Coding Variants in *PNPLA3* and *TM6SF2* Are Risk Factors for Hepatic Steatosis and Elevated Serum Alanine Aminotransferases Caused by a Glucagon Receptor Antagonist

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LY2409021 is a glucagon receptor antagonist that was associated with hepatic steatosis and elevated aminotransferases in phase 2 diabetes studies. We investigated the relationship between selected genetic variants and hepatic steatosis and elevated alanine aminotransferases (ALTs) associated with LY2409021. Patients participated in a 6-week placebo-controlled trial (I1R-MC-GLDI [GLDI], n = 246) and a 52-week placebo- and active comparator-controlled trial (I1R-MC-GLDJ [GLDJ], n = 158). GLDJ had endpoints at 6 months, including measures of hepatic fat fraction (HFF) by magnetic resonance imaging. The five genes tested were patatin-like phospholipase domain containing 3 (*PNPLA3*) (rs738409 and rs738491), transmembrane 6 superfamily member 2 (*TM6SF2*) (rs58542926), peroxisome proliferative activated receptor gamma coactivator 1 alpha (*PPARGC1A*) (rs4361373, rs3774921, rs2970849), adenylate cyclase 3 (*ADCY3*) (rs713586), and insulin-like growth factor 1 (*IGF-1*) (rs1520220). In GLDI, *PNPLA3* I148M ($P = 0.001$) and *TM6SF2* E167K ($P = 0.001$) were significantly associated with an increase in ALT at 6 weeks for LY2409021 but not for placebo. In GLDJ, *PNPLA3* I148M showed the same effect ($P = 0.007$) on ALT at 6 months but the placebo or sitagliptin did not. In GLDJ, both PNPLA3 and TM6SF2 risk-allele carriers showed increases in HFF that were numerically greater but not statistically significant. The carriers of *PNPLA3* and/or *TM6SF2* risk alleles showed significantly increased ALT (GLDI, +13.28 U/L in carriers versus +4.84 U/L in noncarriers, $P = 4 \times 10^{-5}$; GLDJ, +14.6 U/L in carriers versus +1.7 in noncarriers, $P = 0.0018$) and HFF (GLDJ, +5.35% in carriers versus 2.38% in noncarriers, $P = 0.048$). Elevation of transaminase and HFF were also noted in the noncarriers but at a significantly lower degree. *Conclusion:* The carriers of *PNPLA3* and/or *TM6SF2* variant alleles are at risk for hepatic steatosis and elevated ALT levels caused by LY2409021, a glucagon receptor antagonist. More studies are needed to investigate if our observations are generalizable to hepatic steatosis caused by other medications. (*Hepatology Communications* 2018;2:561-570)

Dysregulated glucagon secretion with resultant hepatic glucose overproduction[1-3] is an important pathophysiologic feature that contributes to chronic hyperglycemia in type 2 diabetes (T2D), and glucagon receptor antagonism has been shown to diminish hepatic glucose output and improve both fasting and postprandial hyperglycemia.[4,5] There are currently several molecules in different stages of clinical development that target the inhibition of glucagon action, including small molecule antagonists, humanized antibodies, and antisense oligonucleotides. Although some of these molecules have demonstrated

Abbreviations: ADCY3, adenylate cyclase 3; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BP, blood pressure; CFB, change from baseline; GCGR, glucagon receptor; GLDI, I1R-MC-GLDI; GLDJ, I1R-MC-GLDJ; HbA1c, hemoglobin A1c; HFF, hepatic fat fraction; HSC, hepatic stellate cell; IGF-1, insulin-like growth factor 1; MRI, magnetic resonance imaging; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain containing 3; PPARGC1A, peroxisome proliferative activated receptor gamma coactivator 1 alpha; SNP, single nucleotide polymorphism; T2D, type2 diabetes; TM6SF2, transmembrane 6 superfamily member 2.

promising effects on lowering glucose, they have also shown elevation in serum aminotransferase levels in clinical trials.[6-13] LY2409021, administered orally once daily, is a novel agent with a long half-life (approximately 60 hours) that competitively blocks the glucagon receptor. Recent results from an LY2409021 study have shown not only elevation of aminotransferases but also evidence of hepatic fat accumulation following treatment for 6 months in T2D.[14]

Epidemiologic, familial, and twin studies provide evidence for heritability of hepatic steatosis, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), and NASH cirrhosis.[15,16] Genome-wide association studies have reported genetic variants associated with liver fat accumulation.[17-20] These studies have identified nonsynonymous coding variants in patatin-like phospholipase domain containing 3 (*PNPLA3*) (I148M) and transmembrane 6 superfamily member 2 (*TM6SF2*) (E167K) that are significantly associated with steatosis.[18,19] These genetic variants have recently been extensively studied as risk factors for liver fat accumulation and progression to NASH.[21,22] Because patients carrying risk alleles of *PNPLA3* and *TM6SF2* are at risk for increased liver fat deposition in the general population, it is possible that patients who carry the risk alleles are at a higher risk for liver fat accumulation upon chronic treatment with LY2409021. We investigated the association between *PNPLA3* and *TM6SF2* variants and aminotransferase elevations and hepatic steatosis (as measured by hepatic fat fraction [HFF]) caused by LY2409021 among individuals with T2D who participated in two randomized controlled studies.

Materials and Methods

STUDY POPULATIONS

Patients with T2D enrolled in two phase 2 randomized clinical trials investigating LY2409021 were included in these analyses. The studies were conducted in accordance with regulatory standards of good clinical practice, the Declaration of Helsinki, and all applicable local regulations. Patients who provided written informed consent for genetic testing were included.

I1R-MC-GLDI

I1R-MC-GLDI (GLDI), an ambulatory blood pressure (BP) monitoring study (NCT02091362), was

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a 6-week, phase 2, randomized, crossover study that evaluated the effects of once-daily administration of LY2409021 20 mg versus placebo on systolic BP, diastolic BP, and mean arterial pressure using 24-hour ambulatory BP monitoring. A 4-week washout period was included between the two treatment periods to ensure complete washout of LY2409021 before starting the second period of treatment. More details of the study are reported elsewhere[9] and in Supporting Table S1.

I1R-MC-GLDJ

I1R-MC-GLDJ (GLDJ), a hepatic safety study (NCT02111096), was a phase 2b, randomized, double-blind, placebo- and active comparator-controlled study designed to evaluate changes in liver fat after 6 months of treatment (primary endpoint) and after a total treatment period of 12 months. Adult patients with T2D who were on an optimally effective and stable dose of metformin and a sulfonylurea were recruited into the study. More details of the study are reported elsewhere[14] and in Supporting Table S1. Patients were randomized in a 3:3:2 ratio and received double-blinded LY2409021 (20 mg), placebo, or sitagliptin (100 mg), respectively, administered orally once daily.

OUTCOME MEASUREMENTS

Serum aminotransferases were measured using commercially validated methods and were available in both GLDI and GLDJ studies. Liver fat content was assessed as HFF measured by noncontrast magnetic resonance imaging (MRI) in the GLDJ study. Each baseline MRI was quality reviewed and approved by the core imaging laboratory before randomizing the patient. MRIs performed at baseline, 1, 3, 6, and 12 months from first treatment dose; at early discontinuation (if applicable); and at 4 months posttreatment were used to characterize the extent, time course, and reversibility of changes in HFF. More details about the MRI procedure are described in the original study manuscript.[14] For this analysis, HFF was available at baseline, 1 month, 3 months, and at the time of the primary endpoint at 6 months.

GENOTYPING

All samples were genotyped using the Axiom genotyping array from Affymetrix. A total of 418 samples (256 from GLDI; 162 from GLDJ) along with duplicates and haplotype map controls were used, and data for ~765,000 single nucleotide polymorphisms (SNPs) were generated. Standard metrics of genome-wide data quality control were used, and 404 samples (GLDI, 246; GLDJ, 158) and ~680,000 SNPs passed quality control. Based on our *a priori* knowledge and understanding of disease mechanisms and genetic analysis results from prior phase 1 and phase 2 LY2409021 studies (unpublished data), eight SNPs from five candidate genes were tested for association with elevated aminotransferases and HFF. These were rs713586 for adenylate cyclase 3 (*ADCY3*), rs4361373, rs3774921, and rs2970849 for peroxisome proliferative activated receptor gamma coactivator 1 alpha (*PPARGC1A*), rs738409 and rs738491 for *PNPLA3*, rs1520220 for insulin-like growth factor 1 (*IGF-1*), and rs58542926 for *TM6SF2*. All the tested SNPs were in Hardy–Weinberg equilibrium.

STATISTICAL ANALYSIS

A linear model appropriate for the crossover design of the study was used for the association analyses in GLDI. Because GLDI used a crossover design, all patients received either placebo or LY2409021 at each treatment period. The model included change from baseline (CFB) at 6 weeks as the response variable and consisted of treatment sequence, period, treatment, genotype, genotype-by-treatment interaction, and the baseline measurement as covariates. In GLDJ, analysis of covariance models were used to assess the association between CFB for each endpoint and the SNPs. The model included CFB for each endpoint as the response variable with genotype as a categorical variable, and with treatment, genotype-by-treatment interaction, baseline hemoglobin A1c (HbA1c) stratum ($\leq$8.0%, >8.0%), and baseline measurements of the respective endpoint as covariates. In both studies, the effect of genotype on the endpoint within each treatment arm was also assessed with similar models and covariate adjustments.

For the combined analyses of *PNPLA3* and *TM6SF2* SNPs, a risk-allele carrier status was first defined by the status of carrying at least one risk-allele copy of *PNPLA3* (M allele) or *TM6SF2* (K allele). Two models were run for the combined SNP analyses for each study. First, in GLDI and GLDJ, individual SNP analyses were used except that the carrier status was used as the predictor for the response variable instead of individual genotype effect (Model 1). Second, a regression model was run using additive allelic dosage calculated as the sum of risk alleles from

TABLE 1. BASELINE DEMOGRAPHICS AND PATIENT CHARACTERISTICS

	Study GLDI*	Study GLDJ		
Variable	LY2409021/Placebo (n = 246)	LY2409021 (n = 60)	Sitagliptin (n = 38)	Placebo (n = 60)
Age, years, mean (SD)	58.10 (8.93)	57.3 (8.2)	57.0 (9.0)	57.6 (7.8)
Sex, female, n (%)	104 (42.30)	23 (38.3)	9 (23.7)	25 (41.7)
BMI, kg/m^2, mean (SD)	31.30 (4.31)	32.42 (5.52)	31.82 (6.10)	31.11 (5.12)
Diabetes duration, years, mean (SD)	7.00 (5.31)	12.55 (6.16)	11.08 (6.66)	10.60 (6.3)
HbA1c, %, mean (SD)	7.29 (0.60)	8.15 (1.01)	8.25 (0.94)	8.31 (0.87)
ALT, U/L, mean (SD)	28.69 (18.05)	27.15 (14.1)	32.84 (19.97)	25.20 (14.51)
HFF, %, mean (SD)†	–	12.51 (8.92)	15.49 (9.78)	12.11 (8.92)
Race, n (%)				
Caucasian	165 (67.10)	39 (65.0)	29 (76.3)	46 (76.7)
Black	31 (12.60)	14 (23.3)	6 (15.8)	10 (16.7)
Asian	5 (2.00)	4 (6.7)	2 (5.3)	4 (6.7)
American Indian or Alaska native	36 (14.60)	–	–	–
Ethnicity, n (%)				
Hispanic	113 (45.90)	27 (45.0)	18 (47.4)	40 (66.7)
Non-Hispanic	120 (48.80)	32 (53.3)	19 (50.0)	19 (31.7)

*GLDI was a crossover study and all subjects received LY or placebo in each treatment period, so the demographics of all subjects are reported. †HFF data were collected only in study GLDJ using noncontrast liver serial MRI.
Abbreviations: BMI, body mass index; n, number of patients.

*PNPLA*3 and *TM6SF2* SNPs (Model 2). For comparison, both *P* values from Model 1 and Model 2 are reported.

Bonferroni corrections for multiplicity were done for the primary hypothesis testing; a prespecified adjusted *P* value of 0.10 was considered significant. No multiplicity adjustments were applied in the joint analyses of *PNPLA3* and *TM6SF2* SNPs.

Results

BASELINE ANTHROPOMETRIC AND METABOLIC CHARACTERISTICS

A total of 246 patients from GLDI and 158 from GLDJ (LY2409021, n = 60; placebo, n = 60; sitagliptin, n = 38) were included in the pharmacogenetic analyses. Baseline characteristics of the patients included in these analyses are reported in Table 1. Mean age, body mass index, and baseline aminotransferase levels were comparable between studies. Additional baseline characteristics by genotype groups of *PNPLA3* I148M and *TM6SF2* E167K are listed in Supporting Table S2. Baseline HbA1c levels were similar across the genotypes, while the duration of diabetes was higher in the homozygotes of both *PNPLA3* and *TM6SF2* genotypes. Baseline alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were elevated in *PNPLA3* and *TM6SF2* risk-allele carriers in the GLDI study population but not in the GLDJ study population.

CLINICAL FINDINGS

GLDI

As reported, LY2409021 treatment lowered HbA1c levels with a least squares mean (LSM) difference of −0.49% ($P < 0.001$) versus placebo at 6 weeks in GLDI.[9] Significant increases in aminotransferase levels were observed with LY2409021 treatment ($P < 0.05$ versus placebo). The 24-hour mean systolic BP increased, with an LSM difference of 2.26 mm Hg versus placebo ($P < 0.001$). The CFB for serum cholesterol, high density lipoprotein-cholesterol, low density lipoprotein-cholesterol, and triglyceride levels were significantly higher after the LY2409021 treatment period than after the placebo period at week 6 ($P < 0.001$).

GLDJ

As reported, LY2409021 treatment showed significant HbA1c reductions versus placebo (LSM difference, –0.77%; $P < 0.001$) but not versus sitagliptin (−0.20%; $P = 0.383$) at 6 months.[14] A significant increase in HFF was seen with LY2409021 versus sitagliptin (LSM difference, 3.72%; $P < 0.001$) and placebo (4.44%; $P < 0.001$), accompanied by significant ALT elevations with LY2409021 versus sitagliptin

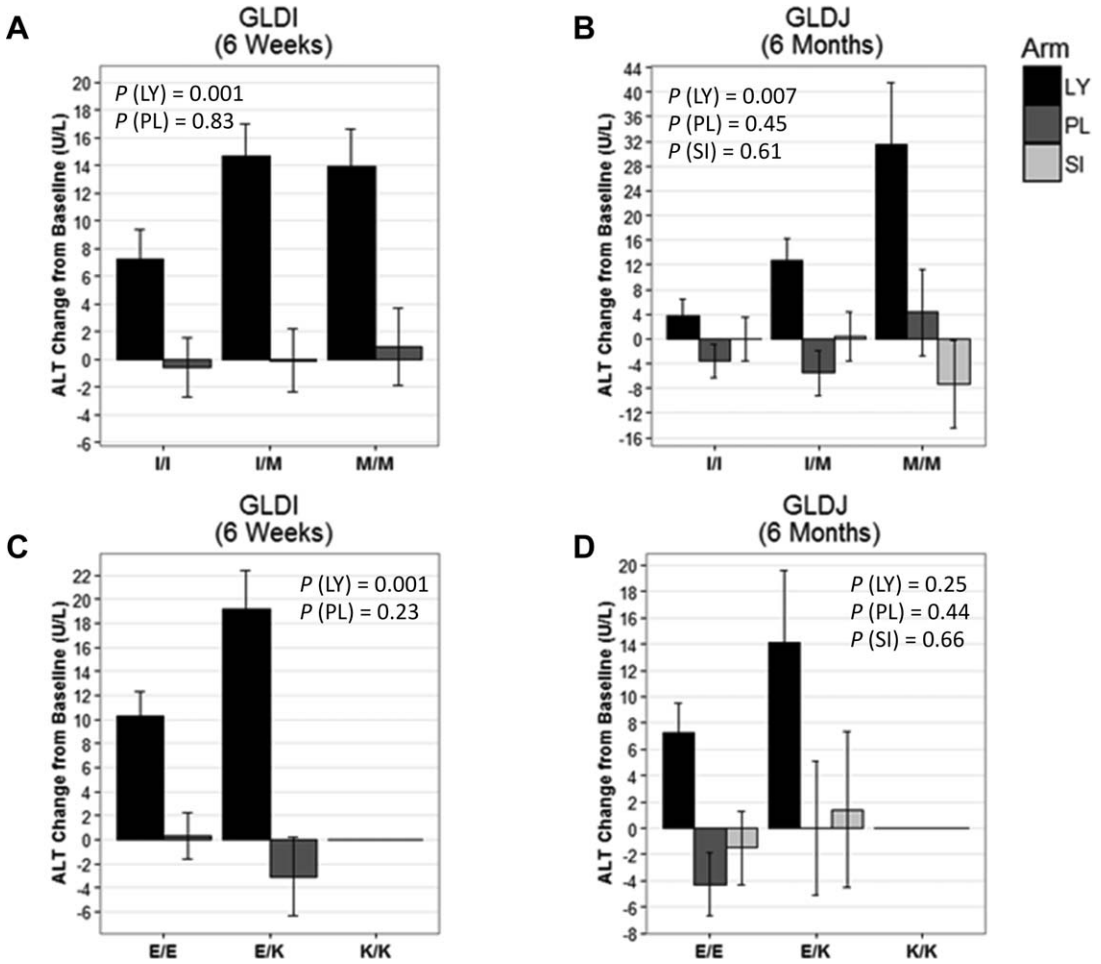

FIG. 1. Association of ALT levels stratified by PNPLA3 (I148M) and TM6SF2 (E167K) genotypes. In both the GLDI and GLDJ studies, ALT levels (change from baseline) are higher in the individuals carrying alternative allele(s) both for PNPLA3 (148M) and TM6SF2 (167K) polymorphisms. (A) Change in ALT levels in GLDI for PNPLA3. (B) Change in ALT levels in GLDJ for PNPLA3. (C) Change in ALT levels in GLDI for TM6SF2. (D) Change in ALT levels in GLDJ for TM6SF2. Abbreviations: E, Glutamate; I, Isoleucine; K, lysine; LY, oral selective glucagon receptor antagonist molecule (LY2409021; 20 mg); M, methionine; PL, placebo; SI, sitagliptin (100 mg).

TABLE 2. GENETIC VARIANTS ASSOCIATED WITH HEPATIC FAT CHANGE FROM BASELINE IN STUDY GLDJ

			Homozygous (Reference Allele)		Heterozygous		Homozygous (Alternate Allele)		*P* Value			
									Main Effect		Interaction	
Outcome	Gene (SNP)	Treatment Arm	n	LSM (95% CI)	n	LSM (95% CI)	n	LSM (95% CI)	Raw	Adj	Raw	Adj
HFF	*PPARGC1A*	LY	30	4.7 (2.9, 6.5)	14	3.8 (1.2, 6.4)	2	–5.6 (–12.5, 1.4)	0.021	0.17		
CFB at	(rs4361373)	PL	29	–0.5 (–2.4, 1.3)	11	–0.5 (–3.5, 2.6)	3	–1.4 (–7.1, 4.2)	0.95		0.330	1.00
6 months		SI	18	0.1 (–2.3, 2.4)	7	1.8 (–1.9, 5.4)	2	–2.6 (–9.4, 4.3)	0.51			
	PNPLA3	LY	27	3.2 (1.2, 5.1)	17	4.6 (2.2, 7.1)	2	8.6 (1.5, 15.7)	0.27			
	(rs738409)	PL	24	–0.4 (–2.4, 1.7)	15	–0.6 (–3.1, 2.0)	4	–1.2 (–6.1, 3.8)	0.95		0.560	1.00
	Ile148Met	SI	11	–1.2 (–4.2, 1.8)	12	1.7 (–1.2, 4.6)	4	0.4 (–4.7, 5.4)	0.40			
	TM6SF2	LY	39	3.4 (1.9, 5.0)	7	6.8 (3.0, 10.5)	–	–	0.11	0.89		
	(rs58542926)	PL	35	–0.6 (–2.3, 1.1)	8	–0.2 (–3.7, 3.3)	–	–	0.84		0.500	1.00
	Glu167Lys	SI	21	0.3 (–1.9, 2.4)	6	0.5 (–3.6, 4.6)	–	–	0.92			

Abbreviations: Adj, adjusted *P* value; CI, confidence interval; LY, LY2409021; n, number of samples observed; PL, placebo; Raw, raw *P* value; SI, sitagliptin.

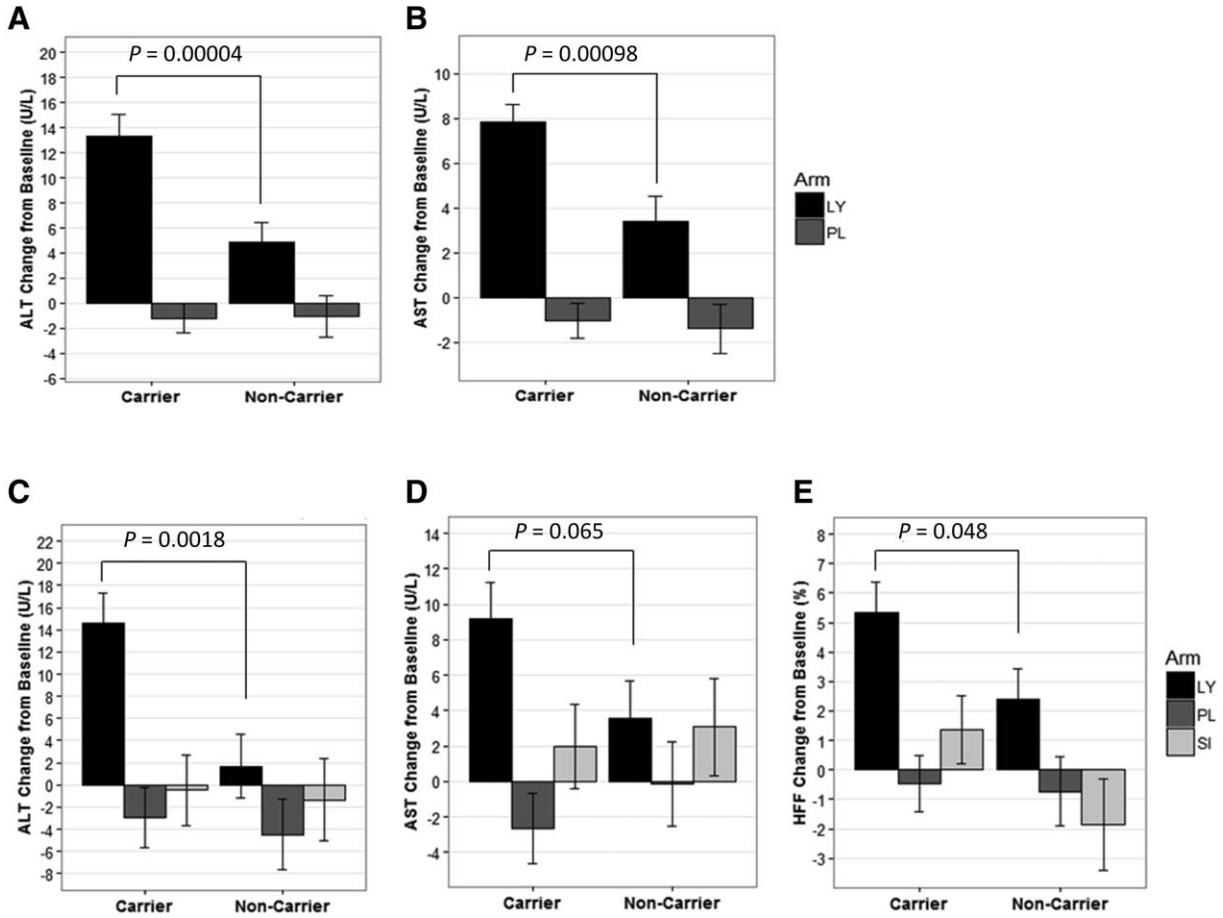

FIG. 2. Relationship between *PNPLA3* (I148M) and *TM6SF2* (E167K) SNPs and aminotransferases and HFF in GLDI and GLDJ studies. All patients were classified as carriers if they carry at least one copy of the risk allele (i.e., methionine for PNPLA3, and/or lysine for TM6SF2). All other patients were defined as noncarriers (statistical significance is shown for Model 1). Evaluation for the additive effects of the risk alleles was also performed for both SNPs (Model 2; see Table 3). (A) Change in ALT levels (from baseline at 6 weeks) in risk-allele carriers versus noncarriers in GLDI. (B) Change in AST levels (from baseline at 6 weeks) in risk-allele carriers versus noncarriers in GLDI. (C) Change in ALT levels (from baseline at 6 months) in risk-allele carriers versus noncarriers in GLDJ. (D) Change in AST levels (from baseline at 6 months) in risk-allele carriers versus noncarriers in GLDJ. (E) Change in HFF (from baseline at 6 months) in risk-allele carriers versus noncarriers in GLDJ. Abbreviations: K, lysine; LY, oral selective glucagon receptor antagonist molecule (LY2409021; 20 mg); M, methionine; PL, placebo; SI, sitagliptin (100 mg).

(6.8 U/L; $P = 0.039$) and placebo (10.7 U/L; $P < 0.001$).

ASSOCIATION OF GENETIC POLYMORPHISMS WITH ALT AND HFF

All SNPs tested were in Hardy–Weinberg equilibrium. The effect of SNPs on ALT and HFF was tested in all treatment groups. Because the changes in ALT and HFF were most prominent and significant in patients treated with LY2409021, the results presented here are focused on LY2409021 unless otherwise specified. Eight SNPs from *ADCY3*, *PPARGC1A*, *IGF-1*, *PNPLA3,* and *TM6SF2* were tested for association with CFB in ALT levels in both studies and with changes in HFF in GLDJ. The results from these analyses are shown in Fig. 1 and Table 2.

In GLDI, *PNPLA3* (observed $P = 0.001$, adjusted $P = 0.005$) and *TM6SF2* (observed $P = 0.001$, adjusted $P = 0.011$) SNPs were significantly associated with increases in ALT at 6 weeks in patients treated with LY2409021 but not in the placebo group. In GLDJ, *PNPLA3* (observed $P = 0.007$, adjusted

TABLE 3. COMBINED EFFECT OF *PNPLA3* I148M AND *TM6SF2* E167K ON CHANGES IN TRANSAMINASES AND HEPATIC FAT FRACTION

			Noncarriers		Carriers		*P* Value	
Study	Endpoint	Treatment	n	LS Mean ± SE	n	LS Mean ± SE	Model 1*	Model 2†
GLDI	ALT	LY2409021	77	4.837 ± 1.635	148	13.280 ± 1.175	4×10^{-5}	1×10^{-5}
		Placebo	76	–1.0312 ± 1.645	143	–1.185 ± 1.192	0.939	0.976
GLDJ	ALT	LY2409021	24	1.680 ± 2.867	25	14.571 ± 2.791	0.002	3×10^{-4}
		Placebo	19	–4.462 ± 3.211	26	–2.974 ± 2.730	0.724	0.284
		Sitagliptin	14	–1.367 ± 3.715	19	–0.463 ± 3.194	0.854	0.693
	HFF	LY2409021	22	2.384 ± 1.062	24	5.353 ± 1.005	0.048	0.021
		Placebo	17	–0.729 ± 1.190	26	–0.470 ± 0.960	0.865	0.850
		Sitagliptin	10	–1.868 ± 1.560	18	1.359 ± 1.151	0.099	0.325

*Model 1, linear model that uses carrier status defined by carriage of at least one minor allele of *PNPLA3* or *TM6SF2* SNPs as the covariate of main interest.
†Model 2, linear model that uses allele dosage defined as the sum of the risk allele counts from the *PNPLA3* and *TM6SF2* SNPs as the covariate.
Abbreviations: LS, least squares; n, number of subjects.

$P = 0.059$) and *PPARGC1A* (rs4361373, observed $P = 0.003$, adjusted $P = 0.021$) SNPs were significantly associated with increased ALT at 6 months in patients treated with LY2409021 but not in the placebo or sitagliptin groups. Heterozygotes for *TM6SF2* E167K had an increase in ALT that was numerically higher but not significant.

In GLDJ, both PNPLA3 and TM6SF2 risk-allele carriers showed increases in HFF that were numerically greater but not significant (Table 2). *PPARGC1A* (rs4361373) SNP was nominally associated with a change in HFF (observed $P = 0.021$, adjusted $P = 0.17$) but did not retain statistical significance after adjustment for multiplicity corrections (Table 2).

We subsequently explored the joint effect of *PNPLA3* and *TM6SF2* SNPs on increases in ALT and HFF (Fig. 2; Table 3). Patients who carry at least one copy of the methionine (M) or lysine (K) allele from PNPLA3 and TM6SF2 SNPs were classified as carriers; those who do not carry M/K alleles were classified as noncarriers. In GLDI, carriers showed an ALT increase of 13.28 ± 1.17 U/L, while the change in noncarriers was 4.84 ± 1.64 U/L ($P = 4 \times 10^{-5}$). Similar to GLDI, in GLDJ, ALT and HFF changes were significantly higher in carriers treated with LY2409021 but not in those receiving placebo or sitagliptin. Carriers showed an ALT increase of 14.57 ± 2.79 U/L, while the change in noncarriers was 1.68 ± 2.87 U/L ($P = 0.002$). For HFF, carriers showed 5.35% ± 1.01% increase, while the increase in noncarriers was 2.38% ± 1.06% ($P = 0.048$) (Table 3). Because weight gain was observed in patients treated with glucagon receptor antagonist in the GLDJ study, we analyzed for the possible association of PNPLA3 and TM6SF2 variants on weight CFB. No significant association was observed (data not shown) between weight change and the SNPs in either study, suggesting that the association observed with ALT/AST may be independent of weight change. We also analyzed the variability of ALT and HFF CFB explained by the SNPs, and the results are shown in Supporting Table S3. PNPLA3 I148M explained more variability in ALT and HFF CFB in general.

Discussion

LY2409021is a novel glucagon receptor antagonist with a significant glucose-lowering effect in patients with T2D but was unexpectedly associated with increases in aminotransferases and hepatic fat in phase 2 clinical trials.[14] We investigated if selected genetic variants linked to NAFLD in the general population are associated with hepatic steatosis and elevated ALT levels associated with LY2409021. Our results demonstrate that patients carrying the risk alleles of *PNPLA3*/*TM6SF2* polymorphisms are at a higher risk for serum aminotransferase elevation and hepatic fat accumulation when exposed to LY2409021. These variants did not show any effect on glycemic efficacy endpoints (data not shown).

The role of *PNPLA3* and *TM6SF2* in NAFLD pathogenesis is not completely understood. *PNPLA3*, also called adiponutrin, encodes a 481-amino acid membrane protein localized in the endoplasmic reticulum and at the surface of lipid droplets. In humans, this protein has the highest expression in hepatic stellate cells (HSCs), retina, and hepatocytes. It functions as both a triglyceride hydrolase (suggesting catabolic lipase activity) and acetyl-coenzyme A-independent

transacylase (suggesting anabolic lipogenic activity).[23-25] TM6SF2 is a 377-amino acid protein of unknown function. It has broad tissue and organ expression with highest relative levels of expression in the small intestine and liver.[18,26] It is speculated that the *PNPLA3* I148M variant is attached on the surface of lipid droplets, reducing triglyceride breakdown leading to lipid retention in the hepatocyte lipid droplet, while the TM6SF2 E167K variant reduces triglyceride secretion through very low-density lipoprotein, leading to hepatocellular retention of lipids.[27-29] PNPLA3 also has a role in HSCs and retinol metabolism. PNPLA3 is shown to have retinyl-palmitate lipase activity in HSCs, and the I148M mutation leads to a loss of this function.[30,31] Retinyl-palmitate content is elevated and the ratio of retinol/retinyl-palmitate was reduced in liver extracts from patients with the homozygous variant genotype of PNPLA3 I148M.[32] The interaction of these variants with glucagon receptor signaling is not clear.

The mechanism for HFF accumulation and increases in hepatic aminotransferase levels by blocking the glucagon receptor remains to be determined. Glucagon exerts hypolipidemic actions in rats,[33] and subcutaneous glucagon administration promotes mobilization of hepatic fat in lactating dairy cows.[34] The hypolipidemic actions of glucagon on hepatocytes is, in part, through a PPARα-dependent pathway.[4] Accelerated development of steatosis was observed in studies with high-fat feeding in glucagon receptor $(GCGR)^{-/-}$ mice.[4] These observations in multiple animal species together with the novel findings reported in humans[4] imply that a threshold level of GCGR signaling might be required for hepatocytes to maintain their metabolic regulatory function. If this is the case, marked attenuation of GCGR signaling by glucagon receptor antagonism could lead to increased risk of developing dyslipidemia and fatty liver.

Glucagon receptor antagonist treatment may have a potentiating effect in patients who are genetically predisposed to dysregulation of liver lipid metabolism, leading to elevated lipid accumulation. These results show that patients who carry the risk alleles of either PNPLA3 or TM6SF2 are at higher risk of the hepatic side effects of a glucagon receptor antagonist. The hepatic lipid accumulation effect of glucagon receptor antagonism can also be observed in nonrisk-allele carriers; however, the severity of this effect may be much higher in risk-allele carriers. The exact mechanism behind this effect is not entirely clear, and it is possible that one or both of these gene products may be directly involved in mediating the known effect of glucagon on hepatic lipid metabolism.

The role of PNPLA3 and TM6SF2 polymorphisms on susceptibility to NAFLD is widely reported. However, the effect of these variants on treatment response is poorly understood. It has recently been reported that PNPLA3 rs738409 variant explained 3.8% of the variability in ALT in remission induction therapy for acute lymphoblastic leukemia.[35] The same variant also has been shown to be associated with significant increases in ALT, AST, and hepatic fat in T2D patients treated with a hepatopreferential insulin, basal insulin peglispro, but not with insulin glargine.[36] In this report, we present a further role of these variants in treatment response in patients with T2D.

Our study has several strengths, including genetic analyses in two independent randomized clinical trials with reasonable sample size to test our hypotheses. To our knowledge, this is the first study to evaluate the genetic basis for drug-induced hepatic steatosis and related aminotransferase elevations.

A few limitations of our study are noteworthy. First, we limited our analyses to a few candidate SNPs, and two other NASH/NAFLD loci with replicated evidence of association in the literature (glucokinase regulator [*GCKR*] and membrane bound O-acyltransferase domain-containing 7– transmembrane channel like 4 [*MBOAT7-TMC4*])[20,37-39] are not included in this analysis. We conducted post-hoc analyses of the effect of the sentinel SNPs in *GCKR* (rs780094) and *MBOAT7-TMC4* (rs2576452, which is in strong linkage disequilibrium with rs641738) on HFF and ALT and did not find any significant meaningful associations (data not shown). Although we have generated genome-wide genotyping data, we have not attempted a traditional genome-wide association study due to sample size limitations. Furthermore, single-variant analyses of *PNPLA3* and *TM6SF2* did not yield significant association with liver fat change at 6 months. The variability of the HFF measurements (indicated by wider confidence intervals) could be one of the reasons. The allele frequency of the TM6SF2 variant was low, and more subjects may be needed to obtain conclusive evidence. However, because both the studied variants are well-established genetic markers for the liver-related traits, we thus capitalized the opportunity to model the joint effect of the two SNPs on liver enzyme elevation and hepatic fat accumulation. Given the low frequency of risk allele along with lower counts for the GLDJ study, this approach is more meaningful if the genetic effect of TM6SF2 is captured along with

PNPLA3 in the joint analyses. Moreover, only one of the studies measured hepatic fat data; it would be helpful to have these results confirmed in other independent settings. Nevertheless, it is worth noting that the hepatic aminotransferase elevations were consistent in both studies with short-term (6 weeks) exposure as well as long-term exposure (6 months). Because our investigations are limited to LY2409021 phase 2 studies, it is unknown if the observed association between *PNPLA3* and *TM6SF2* variants and drug-induced hepatic steatosis is generalizable to other glucagon receptor antagonists or to other drugs, such as mipomersen, lomitapide, or pegylated insulin lispro, which recently have been described to cause hepatic steatosis. Finally, because this is a retrospective pharmacogenetics analysis, the randomization based on genotypes was not possible.

In conclusion, it appears that carriers of *PNPLA3* and *TM6SF2* variant alleles are at risk for hepatic steatosis and elevated ALT levels caused by LY2409021, a novel glucagon receptor antagonist. It is unclear if this genetic predisposition is a class effect of glucagon receptor antagonists or specific to LY2409021. If confirmed, our observations may be used to investigate glucagon receptor antagonists specifically among individuals without at-risk *PNPLA3* and *TM6SF2* alleles.

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