liver and adipose tissue may be present in carriers of the *TM6SF2* polymorphism before onset of type 2 diabetes, which may disappear when diabetes develops.

The relevance of the *TM6SF2* polymorphism for the development of type 2 diabetes has not been reported before. In the present study, HCL had increased markedly in both TM6SF2^{EK} and TM6SF2^{EE} within five years after diabetes diagnosis. By that time, the previously detected differences in liver fat accumulation and whole-body insulin sensitivity between TM6SF2^{EK} and TM6SF2^{EE} had disappeared. We found a trend towards higher HOMA-IR in TM6SF2^{EE} and to lower whole-body insulin sensitivity (insulin-stimulated R_d) in TM6SF2^{EK} after 5 years. In TM6SF2^{EK} changes in HCL correlated negatively with whole-body insulin sensitivity, which in turn associated negatively with changes in BMI. Thus, increase in body mass could primarily account for their decline in whole-body insulin sensitivity. In addition, adipose tissue insulin sensitivity progressively declined in both TM6SF2^{EK} and TM6SF2^{EE} within 5 years. Increased adipose tissue insulin resistance will favor lipid efflux to the liver with subsequent increased HCL accumulation [1], which occurs independently of *TM6SF2* polymorphisms. This combined with the observed metabolic deterioration (worsening

glycemia and lipidemia) in non-carriers may have overcome the initial differences in HCL between TM6SF2^{EK} and TM6SF2^{EE} at 5 years disease duration. Interestingly, changes in HCL associated clearly and inversely with reduced whole-body insulin sensitivity only in TM6SF2^{EK}, but HCL correlated negatively with adipose sensitivity and positively with HOMA-IR in TM6SF2^{EE}. Whether this is due to tissue-specific differences in insulin action among these polymorphisms or due to methodological differences cannot be sorted out by this study. Specific tracer-dilution techniques using stable isotope-labeled glycerol or fatty acid tracers in vivo and/or analyses of lipotoxic metabolites in biopsies in vitro could help to clarify these findings in future studies.

Reductions in circulating TAG, VLDL, and LDL cholesterol were suggested to account for the protection against cardiovascular disease observed in carriers of the *TM6SF2* polymorphism [5,7,34]. Although cholesterol levels and statin therapy were similar between groups at baseline or follow-up in this study, significantly more TM6SF2^{EE} used statin therapy at follow-up than baseline. Our results are in agreement with previous studies, showing that the *TM6SF2* polymorphism associates with lower plasma VLDL cholesterol concentrations [5,7,8], which could reflect less need for statin treatment in the TM6SF2^{EK} group over time during the progression of type 2 diabetes.

The strength of this study is the assessment of tissue-specific insulin sensitivity in well-matched groups within the first year of the diagnosis and 5 years later. This allows to follow the initial course of disease without relevant interference from long-term metabolic alterations associated with diabetes. Furthermore, gold-standard methodology allowed to assess insulin sensitivity, HCL and diabetes-related comorbidities. Limitations include the use of single-step hyperinsulinemic euglycemic clamp for measuring hepatic and adipose tissue insulin sensitivity and the use of non-invasive tests as surrogates of liver fibrosis, as liver biopsies were not available. Glucose-lowering medication will directly or indirectly modulate insulin secretion and sensitivity thereby affecting our analyses. While withdrawal for 3 days before metabolic tests to exclude acute effects on glucose metabolism is established for oral glucose-lowering medications (except for pioglitazone) [26,43], respective data on glucagon-like peptide 1 analogs are scarce. Nevertheless, incretin use neither differed at baseline, nor during follow-up or within each group over time. Excluding persons with incretin treatment from the analyses did not change our main outcomes. Of note, the main limitation of this study is the low sample size of people carrying the *TM6SF2* polymorphism, which may affect the generalizability of the results but can be explained by the overall low frequency of this polymorphism [44]. In addition, the number of suitable participants having all gold-standard measures of HCL and whole-body insulin sensitivity by hyperinsulinemic clamp tests was limited. One previous study performed clamp tests in a smaller cohort (n = 13), but did not measure whole-body insulin sensitivity [13]. Moreover, the GDS cohort includes metabolically well-controlled persons with defined short known duration of type 2 diabetes and has strict exclusion criteria for humans with comorbidities. This also contributes to the small number of carriers. While this limits the generalizability of the results, this design allows to sort out several confounders possibly influencing the primary outcome. Another limitation is the inclusion of males only. The low number of females carrying the *TM6SF2* polymorphism may be due to the observation of higher degree of glycemia (blood glucose and HbA_{1c}) levels in males [21]. Consequently, this study does not allow any conclusions as to women with *TM6SF2* polymorphism. Finally, the inclusion of only male Caucasian participants and the strict exclusion criteria of the GDS [26] does not allow extrapolation to the general or other populations.

In conclusion, these findings indicate that the *TM6SF2* gene polymorphism *rs58542926* dissociates liver steatosis from hepatic as well as from skeletal muscle and adipose tissue insulin resistance in the presented cohort of persons with recent-onset type 2 diabetes. This difference disappears with duration of diabetes, suggesting that diabetes presence could dominate over the *TM6SF2* gene polymorphism during NAFLD progression. In terms of precision diabetology, this study may highlight the need of early treatment with insulin sensitizing drugs, specifically targeting adipose tissue insulin resistance. Both carriers and non-carriers with recent-onset type 2 diabetes could benefit from early prevention of adipose tissue insulin resistance.

Author contributions and guarantor statement

K.B., D.F.M., and M.R. designed the study. K.B. and D.F.M. wrote the article and researched the data. M.B., M.S., B.K., O.P.Z., G.B., Y.K., Yu.K., J.K., R.G., V.S.H., H.A.-H., and J.S. researched the data, and reviewed and edited the article. D.F.M. performed laboratory analyses. K.S. reviewed the power calculation. V.B., R.W., and M.R. contributed to the discussion, and reviewed and edited the article. K.B., D.F.M., and M.R. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors gave final approval of this version to be published.

Data availability

The data sets generated during and/or analyzed during the current study are not publicly available, since they are subject to national data protection laws and restrictions imposed by the ethics committee to ensure data privacy of the study participants. However, they can be applied for through an individual project agreement with the principal investigator of the German Diabetes Study. The study

protocol and methods were published in the cohort profile (Szendroedi J et al. (2016) Cohort profile: the German Diabetes Study (GDS). CardiovascDiabetol15: 59).

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Declaration of competing interest

M.R. is on scientific advisory boards of Boehringer-Ingelheim, Eli Lilly, NovoNordisk and Target RWE, and received investigator-initiated support from Boehringer Ingelheim, Nutricia/Danone and Sanofi–Aventis. All contributions to the manuscript by D.F.M. have been performed when affiliated to the German Diabetes Center, prior to current employment by Boehringer Ingelheim. All other authors have no potential conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2023.06.004.

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