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Ext1 heterozygosity causes a modest effect on postprandial lipid clearance in humans[S]

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Abstract Elevated nonfasting TG-rich lipoprotein levels are a risk factor for CVD. To further evaluate the relevance of LDL-receptor (LDLr) pathway and heparan sulfate proteoglycans (HSPGs) in TG homeostasis, we analyzed fasting and postprandial TG levels in mice bearing combined heterozygous mutations in both *Exostosin (Ext) 1* and *Ldlr*, in subjects with hereditary multiple exostosis (HME) due to a heterozygous loss-of-function mutation in *EXT1* or *EXT2* (N = 13), and in patients with heterozygous mutations in *LDLR* [familial hypercholesterolemia (FH)] and SNPs in major HSPG-related genes (n = 22). Mice bearing a homozygous mutation in hepatic *Ext1* exhibited elevated plasma TGs similar to mice lacking other key enzymes involved in HSPG assembly. Compound heterozygous mice lacking *Ldlr* and *Ext1* showed synergy on plasma TG accumulation and postprandial clearance. In human subjects, a trend was observed in HME patients toward reduced postprandial TG clearance with a concomitant reduction in chylomicron clearance [area under the curve (AUC)-retinyl ester (RE) HME, 844 ± 127 vs. controls, 646 ± 119 nM/h, $P = 0.09$]. Moreover, in FH subjects with a high HSPG gene score, retinyl palmitate excursions were higher (AUC-RE, 2,377 ± 293 vs. 1,565 ± 181 nM/h, $P < 0.05$). Incremental AUC-apoB48 was similar between the groups. In conclusion, the data are supportive for a minor yet additive role of HSPG in human postprandial TG clearance, and further studies are warranted.—Mooij, H. L., S. J. Bernelot Moens, P. L. S. M. Gordts, K. I. Stanford, E. M. Foley, M. A. W. van den Boogert, J. J. Witjes, H. C. Hassing, M. W. Tanck, M. A. J. van de Sande, J. H. Levels, J. J. P. Kastelein, E. S. G. Stroes, G. M. Dallinga-Thie, J. D. Esko, and M. Nieuwdorp. **Ext1 heterozygosity causes a modest effect on postprandial lipid clearance in humans. *J. Lipid Res.* 2015.** 56: **665–673.**

Supplementary key words triglycerides • heparan sulfates • hereditary multiple exostoses • familial hypercholesterolemia

The presence of increased TGs, defined as plasma TG levels >1.7 mM (150 mg/dl), occurs in 10–20% of the population in Western countries and is associated with increased risk of CVD (1). The etiology of hypertriglyceridemia is complex, and current therapeutic strategies to lower TG levels, including fibrates, PUFAs, and statins are available, yet their efficacy with regard to lower CVD risk is not fully elucidated. Hence, the need for identification and characterization of additional pathways controlling synthesis and clearance of triglyceride-rich lipoproteins (TRLs) is ongoing, aiming to identify selective and potent targets for interventions. Although TG levels are routinely measured in a fasting state, observational studies have substantiated that nonfasting TG levels are in fact superior in predicting CVD risk (1–6). In this regard, the most important players involved in hepatic removal of TRLs are the LDL receptor (LDLr), the LDL receptor related protein 1 (LRP1), and heparan sulfate proteoglycans (HSPGs) (7–9). Of note, heterozygous

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Abbreviations: AUC, area under the curve; EXT, exostosin; FH, familial hypercholesterolemia; *GLCE*, glucuronic acid epimerase; HDL-C, HDL-cholesterol; HME, hereditary multiple exostosis; HSPG, heparan sulfate proteoglycan; iAUC, incremental area under the curve; IQR, interquartile range; LDL-C, LDL-cholesterol; LDLr, LDL receptor; LOF, loss-of function; NDST1, N-deacetylase/N-sulfotransferase 1; RE, retinyl ester; RP, retinyl palmitate; Sdc1, syndecan-1; *SULF2*, glucosamine-6-*O*-endosulfatase 2; tagSNP, tagging SNP; TRL, triglyceride-rich lipoprotein.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of four tables.

as well as homozygous familial hypercholesterolemia (FH) patients with deleterious mutations in the LDLr (*LDLR*) have at best modest elevations of TRL levels (10–13).

HSPGs were identified as a class of receptors that in animal models mediate the clearance of TRLs in the liver in conjunction with LDL and LRP1 receptors (14). The in vivo role of HSPGs in TRL clearance in mice was initially illustrated by injecting heparinase, a heparan sulfate degrading enzyme, into the portal vein, which resulted in decreased TRL clearance in mice (15). Subsequent genetic studies showed that syndecan-1 (Sdc1), a type of transmembrane HSPG that resides on the basal membrane of hepatocytes exposed to the Space of Disse, binds to TRLs by way of the sulfated heparan sulfate chains on the proteoglycan and apoE and apoAV on the TRL particles (16, 17). Mutations affecting the biosynthesis of the heparan sulfate chains or in the expression of *Sdc1* in murine models were invariably associated with delayed postprandial TRL clearance and validated the concept that Sdc1 and specific HSPG-sulfation patterns are critical determinants for murine hepatic TRL clearance (18, 19). Hodoğlugil et al. (20) showed that SNPs in glucuronic acid epimerase (*GLCE*), a heparan sulfate biosynthetic enzyme, correlated with TG levels in Turkish families, and the association was stronger in APOE2/3 subjects (apoE2 has reduced binding to HSPGs). In line, these authors demonstrated a causal role for the enzyme in TRL metabolism of $Glce^{+/-}$ mice. In patients with hereditary multiple exostosis (HME), heparan sulfate synthesis is disrupted, leading to shortened chains and growth of multiple bony tumors (exostoses or osteochondromas) (21). The HME phenotype is linked to heterozygous loss-of-function (LOF) mutations in *EXT1* and *EXT2*, which encode the copolymerase involved in the formation of the backbone of heparan sulfate (22, 23).

In the present study, we set out to evaluate the role of HSPGs in postprandial TRL clearance in subjects with a single LOF mutation in *EXT1* or *EXT2*. To further address a potential additive effect of HSPG and LDLr on postprandial TRL clearance, we examined postprandial TRL metabolism in compound heterozygous mice lacking *Ext1* and *Ldlr* and in subjects with heterozygous FH with multiple tagging SNPs (tagSNPs) in *HSPG* genes.

METHODS

Mice and animal husbandry

$Ext1^{+/-}$ mice were described previously and were fully backcrossed into C57Bl/6 background (24). $Ext1^{f/f}$ mice were described previously (25). Alb*Cre* mice and $Ldlr^{-/-}$ mice were purchased from Jackson Labs. All animals were housed and bred in vivaria approved by the Association for Assessment and Accreditation of Laboratory Animal Care located in the School of Medicine at the University of California following standards and procedures approved by the Institutional Animal Care and Use Committee. Mice were weaned at 3 weeks, maintained on a 12 h light cycle, and fed ad libitum with water and standard rodent chow during the experimental period (Harlan Teklad).

Study population

We enrolled subjects with a clinically established diagnosis of HME (23) who were recruited via the website of the Dutch HME foundation (www.hme-mo.nl) and unaffected, age- and gender-matched controls (26). Exclusion criteria were current pregnancy, malignancy with limited life span, and/or overt CVD. Subjects were asked to stop any cardiovascular medication before the oral lipid load test (ATII/ACE inhibitors 5 days and statins 4 weeks prior to the clinical investigations). For the selection of FH patients, we used a previously described cohort [Genetic Identification of Risk Factors in Familial Hypercholesterolemia (GIRaFH)] (27) including DNA samples of 2,345 FH patients with heterozygous LOF mutations in *LDLR* (heFH) from 27 lipid clinics throughout the Netherlands. The FH diagnostic criteria were based on internationally established criteria (28). For 1,600 FH patients, we had the complete plasma lipid profiles and DNA for SNP genotyping. Exclusion criteria for participation in the postprandial substudy were the use of any lipid-lowering medication in 4 weeks preceding the fat tolerance test, clinical signs of malabsorption (e.g., diarrhea), or exogenous insulin treatment. Both studies were approved by the Institutional Review Board and conducted at the Academic Medical Center Amsterdam in accordance with the Declaration of Helsinki. Each patient provided written informed consent.

Selection of tagSNPs in HSPG-related genes and gene score calculation

We selected tagSNPs in genes involved in HSPG biosynthesis such as *N-deacetylase/N-sulfotransferase* 1*(NDST1)*, *EXT1*, *EXT2*, *SDC1*, and *heparan sulfate 2-O-sulfotransferase 1 (HS2ST1)*. TagSNPs were selected using the HAPMAP database and the TAGGER algorithm as presented earlier (29). We limited our search to SNPs with a minor allele frequency >5% using a pairwise tagging approach with an r^2 cutoff level >0.8. Genotyping of SNPs was carried out on an ABI 7900 system, using Assay by Design™ (Applied Biosystems, Foster City, CA). Allelic discrimination was performed using either FAM (6-carboxyfluorescein) or VIC as fluorophore (Life Technologies Inc.). PCR conditions were denaturation for 10 min at 95°C, followed by 40 cycles (30 s at 92°C, 45 s at 60°C). PCR assay mix was obtained from Applied Biosystems. All SNPs were in Hardy-Weinberg equilibrium.

We then calculated a gene score including all analyzed tagSNPs. For each individual, the TG-specific gene score was calculated using the weighted sum of TG-raising alleles. The weights used were the corresponding calculated per allele β coefficients from a multiple regression model with (log-transformed) TG levels as outcome and the tagSNP as predictor variables. The average predicted plasma TG levels, calculated with the weighted gene risk score, in the low and high HSPG gene score groups were 1.23 [0.96–1.75] mM (n = 146) and 1.75 [1.26–2.42] mM (n = 148), respectively ($P < 0.001$). Patients from the upper and lower 10th percentile of these predicted TG levels (i.e., low or high HSPG gene score) were selected to participate in the fat load test.

Vitamin A fat tolerance testing in mice

Vitamin A fat tolerance testing was done essentially as described previously (16). Briefly, 27 μCi [11,12-3H]retinol (44.4 Ci/mmol; PerkinElmer) in ethanol was mixed with 1 ml of corn oil (Sigma-Aldrich). Each mouse received 200 μl of the mixture by oral gavage. Blood was sampled at the times indicated by tail vein bleed, and radioactivity was measured in triplicate (10 μl serum) by liquid scintillation counting.

Vitamin A fat tolerance testing in humans

Selected participants were asked to refrain from alcohol intake the day before. Participants were admitted at 7:30 AM after an overnight fast. Cream, consisting of 40% fat (wt/vol) with a polyunsaturated fat to saturated fat ratio of 0.06, 0.001% cholesterol (wt/vol), and supplemented with 60,000 IU/m^2 body surface of retinyl palmitate (RP; Department of Clinical Pharmacy, AMC) for specific monitoring of dietary derived lipids, was administered in a dose of 35 g fat/m^2 body surface as described previously (30). Pre- and postprandial blood samples were drawn at 0, 2, 3, 4, 5, 6, and 8 h. Subsequently, the catheter was removed, and the subject was discharged. Venous blood was collected into EDTA-containing tubes, which were placed on ice and protected from light. Plasma was separated within 30 min by centrifugation at 3,000 rpm for 20 min at 4°C. Aliquots of plasma were protected from light and frozen at −80°C for subsequent analysis of TG and RP.

Biochemical analyses

Total cholesterol, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), and TG were measured by standard enzymatic methods (Roche Diagnostics, Basel, Switzerland) on a COBAS MIRA automated spectrophotometric analyzer (Roche Diagnostica). ApoB was analyzed with a turbidimetric assay (Randox) on a Cobas Mira autoanalyzer. Glucose was assessed using the hexokinase method (Gluco-quant, Hitachi 917; Hitachi). Plasma insulin was measured by an immunoluminimetric assay (Immulite insulin)

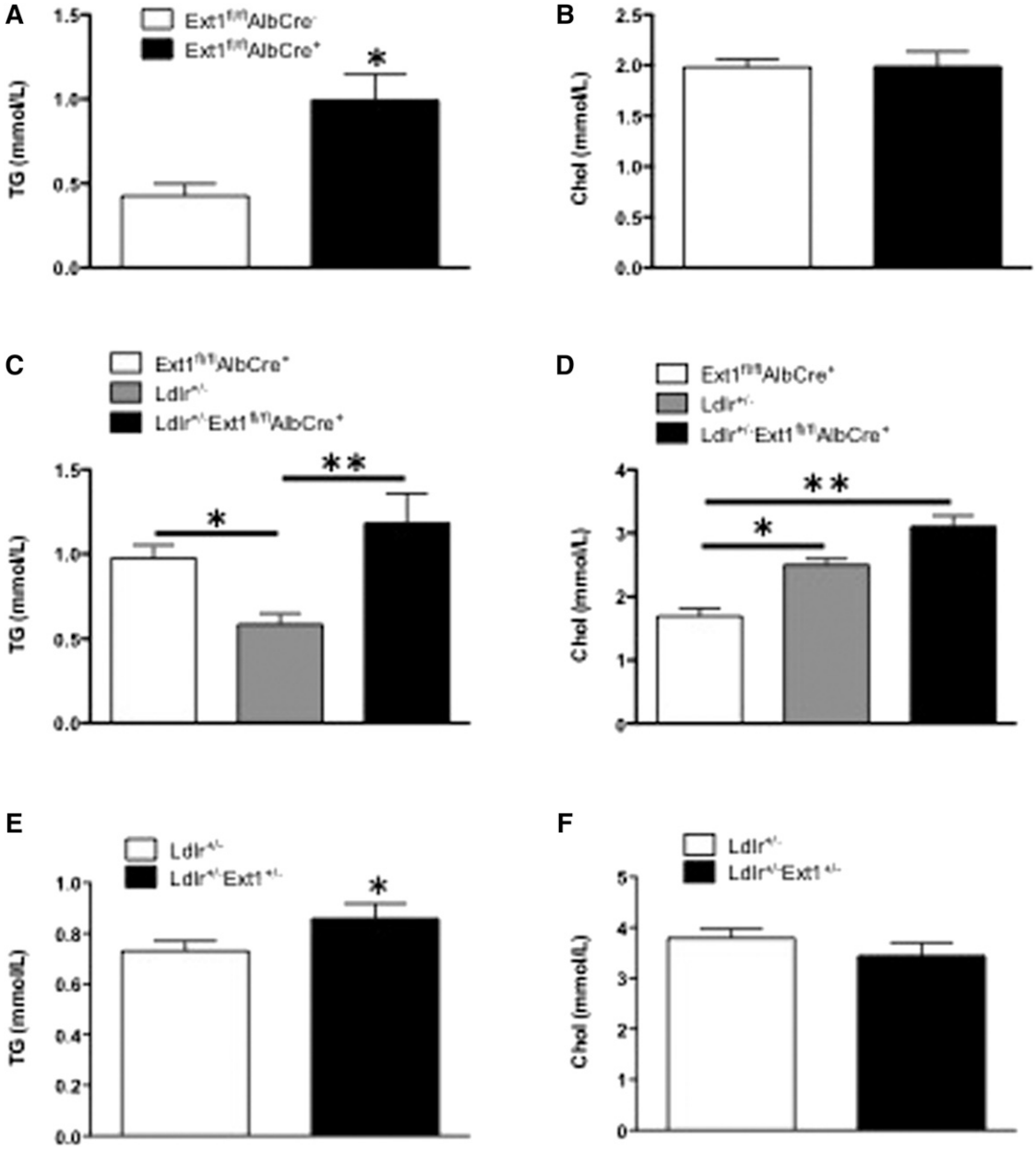

Fig. 1. Fasting plasma lipid levels in mice with mutations affecting HSPG chain length. A, B: TG and cholesterol levels in *Ext1*$^{f/f}$*AlbCre*$^{+}$ and wild-type control mice (*Ext1*$^{f/f}$*AlbCre*$^{-}$; n = 4/group). C, D: TG and cholesterol levels in *Ext1*$^{f/f}$*AlbCre*$^{+}$, *Ldlr*$^{+/-}$, and *Ldlr*$^{+/-}$*Ext1*$^{f/f}$*AlbCre*$^{-}$ mice (n = 14–26/group). E, F: TG and cholesterol levels in *Ldlr*$^{+/-}$ and *Ldlr*$^{+/-}$*Ext1*$^{+/-}$ mice (n = 5–6 mice/group). Data are presented as mean ± SEM. Blood was drawn after an overnight fasting period. Data were tested using two-tailed *t*-test statistics (A) and Kruskal-Wallis test statistics (C, D), whereas post hoc analyses were performed using Dunn's multiple comparison test. Data presented in E were tested using two-tailed *t*-test statistics. * $P < 0.01$ and ** $P < 0.001$.

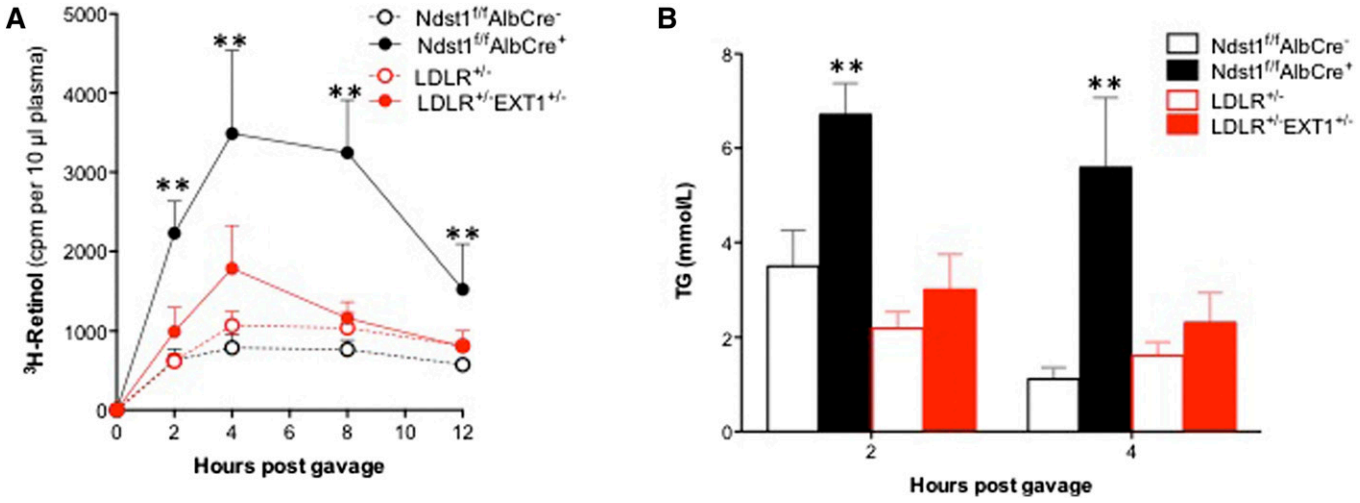

Fig. 2. Postprandial lipid clearance in mice with compound heterozygous loss of *Ext1* and *Ldlr*. Mice were fasted for 4 h prior to the start of the study. A: Postprandial RE excursions in wild-type (*Ndst1*$^{f/f}$*AlbCre*$^{-}$), *Ndst1*$^{f/f}$*AlbCre*$^{+}$, *Ldlr*$^{+/-}$, and *Ldlr*$^{+/-}$*Ext1*$^{+/-}$ mice. B: Postprandial TG levels 2 and 4 h after oral gavage of corn oil in wild-type (*Ndst1*$^{f/f}$*AlbCre*$^{-}$), *Ndst1*$^{f/f}$*AlbCre*$^{+}$, *Ldlr*$^{+/-}$, and *Ldlr*$^{+/-}$*Ext1*$^{+/-}$ mice (n = 3–5 mice/group). Data are presented as mean ± SEM. The data in A were analyzed with a two-way ANOVA with post hoc *t*-testing against the wild type of the iAUC data. The data in B were tested with a two-way ANOVA with post hoc Bonferroni testing. * $P < 0.05$ and ** $P < 0.01$.

on Immulite 2000 (Diagnostic Products). HbA1C was measured by HPLC (Reagens Bio-Rad Laboratories, Veenendaal, the Netherlands) on a Variant II (Bio-Rad Laboratories). Plasma RP was analyzed using reversed phase HPLC in 200 µl plasma after extraction of retinyl esters (REs) using chloroform, methanol, and water as described (31, 32). RP was separated by HPLC on an Inertsil ODS-3 reversed phase column (GL Sciences, Tokyo, Japan). All samples were protected against light during the analysis. Retinyl propionate (Sigma Chemicals, St. Louis, MO) was used as internal standard; methanol-ethylacetate (70:30, v/v, HPLC grade; Biosolve, Valkenswaard, the Netherlands) was used as isocratic mobile phase at a flow rate of 0.425 ml/min, and the effluent was monitored at 332/484 nm (ex/emm). Peak heights were measured and used for calculations of the absolute retinyl propionate values. Plasma apoB48 was analyzed using a commercially available ELISA from Shibayagi (Japan; AKJB48).

Power calculation and statistical analysis

Based on our previous experience assessing postprandial TRL clearance in subjects stratified for glucosamine-6-*O*-endosulfatase 2 (*SULF2*) genotype (a heparan sulfate modifying enzyme) (30), the difference between the mean incremental area under the curve (iAUC)-TG for the homozygous carriers of the major allele (6.4 ± 3.8 mM/h) and the homozygous carriers of the minor allele (2.9 ± 2.9 mM/h) was 3.5 mM/h. Based on an SD of 3.35, and assuming a two group *t*-test with a 0.05 two-sided significance level and a power of 80%, we aimed to include at least 11 subjects in each genetic group for the lipid load tests. Data are presented as mean ± SD or medians with interquartile range (IQR) unless stated otherwise. All postprandial data are presented as mean ± SEM. Normally distributed continuous outcomes at baseline were compared using a Student's *t*-test. Differences in TG levels were assessed using the nonparametric Mann-Whitney *U*-test. Postprandial TG and RP excursions were calculated as total area under the curve (AUC) calculated by the trapezoid rule. iAUC was obtained by subtracting the fasting plasma level from each postprandial time point. Differences were assessed using nonparametric Mann-Whitney *U* statistical analysis. Data in the mice were tested using two-tailed *t*-test statistics or the Kruskal-Wallis test as specified in the figure legends. Two-sided probability values of <0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 21.0.

RESULTS

Mice with hepatic Ext1 deficiency

Hepatic deletion of HSPG-associated genes such as *Ndst1* and *Sdc1* in murine models resulted in delayed postprandial

TABLE 1. Baseline characteristics of the HME patients and the matched controls

	Healthy Controls	HME Cases	*P*
N	11	13	
Age (years)	43 ± 15	41 ± 14	
Gender (% male)	4 (40%)	5 (40%)	
BMI	24.9 ± 3.4	25.9 ± 4.3	
Cholesterol (mM)	4.8 ± 0.8	4.9 ± 1.4	0.79
HDL-C (mM)	1.42 ± 0.26	1.37 ± 0.27	0.67
LDL-C (mM)	2.5 ± 0.5	2.6 ± 0.9	0.75
TG (mM)	0.9 [0.7–1.1]	1.1 [0.8–1.4]	0.13
Glucose (mM)	4.9 ± 0.7	4.8 ± 0.5	0.63
HbA1C (mmol/mol)	36 ± 4	39 ± 4	0.70
Insulin (pM)	49 ± 33	39 ± 28	0.40
HOMA-IR	1.5 ± 1.1	1.2 ± 1.0	0.33

Data are means ± SD or median [IQR]. HOMA-IR = insulin (pM)/6.945 × glucose (mM)/22.5. Differences were tested using *t*-test statistics and for plasma TG a Mann-Whitney test.

TRL clearance (16, 18). To examine whether reduction of HSPG chain length affects lipid metabolism in mice, we analyzed fasting plasma lipid levels in $Ext1^{f/f}AlbCre^{+}$ and wild-type control mice ($Ext1^{f/f}AlbCre^{-}$). Hepatic inactivation of *Ext1* induced significantly elevated plasma TG levels ($P < 0.01$; **Fig. 1A**) without affecting total plasma cholesterol levels (Fig. 1B). Crossbreeding of $Ext1^{f/f}AlbCre^{+}$ mice to $Ldlr^{+/-}$ mice exacerbated the effect and revealed enhanced TG as compared with $ldlr^{+/-}$ ($P < 0.001$; Fig. 1C) but not as compared with the $Ext1^{f/f}AlbCre^{+}$ mice. The heterozygous deletion of Ldlr resulted in a significant increase in total plasma cholesterol in all conditions as expected (Fig. 1D; $P < 0.01$). However, compounding the mutations resulted in a mild elevation of fasting plasma TG levels (Fig. 1E, $P < 0.05$) but no effect on plasma cholesterol levels (Fig. 1F) and postprandial TRL clearance (**Fig. 2**). For comparison, complete loss of hepatic Ndst1 ($Ndst1^{f/f}AlbCre^{+}$) caused greater fasting plasma TG levels, and postprandial TRL clearance was dramatically affected.

Patients with HME

HME was established by identification of the LOF mutations in *EXT1* or *EXT2* (supplementary Table 1). Anthropometric data as well as plasma biochemistry were not different between HME patients (n = 13) and controls (n = 11) (**Table 1**). Fasting plasma TG levels were not significantly different between patients and controls (HME, 1.1 [0.8–1.4] mM; controls, 0.9 [0.7–1.3] mM; NS). Compared with controls, postprandial TG curves

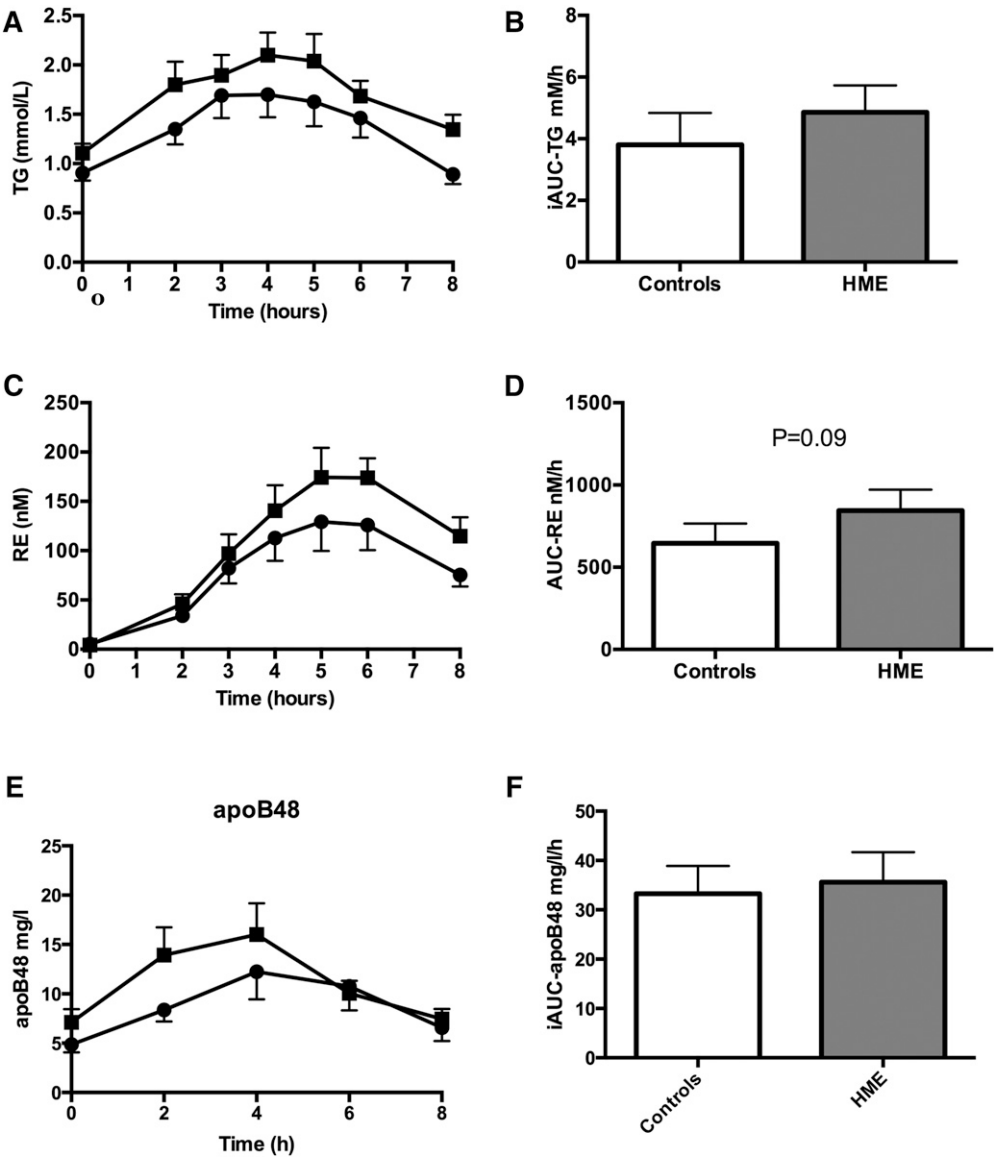

Fig. 3. Postprandial lipid clearance in patents with HME and healthy controls. A, B: Postprandial TG excursions and iAUC-TG after an oral fat load of cream in HME patients (filled squares) and controls (filled circles). Data are presented as mean ± SEM. C, D: Postprandial RE excursions and iAUC-RE in HME patients and controls. E, F: Postprandial apoB48 excursions and iAUC-apoB48 in HME patients and controls. Data are presented as mean ± SEM. Data were tested using a nonparametric Kruskal-Wallis test.

in HME patients showed a trend toward delayed clearance (iAUC-TG, 4.85 ± 0.88 mM/h; controls, 3.80 ± 1.04 mM/h; **Fig. 3A**, B) and delayed postprandial RE excursions (AUC-RE HME, 844 ± 127 nM/h vs. controls, 646 ± 119 nM/h, $P = 0.09$; Fig. 3C, D). No difference was found in postprandial plasma apoB48 excursions (Fig. 3E, F).

Patients with heterozygous FH

Baseline characteristics of the patients in the GiRaFH cohort are presented in supplementary Table 2. TagSNPs in HSPG genes *EXT1*, *EXT2*, *NDST1*, *SDC1*, and *HS2ST1* (supplementary Table 3) were analyzed in the GiRaFH cohort and tested for association with plasma TG levels (supplementary Table 4). A weighted gene risk score was calculated based on these associations. We randomly selected patients from the upper and lower 10th percentile of these predicted TG levels (i.e., low or high HSPG gene score) to participate in the fat load test. Anthropometric data as well as plasma lipids, glucose, and insulin were similar among the two groups (**Table 2**). We observed a significantly impaired postprandial remnant clearance in patients with FH as compared with the controls reflected by an increased iAUC-TG of 6.9 ± 0.98 mM/h in FH versus 3.80 ± 1.04 mM/h in healthy controls (Fig. 3B; $P = 0.026$) as well as increased AUC-RE of 1,971 ± 190 in heterozygous FH versus 646 ± 119 nM/h in controls (Fig. 4D; $P < 0.0001$). The iAUC-apoB48 was also higher in FH patients; however, this did not reach statistical significance (Fig. 4E, F). Postprandial plasma TG excursions between the two HSPG gene score groups were, however, similar (iAUC-TG, 7.0 ± 1.2 mM/h in low gene score group vs. 6.9 ± 1.7 mM/h in the high gene score group; NS; Fig. 4A, B). In contrast, postprandial RE excursions were significantly increased in FH patients with a high gene score, suggesting a delay in TRL clearance (Fig. 4C, D) (iAUC-RE, 2,377 ± 294 nM/h for the high gene score group vs. 1,565 ± 181 nM/h in the low gene score group, $P < 0.05$), and were significantly higher as compared with controls ($P < 0.05$; Fig. 4D). Postprandial plasma apoB48 levels were not different between both FH groups.

DISCUSSION

In HME patients, an LOF mutation in *EXT1* or *EXT2* was associated with a trend toward impaired postprandial TRL clearance, reflected by increased postprandial RE excursions. Subsequently, in untreated FH patients, the presence of an *LDLR* mutation leads to significantly increased postprandial remnant excursions as compared with healthy controls. Stratification for the presence of multiple SNPs in pivotal HSPG genes had no additional effect on postprandial plasma TG levels, whereas a significant increase in postprandial plasma RE levels was observed in untreated FH patients with high HSPG gene score. Our translational data suggest that HSPGs only modestly contribute to hepatic TRL clearance.

TRL clearance in FH

Hepatic uptake of TRL involves the participation of three different receptors (33). In this respect, LDLr's play a role in hepatic remnant clearance. Heterozygous FH patients are characterized by a partial LOF of the LDLr protein, which is expected to lead to increased circulating plasma TG levels and impaired postprandial handling of dietary fat. In line with previous publications (13, 34, 35), we found that plasma TG levels and postprandial TG clearance is indeed impaired in heterozygous FH patients as compared with the controls, illustrating the role for the LDLr in remnant clearance (36). However, the increase in plasma TG levels is only modest, confirming earlier reports showing minor TG elevations in FH homozygous patients (37). These findings underscore the likely participation of other receptors in the uptake of TRL in the liver (37, 38).

TRL clearance in HME

Based on genetic studies in mice, Sdc1 has been implicated in TRL clearance (39). These proteoglycan receptors work in parallel to, but independently of, the LDLr. The two families of receptors clear distinct particles and can compensate to a limited extent for each other (9). Thus, we wondered if partial deficiency in the LDLr might sensitize mice to partial LOF of HSPGs. Indeed, mice bearing

TABLE 2. Baseline characteristics of the FH patients participating in the fat load study

	Low Gene Score	High Gene Score	*P*
N	11	11	
Gender (% male)	5 (50%)	5 (50%)	
Age (years)	26 ± 2	26 ± 2	
BMI	25.8 ± 3.6	26.4 ± 4.0	0.55
HbA1C (mmol/mol)	37 ± 2.6	38 ± 3.5	0.39
Cholesterol (mM)	8.1 ± 1.4	8.6 ± 1.4	0.19
LDL-C (mM)	5.8 ± 1.0	6.2 ± 1.4	0.06
HDL-C (mM)	1.46 ± 0.45	1.45 ± 0.45	0.39
TG (mM)	1.3 [1.0–1.9]	1.1 [0.79–2.2]	0.66
Glucose (mM)	5.1 ± 0.5	5.3 ± 0.7	0.15
Insulin (pM)	35 ± 32	40 ± 32	0.51
HOMA-IR	1.20 ± 1.26	1.46 ± 1.30	0.63

Data are presented as mean ± SD. TGs are presented as median [IQR]. HOMA-IR = insulin (pM)/6.945 × glucose (mM)/22.5. Differences were tested using *t*-test statistics and for plasma TG a Mann-Whitney test.

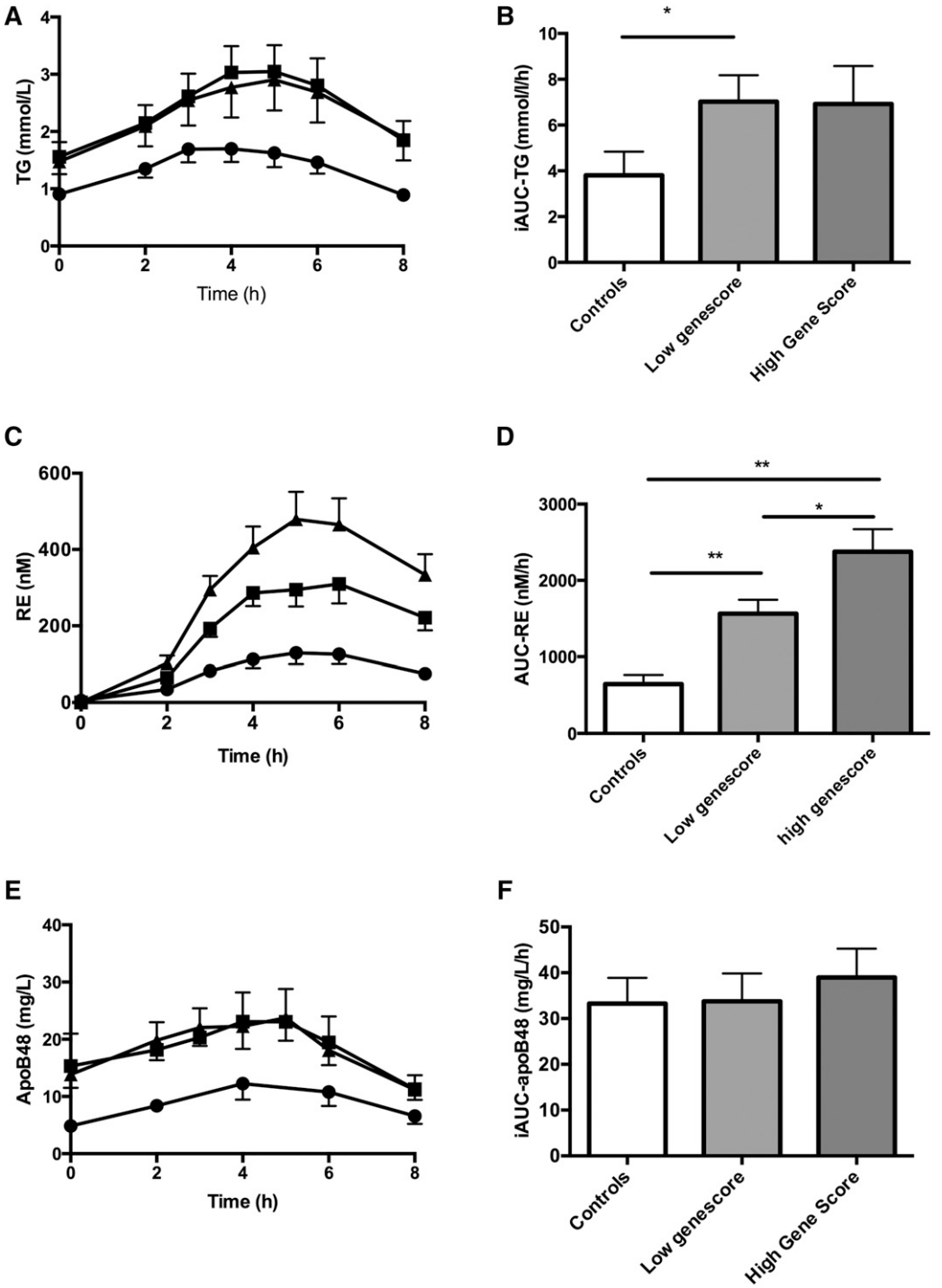

Fig. 4. Postprandial lipids after an oral fat load in selected FH with either a low gene score or a high gene score. A, B: Postprandial plasma TG excursions and iAUC-TG. C, D: Postprandial RE excursions and iAUC-RE. E, F: Postprandial apoB48 excursions and iAUC-apoB48 [controls (filled circles), low gene score group (filled squares), and high gene score group (filled triangles)]. Data are presented as mean ± SEM. Data were tested using nonparametric Kruskal-Wallis statistics. * $P < 0.05$; ** $P < 0.001$

compound heterozygous loss of *Ldlr* and *Ext1*, fed a normal chow diet, demonstrate mild hypertriglyceridemia both under fasting and postprandial conditions, yet postprandial lipid excursions were not different. This is in contrast to our earlier findings in the hepatic conditional *Ndst1*$^{-/-}$ knockout mice. It may thus be that in HSPG mediated remnant clearance sulfation grade of HSPG seems to be much more important than HSPG chain length.

Data on the role of HSPGs in human lipoprotein metabolism are scarce. Patients with Knobloch syndrome, caused by a heterozygous LOF mutation in vascular collagen XVIII (*COL18A1*), were reported to have increased fasting TG (40). In line with this finding, SNPs in *GLCE*,

which encodes an enzyme involved in heparan sulfate assembly, correlated with plasma TG in a Turkish population (20). Our previous findings in hypertriglyceridemic diabetic mice showed that inhibition of Sulf2, an enzyme that removes the 6-*O* sulfate groups from HSPG, normalized the impaired TG clearance, implying that sulfation of HSPGs is essential for proper (postprandial) TRL clearance (41). Moreover, it was recently shown that in humans, a genetic variant in *SULF2* predisposes to lower fasting TG and postprandial TRL levels in type 2 diabetes mellitus patients (30) as well is in healthy controls (42). HME patients have a diagnosed heterozygous LOF mutation in either *EXT1* or *EXT2* gene resulting in altered HSPGs with reduced length of the heparan sulfate chain, albeit with a normal sulfation pattern (34). Mutations in *EXT1* are associated with a higher disease burden of HME (43, 44) due to a more pronounced biological function for EXT1, albeit that EXT2 is required for the proper function of EXT1 (23) and thus allows us to combine both mutation carrier groups. Based on the results obtained in our study, it can be concluded that HSPG chain length differences (as seen in the presence of LOF mutations in *EXT1* or *EXT2*) do not have a large impact on postprandial lipid metabolism in humans. We then questioned the possibility that FH individuals (with heterozygous mutation in LDLr) might provide a sensitized background to detect the contribution of HSPGs to TRL clearance in humans.

TRL clearance in FH according to HSPG gene score. To test if FH individuals with elevated TGs might harbor SNPs in genes involved in HSPG formation, we genotyped tagSNPs reflecting the total genetic variation in each HSPG gene and tested for association with plasma TG levels (29). However, we found only minor associations in this limited data set. We also calculated a weighted HSPG gene score and predicted plasma TG levels to categorize FH patients with a low burden in genetic variation in HSPG genes and those with a high number of genetic variants in HSPG genes. Although predicted plasma TG levels were significantly higher in the high HSPG gene score group, subsequent functional testing showed no significant differences in postprandial plasma TG and apoB48 plasma levels in these FH patients. Interestingly, we did find a significantly altered postprandial remnant (RE) clearance (reflecting TRL clearance) in FH subjects with HSPG SNPs, which is in line with the previously published murine data (18, 19), and further (stable) isotope-based studies investigating chylomicron clearance in these FH subjects are warranted.

Study limitations. A limitation is the lack of available plasma HPLC profiles in our murine studies that preclude more detailed studies in LDL and/or HDL changes in the different models. Moreover, the relatively small number of participants in our human postprandial lipid loading studies (predominantly due to low number of HME patients with a proven mutation in the Netherlands as well as the limited number of FH patients with a high HSPG gene score) might have affected the outcomes of our study. However, based on the robustness of our functional tests, we feel that these translational data still provide an interesting insight into the human relevance of HSPGs in (postprandial) lipid metabolism (18, 19). Second, although REs are widely used in postprandial studies as marker for chylomicron clearance, it has been suggested that RE may exchange between chylomicrons and VLDL resulting in a less specific determination of postprandial TG handling (45). As this exchange is estimated to be ~5% in 5 h, it is not very likely that RE exchange between lipoprotein particles contributed significantly to the differences in the 8 h postprandial curves of our subjects.

In conclusion, our studies in mice and in humans support the concept that although minor, HSPGs do contribute to human hepatic TRL clearance. Further studies into the role of hepatic HSPGs in postprandial TRL clearance are currently under investigation.

Note added in proof

This manuscript was submitted and accepted for publication in *the Journal* with a different title, "Genetic variation in heparan sulfate proteoglycans modestly affect postprandial lipid clearance in humans." The title was changed by the authors post-acceptance.

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