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# Impaired Very-Low-Density Lipoprotein catabolism links hypoglycemia to hypertriglyceridemia in Glycogen Storage Disease type Ia

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## Abstract

Prevention of hypertriglyceridemia is one of the biomedical targets in Glycogen Storage Disease type Ia (GSD Ia) patients, yet it is unclear how hypoglycemia links to plasma triglyceride (TG) levels. We analyzed whole-body TG metabolism in normoglycemic (fed) and hypoglycemic (fasted) hepatocyte-specific glucose-6-phosphatase deficient (L-$G6pc^{-/-}$) mice. *De novo* fatty acid synthesis contributed substantially to hepatic TG accumulation in normoglycemic L-$G6pc^{-/-}$ mice. In hypoglycemic conditions, enhanced adipose tissue lipolysis was the main driver of liver steatosis, supported by elevated free fatty acid concentrations in GSD Ia mice and GSD Ia patients. Plasma very-low-density lipoprotein (VLDL) levels were increased in GSD Ia patients and in normoglycemic L-$G6pc^{-/-}$ mice, and further elevated in hypoglycemic L-$G6pc^{-/-}$ mice. VLDL-TG secretion rates were doubled in normo- and hypoglycemic L-$G6pc^{-/-}$ mice, while VLDL-TG catabolism was selectively inhibited in hypoglycemic L-$G6pc^{-/-}$ mice. In conclusion, fasting-induced hypoglycemia in

L-*G6pc*$^{-/-}$ mice promotes adipose tissue lipolysis and arrests VLDL catabolism. This mechanism likely contributes to aggravated liver steatosis and dyslipidemia in GSD Ia patients with poor glycemic control and may explain clinical heterogeneity in hypertriglyceridemia between GSD Ia patients.

**KEYWORDS**

Glycogen Storage Disease type Ia, hepatic steatosis, hypertriglyceridemia, metabolic control, translational research

## 1 | INTRODUCTION

Glycogen Storage Disease type Ia (GSD Ia) is an inborn error of carbohydrate metabolism caused by a deficiency of the catalytic subunit (G6PC) of the glucose-6-phosphatase (G6Pase) complex G6PC, selectively expressed in liver, kidney, and intestine, which catalyzes the final step in gluconeogenesis and glycogenolysis by hydrolyzing glucose-6-phosphate to glucose and is the key enzyme for glucose homeostasis during fasting.[1]

GSD Ia patients clinically present with severe fasting intolerance and hepatomegaly, biochemically characterized by nonketotic hypoglycemia, hyperlactacidemia, hyperuricemia, hypercholesterolemia, hepatic steatosis, and hypertriglyceridemia.[2,3] Currently, dietary management still is the cornerstone of treatment for GSD patients to maintain normoglycemia and prevent secondary metabolic derangement (such as hyperlipidemia) as well as long-term complications. Therefore, GSD Ia patients have to adhere to a strict diet, consisting of frequent meals with restriction of simple sugars, relatively small doses of uncooked cornstarch (UCCS) during the day and either UCCS doses or continuous (naso)gastric drip feeding through the night.[3-5] Although strict dietary management and compliance have significantly improved outcomes,[6-8] long-term complications of GSD Ia,[4,7,9,10] among which the development of liver adenomas in young adulthood,[9-12] still occur.

Triglyceride (TG) concentrations are currently regarded as an important biomarker for metabolic control in GSD Ia patients.[4,13,14] Importantly, "Uncertainties regarding optimal metabolic control both clinically and biochemically (like lactate, ketones, and/or lipids) in hepatic GSDs" is among the top research priorities that were recently established by the international GSD priority setting partnership.[15] We previously reported large phenotypic heterogeneity in TG levels between GSD Ia patients ([16,17]); however, the mechanisms underlying (variations in) hypertriglyceridemia in GSD Ia are as yet incompletely understood. Such insight requires an in-depth analysis of the different physiological pathways involved in systemic TG metabolism. Mouse models for GSD Ia used in preclinical studies are commonly investigated under specific,

**Synopsis**

This work shows that fasted hepatocyte-specific G6pc knockout mice show a concomitant increase in VLDL-TG production and reduction in VLDL catabolism, resulting in more pronounced hypertriglyceridemia in hypoglycemic Glycogen Storage Disease type Ia.

controlled fasting conditions. Such studies at one hand limit transability of preclinical findings as dietary management in GSD Ia patients involves frequent intake of small meals, while they do not accommodate the extreme ends of the glycemic condition that GSD Ia patients face, that is, normoglycemia and severe hypoglycemia.

Because hypertriglyceridemia and poor glycemic control are associated with long-term complications such as liver adenoma progression,[14,18-20] detailed understanding of the mechanistic link between glycemic control and hyperlipidemia is of great importance to further improve and personalize GSD Ia patient care. Previous work in tissue-specific *G6pc* knockout mice have shown that hepatocyte-specific deficiency of G6PC is the major determinant of hypoglycemia and hypertriglyceridemia in GSD Ia.[21] In the current study, we therefore performed a systematic analysis of whole-body TG metabolism in normoglycemic (fed) and in hypoglycemic (fasted) hepatocyte-specific *G6pc* knockout (L-*G6pc*$^{-/-}$) mice. Our preclinical findings indicate that severe hypertriglyceridemia in GSD Ia patients with poor glycemic control is most likely due to impaired catabolism of TG-rich lipoproteins.[21]

## 2 | RESULTS

### 2.1 | Hyperlipidemia and hepatic steatosis are aggravated in hypoglycemic L-*G6pc*$^{-/-}$ mice

Fast protein liquid chromatography (FPLC) analysis revealed that excess TGs and cholesterol in serum of

**FIGURE 1** Hyperlipidemia and hepatic steatosis are aggravated in hypoglycemic L-$G6pc^{-/-}$ mice. Triglyceride and total cholesterol concentrations in lipoprotein profiles of (A) GSD Ia patients (n = 3) and healthy control subject (n = 1) and (B) L-$G6pc^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 1). C, Representative pictures of Oil-red-O staining and (D) quantification of lipid droplet area in livers of L-$G6pc^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 6-8). E, Hepatic triglyceride levels and (F) absolute values of hepatic total fatty acids in L-$G6pc^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 6-8). GSD Ia, Glycogen Storage Disease type Ia
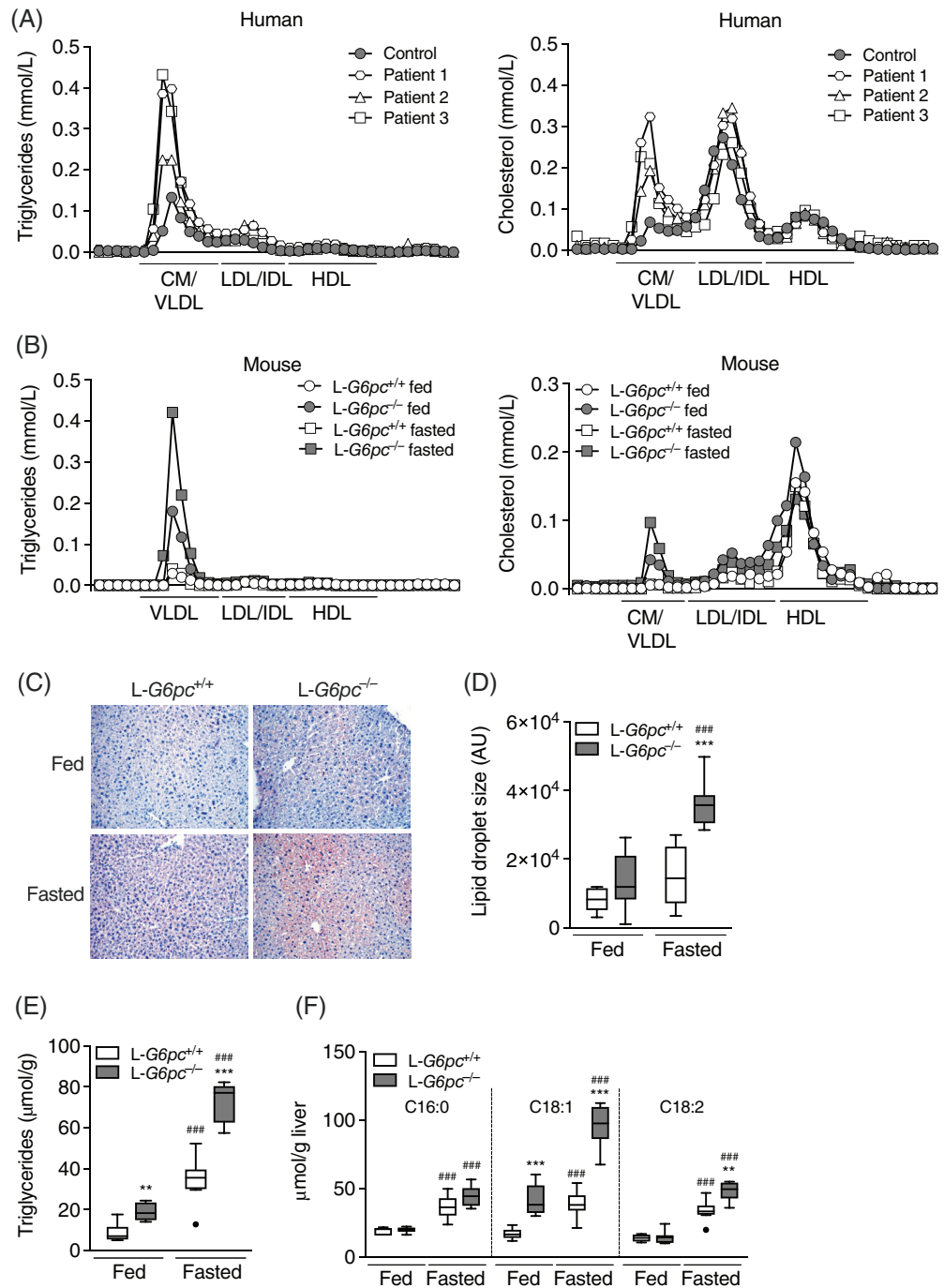
normoglycemic GSD Ia patients were mainly associated with VLDL/chylomicrons (Figure 1A, Table S1). Since hypoglycemia and hyperlipidemia in GSD Ia are caused by the loss of G6PC activity,[21] we characterized TG metabolism in L-$G6pc^{-/-}$ mice. Consistent with observations in patients, plasma TG and cholesterol levels were increased in fed (normoglycemic) L-$G6pc^{-/-}$ mice as compared to wildtype (L-$G6pc^{+/+}$) littermates (Figure 1B, Table 1). Overnight fasting resulted in hypoglycemia in L-$G6pc^{-/-}$ mice with additional increases in plasma TG levels (Figure 1B, Table 1). Under both conditions, L-$G6pc^{-/-}$ mice exhibited increased lipid accumulation as

compared to wild-type controls (Figure 1C,E), due to the hepatic accumulation of TGs and cholesterol esters (CEs; Figures 1E and S1A,B). Similar to what was observed for plasma TG levels, the largest increase in hepatic TG and CE content was observed in hypoglycemic L-$G6pc^{-/-}$ mice (Figures 1C,E and S1B). Hepatic G6P and glycogen contents were most markedly increased in hypoglycemic L-$G6pc^{-/-}$ mice as compared to wild-type controls (Table 1). Exacerbated liver steatosis of hypoglycemic L-$G6pc^{-/-}$ mice was associated with an increase in hepatic lipid droplet area (Figure 1D). Oleate (C18:1) was the most abundant hepatic fatty acid in both normo- and
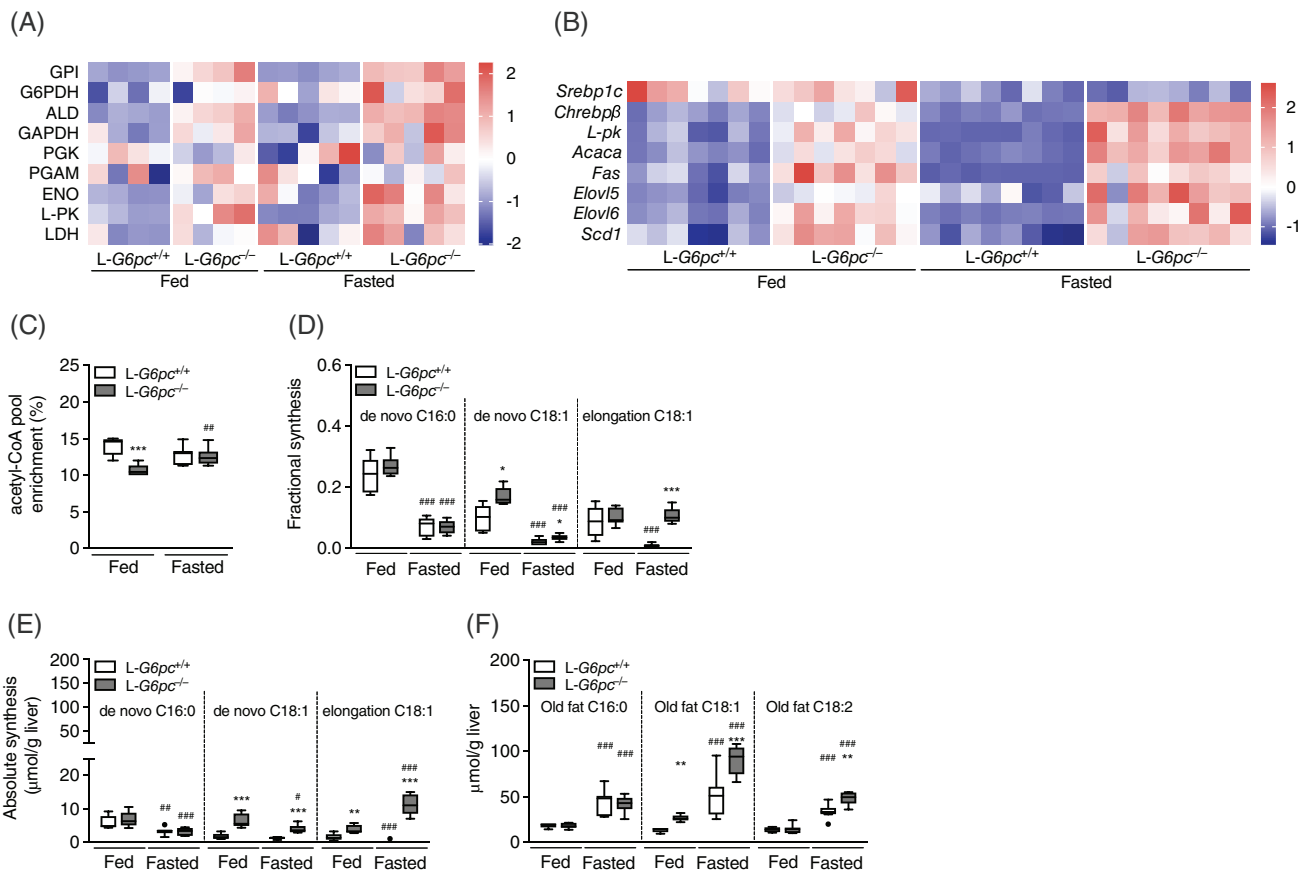
**FIGURE 2** Differential contribution of _de novo_ lipogenesis in fed and fasted L-_G6pc_$^{-/-}$ mice. Heatmaps presenting Z-score normalized (A) hepatic enzymatic activities and (B) hepatic gene expression levels in L-_G6pc_$^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 4-8). C, Acetyl-CoA pool enrichment and (D) fractional and (E) absolute _de novo_ synthesis and chain elongation of palmitate (C16:0), oleate (C18:1) in L-_G6pc_$^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 5-7). F, Hepatic palmitate (C16:0), oleate (C18:1), and linoleate (C18:2) derived from old fat in L-_G6pc_$^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 5-8). Table S3 contains raw values and statistics for data presented in heatmaps

hypoglycemic L-_G6pc_$^{-/-}$ mice, with 3-fold increases in its content in hypoglycemic compared to normoglycemic L-_G6pc_$^{-/-}$ mice (Figure 1F, Table S2), reflecting the higher degree of TG and CE accumulation under hypoglycemic conditions. Linoleate (C18:2), an essential fatty acid that cannot be synthesized _de novo_, was selectively increased in hypoglycemic L-_G6pc_$^{-/-}$ mice, while hepatic palmitate (C16:0) content was similar in L-_G6pc_$^{+/+}$ mice and L-_G6pc_$^{-/-}$ mice (Figure 1F). These findings suggest that part of the hepatic fatty acids accumulated in hypoglycemic L-_G6pc_$^{-/-}$ mice is derived from extrahepatic sources, and not from _de novo_ lipogenesis.

## 2.2 | Differential contribution of _de novo_ lipogenesis in normo- and hypoglycemic L-_G6pc_$^{-/-}$ mice

It is generally assumed that increased glycolysis and _de novo_ lipogenesis have major contributions to hepatic

steatosis in GSD Ia. The enzymatic activities of the glycolytic enzymes GPI, ALD, GAPDH, L-PK, and LDH were significantly increased in the liver of L-_G6pc_$^{-/-}$ mice, irrespective of feeding state (Figure 2A, Table S2). To relate the activity of _de novo_ lipogenesis to hepatic steatosis in normo- and hypoglycemic L-_G6pc_$^{-/-}$ mice, we quantified the hepatic mRNA levels of transcription factors _Srebp1c_ and _Chrebpβ_ as well as of their glycolytic and lipogenic target genes (Figure 2B). Hepatic _Srebp1c_ mRNA expression was lower in the hypoglycemic state but not affected by genotype, while _Chrebpβ_ expression was 3-fold induced in normoglycemic L-_G6pc_$^{-/-}$ mice and 7-fold in hypoglycemic L-_G6pc_$^{-/-}$ mice compared to fed wild-type controls. Similarly, the hepatic expression levels of _L-pk_, _Acaca_, _Fas_, _Elovl5_, _Elovl6_, and _Scd1_ were increased under both conditions in L-_G6pc_$^{-/-}$ mice as compared to wild-type controls, with largest increments in hypoglycemic L-_G6pc_$^{-/-}$ mice (Figure 2B).

To establish the contribution of lipogenesis to hepatic steatosis in L-_G6pc_$^{-/-}$ mice, we quantified hepatic

*de novo* lipogenic fluxes using $^{13}$C-acetate administered via drinking water during 24 hours prior to sacrifice. A significant reduction in acetyl-CoA precursor pool enrichment was observed in normoglycemic L-*G6pc*$^{-/-}$ mice, indicative of increased acetyl-CoA turnover which is compatible with enhanced glycolysis (Figure 2C).[22,23] As expected, hepatic fractional *de novo* fatty acid synthesis of palmitate (C16:0) and oleate (C18:1), two major nonessential TG-associated fatty acids, was significantly higher in fed compared to fasted wild-type mice (Figure 2D). The accumulation of hepatic lipids in normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice was accompanied by an increase in fractional synthesis of oleate, while fractional synthesis of palmitate remained unchanged (Figure 2D). Absolute hepatic oleate synthesis via elongation of pre-existing palmitate was increased in both normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice (Figure 2E), from which the latter was more pronounced. Similarly, fractional and absolute *de novo* synthesis of stearate (C18:0) were significantly increased in normoglycemic L-*G6pc*$^{-/-}$ mice compared to wild-type controls, while chain elongation was significantly increased in hypoglycemic L-*G6pc*$^{-/-}$ mice only (Table S4). In terms of absolute hepatic fatty acid synthesis, lipid accumulation in normoglycemic L-*G6pc*$^{-/-}$ mice mainly resulted from *de novo* oleate synthesis (Figure 2E). In contrast, elongation of pre-existing palmitate to oleate was a major contributor of lipid accumulation in hypoglycemic L-*G6pc*$^{-/-}$ mice (Figure 2E). The contribution of *de novo* lipogenesis to hepatic lipid accumulation in normoglycemic L-*G6pc*$^{-/-}$ mice accounted for up to 20% (Figure 2D), while in hypoglycemic L-*G6pc*$^{-/-}$ mice about 10% of the excess oleate synthesis was derived from elongation of pre-existing palmitate (Figure 2D). Yet, the majority of excess hepatic fatty acids in both normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice was derived from "old fat" (Figure 2F, Table S2).

## 2.3 | Adipose tissue lipolysis is enhanced in hypoglycemic L-*G6pc*$^{-/-}$ mice

To investigate the origin of old fat accumulation in the liver of hypoglycemic L-*G6pc*$^{-/-}$ mice, we analyzed alternative sources of hepatic TG input and output, that is, adipose tissue lipolysis, hepatic mitochondrial β-oxidation and VLDL-TG secretion and VLDL-remnant uptake. Circulating nonesterified fatty acids (NEFAs), including oleate, were increased in both normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice compared to wild-type controls, with highest concentrations in hypoglycemic L-*G6pc*$^{-/-}$ mice (Figure 3A, Table S5). In normoglycemic GSD Ia patients, the concentrations of circulating NEFAs were also

increased (Figure 3B, Table S5). Ex vivo glycerol release from adipose tissue was enhanced in hypoglycemic L-*G6pc*$^{-/-}$ mice only (Figures 3C and S2A,B) and the phosphorylation of hormone sensitive lipase (HSL) on serine 563 was also increased in hypoglycemic L-*G6pc*$^{-/-}$ mice compared to wild-type littermates (Figure 3D-F). In fasted L-*G6pc*$^{-/-}$ mice, ex vivo glycerol release from adipose tissue positively correlated to pHSL S563 protein expression levels (Figure S2C). Adipose triglyceride lipase (ATGL) and perilipin (PLIN) protein levels in adipose tissue, essential for lipid mobilization, were not altered in L-*G6pc*$^{-/-}$ mice (Figure 3F). Plasma levels of fibroblast growth factor 21 (FGF21) were increased in normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice as compared to wild-type controls (Figure 3G). Increased adipose tissue lipolysis in fasted L-*G6pc*$^{-/-}$ mice did not result in significant reductions in adipose tissue mass or whole-body energy expenditure as compared to wild-type controls (data not shown). Plasma ketone body concentrations were increased in both normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice as compared to wild-type littermates, while total ketone body levels were significantly higher in hypo- vs normoglycemic L-*G6pc*$^{-/-}$ mice (Figure 3H). Ex vivo hepatic mitochondrial β-oxidation capacity was significantly increased in normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice (Figures 3I and S2D). Total hepatic acylcarnitine levels were not different between genotypes in fed or fasted state; however, C2 levels, the end product of β-oxidation, were increased in hypoglycemic L-*G6pc*$^{-/-}$ mice (Figure 3J, Table S6). Amino acid-derived acylcarnitines C4 and C5 were increased in both normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice, while medium- and long-chain acylcarnitines were not different (Table S6).

To investigate whether altered hepatic VLDL-TG secretion contributed to exacerbated liver steatosis or to hypertriglyceridemia in L-*G6pc*$^{-/-}$ mice, we quantified hepatic VLDL-TG secretion rates. VLDL-TG secretion was doubled in L-*G6pc*$^{-/-}$ mice as compared to controls, independent of feeding status (Figure 3K). The enhanced VLDL-TG secretion rate in L-*G6pc*$^{-/-}$ mice was associated with similar increases in the TG-to-Apolipoprotein B (ApoB) ratios, with a most marked effect on the TG-to-Apolipoprotein B48 ratio (Figures 3L and S2E). Hepatic ApoB protein and mRNA levels were elevated in normo- and hypoglycemic L-*G6pc*$^{-/-}$ mice as compared to wild-type controls (Figures 3M and S2F). Hepatic ApoB protein levels were further increased in hypoglycemic compared to normoglycemic L-*G6pc*$^{-/-}$ mice (Figure 3M), while plasma ApoB levels were similar in L-*G6pc*$^{+/+}$ and L-*G6pc*$^{-/-}$ mice under both conditions (Figure 3N). mRNA and protein levels of MTTP and TM6SF2, both involved in VLDL assembly, were increased in L-*G6pc*$^{-/-}$ vs L-*G6pc*$^{+/+}$ mice, but unaffected by fasting (Figures 3M and S2F). Altogether, these data indicate that in
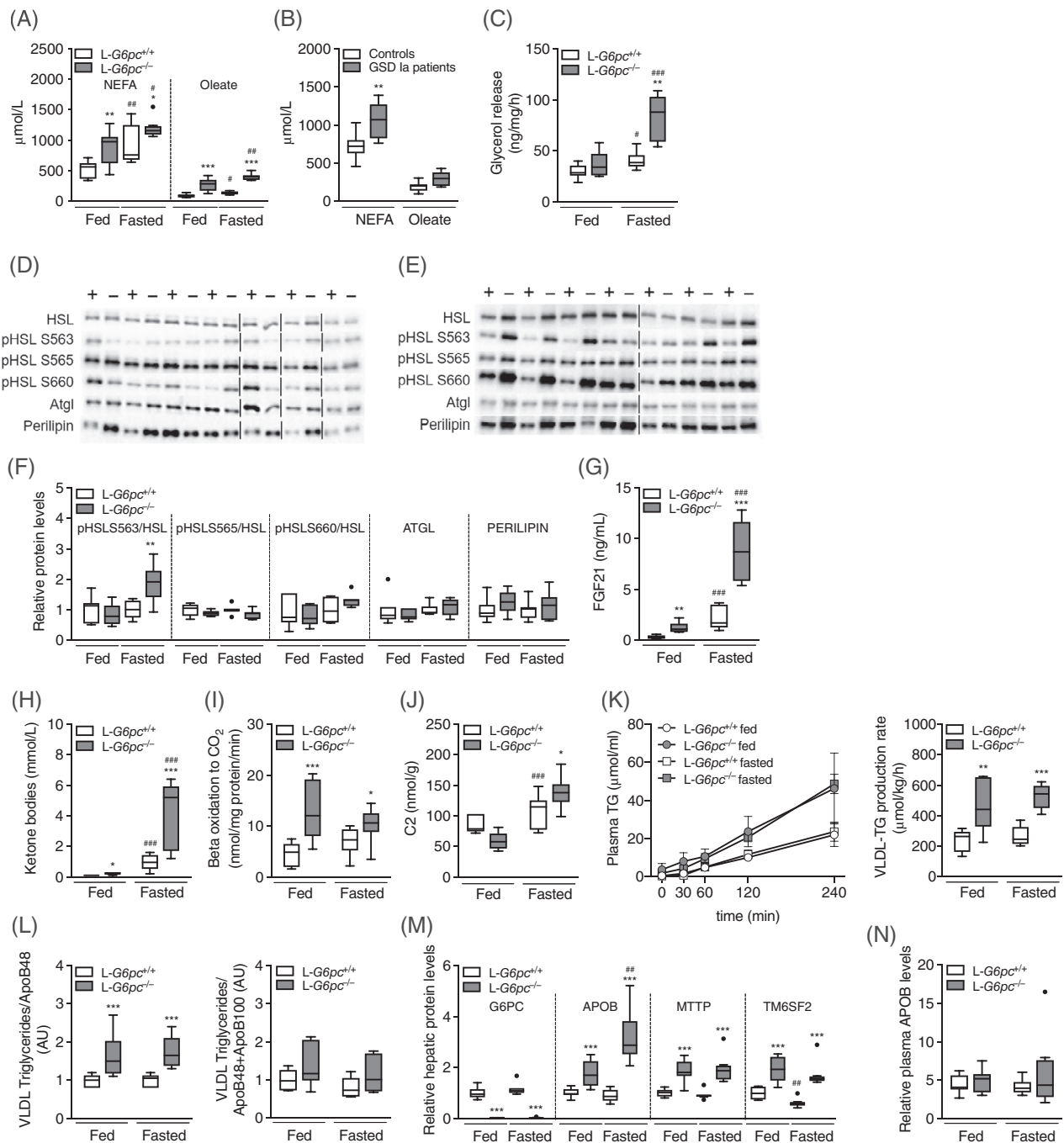
**FIGURE 3**  Adipose tissue lipolysis is induced in fasted L-*G6pc*⁻/⁻ mice. Plasma levels of nonesterified fatty acids (NEFAs) and oleate in (A) L-*G6pc*⁻/⁻ mice and wild-type littermates under fed and fasted conditions (n = 5-8) and in (B) normoglycemic GSD Ia patients and age- and sex-matched controls (n = 7). C, Ex vivo glycerol release from adipose tissue isolated from L-*G6pc*⁻/⁻ mice and wild-type littermates under fed and fasted conditions (n = 6-7). Representative Western blots from adipose tissue of (D) fed and (E) fasted L-*G6pc*⁻/⁻ mice (indicated by −) and wild-type littermates (indicated by +) for indicated proteins (n = 7), with boundaries between different sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) gels indicated by vertical dashed lines. F, Quantification of Western blots for phosphorylated HSL at different sites normalized to total HSL, and ATGL and PLIN (n = 7). Plasma levels of (G) FGF21 and (H) total ketone bodies in L-*G6pc*⁻/⁻ mice and wild-type littermates under fed and fasted conditions (n = 5-8). I, Ex vivo β-oxidation capacity and (J) hepatic acylcarnitine C2 levels in L-*G6pc*⁻/⁻ mice and wild-type littermates under fed and fasted conditions (n = 5-8). K, very-low-density lipoprotein-triglyceride (VLDL-TG) production curves (left) and production rates (right); L, Ratios of TG/ApoB48 (left) and TG/ApoB48 +ApoB100 (right) in isolated VLDL as assessed by Western blot analysis (for full blots, see Figure S2E). M, Hepatic and (N) plasma protein levels assessed by targeted proteomics in L-*G6pc*⁻/⁻ mice and wild-type littermates under fed and fasted conditions (n = 6-8), with APOB protein levels representing the sum of APOB48 and APOB100. ATGL, adipose triglyceride lipase; FGF21, fibroblast growth factor 21; GSD Ia, Glycogen Storage Disease type Ia; HSL, hormone sensitive lipase; PLIN, perilipin
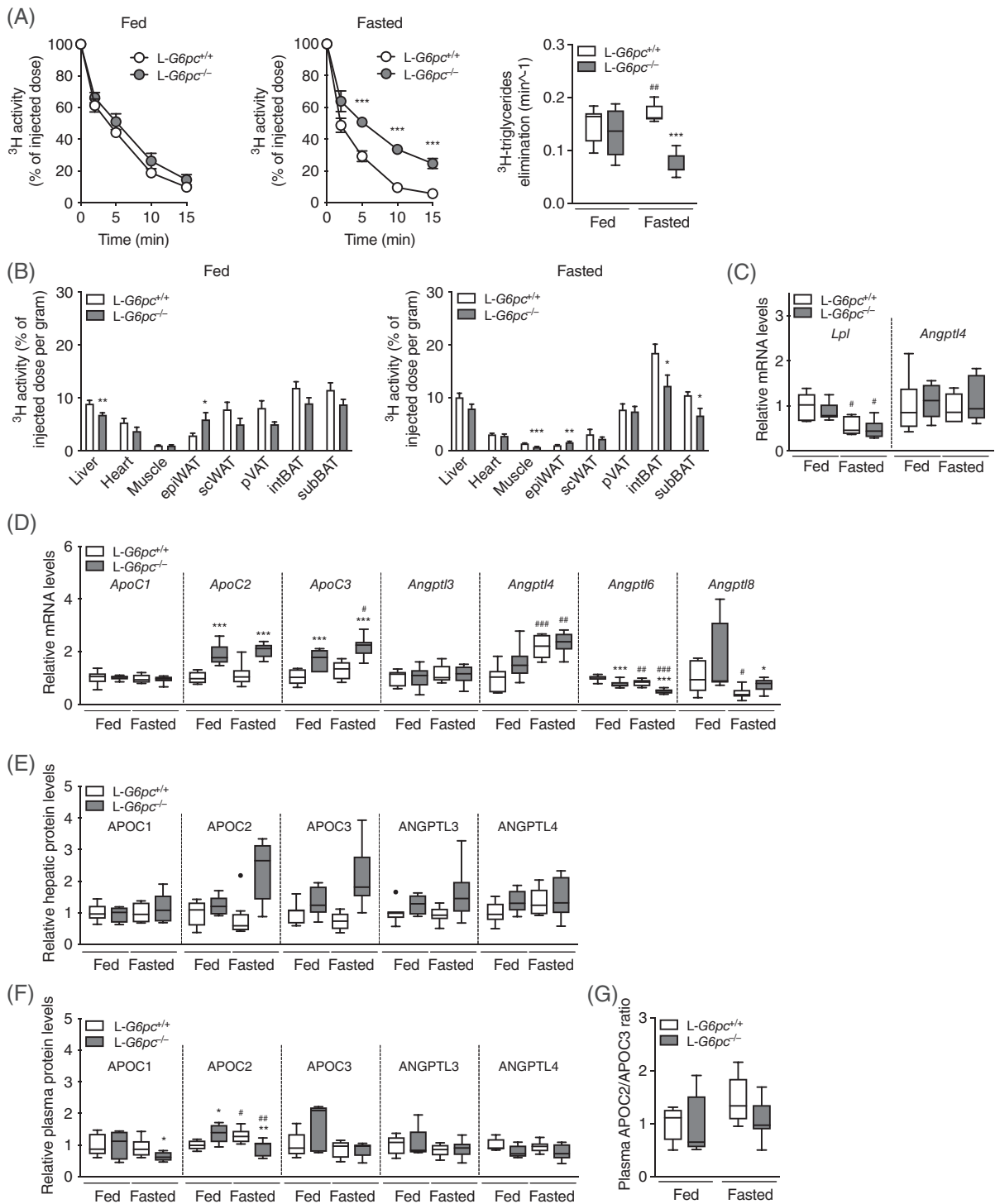
**FIGURE 4** VLDL catabolism is impaired in fasted L-$G6pc^{-/-}$ mice. A, Plasma clearance and plasma elimination rate of glycerol tri-[$^3$H] oleate-labeled VLDL-like particles in L-$G6pc^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 6-8). B, $^3$H activity in various organs and expressed as percentage of the injected dose of glycerol tri[$^3$H]oleate-labeled very-low-density lipoprotein (VLDL)-like particles per gram wet tissue weight (n = 7-8). C, White adipose tissue mRNA levels of L-$G6pc^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 5-6). D, Hepatic gene expression and (E) hepatic protein levels assessed by targeted proteomics in L-$G6pc^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 6-8). F, Plasma protein levels assessed by targeted proteomics and (G) Plasma APOC2/APOC3 protein ratio in L-$G6pc^{-/-}$ mice and wild-type littermates under fed and fasted conditions (n = 5-8)

acutely fasted L-*G6pc*$^{-/-}$ mice, exacerbated liver steatosis was paralleled by enhanced adipose tissue lipolysis, but not by impaired β-oxidation or reduced VLDL-secretion.

## 2.4 | VLDL catabolism is impaired in hypoglycemic L-*G6pc*$^{-/-}$ mice

Because VLDL-TG levels were increased in hypo- vs normoglycemic L-*G6pc*$^{-/-}$ mice in the absence of an additional increase in VLDL-TG secretion, we quantified VLDL catabolism in fed and fasted L-*G6pc*$^{-/-}$ and L-*G6pc*$^{+/+}$ mice. In the fed state, the plasma decay of glycerol tri[$^3$H]oleate-labeled VLDL-like particles was not different between L-*G6pc*$^{-/-}$ mice and wild-type littermates (Figure 4A,B). However, in the fasted state, elimination rate of labeled VLDL-like particles was decreased by about 50% in L-*G6pc*$^{-/-}$ mice compared to controls (Figure 4A). In the fasted state, uptake of TG-derived fatty acids by muscle, epididymal white adipose tissue, and brown adipose tissue depots was significantly reduced (Figure 4B). VLDL-TG catabolism is largely dependent on the activity of lipoprotein lipase (LPL), which is in turn controlled by apolipoproteins and angiopoietin-like proteins.[24,25] *Lpl* mRNA levels in white adipose tissue were decreased in the fasted state yet were comparable in L-*G6pc*$^{-/-}$ mice and wild-type littermates (Figure 4C), while *Angptl4* mRNA levels were unchanged (Figure 4C). Hepatic protein levels of APOC1 were unaffected in hypoglycemic L-*G6pc*$^{-/-}$ mice while hepatic mRNA and protein levels of APOC2 were increased in both normoglycemic and hypoglycemic L-*G6pc*$^{-/-}$ mice compared to controls (Figure 4D,E). Plasma protein levels of APOC3, ANGPTL3 and ANGPTL4, and APOC2/APOC3 ratios were not affected by genotype or feeding state (Figure 4F,G).

## 3 | DISCUSSION

In the current study, we investigated the physiological mechanisms that link glycemia to hyperlipidemia in GSD Ia, by comparing fed (normoglycemic) to acutely fasted (hypoglycemic) L-*G6pc*$^{-/-}$ mice. We found that excess TGs and cholesterol in GSD Ia patient serum were specifically associated with VLDL/chylomicrons, and that more severe liver steatosis and hypertriglyceridemia in hypoglycemic L-*G6pc*$^{-/-}$ mice were associated with enhanced adipose tissue lipolysis and impaired VLDL-TG catabolism, respectively (Figure S3). Altogether, our data suggest that increased VLDL-TG secretion by the liver contributes to elevated serum TG concentrations in GSD Ia patients,[17] and that impaired catabolism of TG-rich lipoproteins explains the severe hyperlipidemia in patients with poor glycemic control.[23,26,27]

It is commonly assumed that hepatic steatosis and hypertriglyceridemia in GSD Ia result from an increase in hepatic *de novo* lipogenesis and VLDL secretion driven by a high glycolytic flux.[22,23,26,28] Our findings indicate that hepatic TG accumulation in normoglycemic L-*G6pc*$^{-/-}$ mice mainly, but not exclusively, results from increased *de novo* lipogenesis. It should be noted that the contribution of lipogenesis was quantified over a 24 hour period and reached up to 20%, a value comparable to that observed in humans in the postprandial state.[29] In fasted, hypoglycemic L-*G6pc*$^{-/-}$ mice, enhanced adipose tissue lipolysis and subsequent uptake and elongation of fatty acids released by the adipose tissue were found to be the most predominant causes of hepatic TG accumulation. The increased contribution of adipose tissue-derived circulating NEFAs to liver steatosis was further supported by the accumulation of linoleate (an essential fatty acid) which exclusively occurred in livers of hypoglycemic L-*G6pc*$^{-/-}$ mice.

Adipose tissue lipolysis is, among other factors, controlled by HSL.[30] Our data show increased phosphorylation of HSL at Ser-563 and a tendency for increased phosphorylation at Ser-660 (marking HSL activation) in hypoglycemic L-*G6pc*$^{-/-}$ mice, while the inhibitory phosphorylation at Ser-565 remained unaffected. These findings are in agreement with the increase in ex vivo adipose tissue lipolysis in these animals. Glucagon, catecholamines, and glucocorticoids, which are elevated in response to hypoglycemia, are well-established enhancers of HSL-mediated adipose tissue lipolysis via cAMP and β-adrenergic signaling.[30-34] In hypoglycemic L-*G6pc*$^{-/-}$ mice, plasma glucagon levels were significantly increased, and the insulin-to-glucagon ratio was reduced (Table 1). We hypothesize that a lower insulin-to-glucagon ratio and/or increased catecholamine/glucocorticoid levels in fasted hypoglycemic L-*G6pc*$^{-/-}$ mice[35-37] increases adipose tissue lipolysis via enhanced HSL phosphorylation.[31,34,38] We found that enhanced adipose tissue lipolysis in fasted L-*G6pc*$^{-/-}$ mice was not associated with reduced adipose tissue mass (data not shown). Yet, it has been reported that L-*G6pc*$^{-/-}$ mice display failure to thrive starting from 9 months after gene deletion,[39] comparable to what is observed in patients. It is conceivable that prolonged (hepatic) *G6pc* deficiency imposes recurrent catabolic states that on the long run are reflected in reduced body weight or adiposity, while the catabolic response induced by a single fast within 2 weeks after hepatic *G6pc* deletion in the current study was relatively modest and did not (yet) translate into lower fat mass or body weight.

Our data highly indicate that enhanced VLDL-TG secretion in L-*G6pc*$^{-/-}$ mice mainly resulted from enhanced VLDL lipidation, rather than an increase in VLDL particle number. Consistent with these findings,[40,41] we observed that hepatic MTTP and TM6SF2 protein levels were increased and that circulating ApoB levels remained

unchanged in L-$G6pc^{-/-}$ mice. Importantly, our data also show that the increase in VLDL-TG secretion was paralleled by impaired VLDL-TG clearance in hypoglycemic L-$G6pc^{-/-}$ mice only. This suggests that the activity of lipolytic enzymes such as LPL or hepatic lipase (HL) was reduced by a circulating factor that is specifically altered under fasted, hypoglycemic conditions, as was proposed previously.[42] Among others, LPL and HL activities are regulated by apolipoproteins.[25,43] It has been reported that the apoC2/C3 ratio is reduced in GSD Ia VLDL,[44] which may contribute to reduced LPL activity.[45,46] However, the data presented in the current study do not confirm a solid relationship between feeding state, apoC2/C3 ratios and VLDL levels or VLDL catabolism. Yet, this does not exclude the possibility that reduced availability of apoC2 to LPL contributed to impaired TG clearance and more severe hypertriglyceridemia in fasted L-$G6pc^{-/-}$ mice. Interestingly, it has been proposed that circulating NEFAs inhibit LPL and TG hydrolysis, hence exerting product inhibition on its activity.[42,47-50] Oleate, one of the most prevalent NEFAs, dose-dependently inhibits LPL activity,[47] probably by preventing ApoC2-LPL interaction.[51,52] The observed increases in plasma NEFA and oleate concentrations in hypoglycemic L-$G6pc^{-/-}$ mice may hence contribute to impaired VLDL catabolism independent of changes in ApoC2/C3 levels. This mechanism also likely explains previous findings from our laboratory in which plasma TG levels were increased in hypoglycemic rats treated with a glucose-6-phosphate transporter inhibitor, thereby acutely inducing GSD type 1b.[22] In that study, VLDL-TG secretion was not enhanced, yet NEFA concentrations were increased and presumably resulted in rapid inhibition of VLDL catabolism and hypertriglyceridemia.

Experimental data in GSD Ia patients suggest that altered VLDL metabolism may contribute to hypertriglyceridemia in GSD Ia.[23,26,42,44,53,54] It has been reported that ApoB turnover VLDL secretion is unchanged in GSD Ia patients in normoglycemic state.[44,54] Bandsma et al reported reduced ApoB100 turnover rates in two GSD Ia patients, and a higher turnover in another patient as compared to healthy controls.[23] Our current data suggest that hepatic VLDL particle secretion remains (largely) unaffected in L-$G6pc^{-/-}$ mice. Assessment of ApoB turnover rates therefore likely does not reveal increased VLDL-TG secretion in GSD Ia. Our finding that excess TGs and cholesterol in patients are almost exclusively associated with TG-rich lipoproteins is in agreement with our finding in mice that impaired VLDL catabolism is a major determinant of hyperlipidemia in GSD Ia. Although impaired VLDL catabolism has been proposed to contribute to hyperlipidemia in normoglycemic GSD Ia patients,[23,42,44,53,55,56] we show that VLDL catabolism was selectively inhibited in *fasted* L-$G6pc^{-/-}$ mice. Besides the fact that the observed difference

in lipid composition (Figure 3L) and basal plasma TG levels is not accounted for when using these model particles, it is unclear whether apoB, which is elevated in plasma of GSD Ia patients[44,55,57] and livers of GSD Ia mice (Figure 3M), is exchanged with VLDL-like particles. One methodological limitation of our study is therefore that we may not have accounted for altered lipid and apolipoprotein levels on VLDL catabolism in L-$G6pc^{-/-}$ mice.

It should be noted that some differences exist between our preclinical findings and observations in GSD Ia patients. These may be related to multiple factors, such as (a) the use of hepatocyte-specific instead of whole body $G6pc$ knockout mice, (b) a lack of extra carbohydrate intake during the inactive phase in mice while GSD Ia patients regularly ingest complex carbohydrates during both day and night, (c) species differences between mice and humans, and (d) the experimental setup. In our study, hepatic $G6pc$ deletion was induced during adulthood and analysis of TG metabolism was performed within 2 weeks after gene deletion, which provides insights into an early disease stage and does not account for pathophysiological adaptations occurring over a longer timeframe. In hypoglycemic L-$G6pc^{-/-}$ mice, we observed enhanced β-oxidation capacity and increased levels of ketone bodies as well as hepatic C2 acylcarnitines, suggesting that fatty acid oxidation was increased under hypoglycemic conditions. Based on these findings, we hypothesize that enhanced hepatic NEFA influx in hypoglycemic L-$G6pc^{-/-}$ mice promoted hepatic fatty acid elongation, β-oxidation, and ketogenesis. In contrast, hepatic C2 acylcarnitine levels were modestly reduced and plasma ketone body levels were only slightly increased in normoglycemic L-$G6pc^{-/-}$ mice, despite an increase in hepatic β-oxidation capacity ex vivo. These data suggest that in vivo β-oxidation was not increased under fed conditions, consistent with findings in normoglycemic GSD Ia patients.[23] Unlike fasted L-$G6pc^{-/-}$ mice, GSD Ia is traditionally considered to cause nonketotic or hypoketotic hypoglycemia[58,59] despite elevated NEFA levels,[60,61] although GSD Ia patients may display elevated ketone concentrations.[62,63] This seemingly inconsistency may be related to the physiological state during which ketone levels were analyzed. With respect to plasma lactate levels, these tended to increase in fed L-$G6pc^{-/-}$ mice as compared to wild-type controls but were only slightly elevated in fasted L-$G6pc^{-/-}$ mice, while circulating lactate levels are elevated in GSD Ia patients[10,62,64] and in whole-body $G6pc^{-/-}$ mice.[65] We speculate that these discrepancies in plasma ketone body and lactate levels are related to an impairment of renal lactate excretion in GSD Ia patients and whole-body $G6pc$ knockouts,[64,65] that does not occur in hepatocyte-specific $G6pc$ knockout mice.[21] Elevated lactate levels in GSD Ia

patients and whole-body *G6pc* knockout mice, in turn, likely inhibit ketogenesis.[66] L-*G6pc*$^{-/-}$ mice, on the other hand, retain functional renal lactate excretion, hence permitting ketogenesis, especially in hypoglycemic states. Our current data show a selective increase in adipose tissue lipolysis and inhibition of VLDL catabolism in fasted hypoglycemic L-*G6pc*$^{-/-}$ mice. Previous studies have shown enhanced adipose tissue lipolysis in both normo- and hypoglycemic GSD Ia patients,[60,61] while TG hydrolysis activities were reduced under normoglycemia.[42,44,53,56,67] The fact that adipocyte lipolysis and VLDL catabolism are also altered in well-controlled patients is most likely explained by the presence of insulin resistance associated with dietary overtreatment,[68] which also enhances adipose tissue lipolysis and circulating NEFA levels that may, in turn, inhibit VLDL catabolism.[42,47-49,69,70]

According to the European Study on Glycogen Storage Disease type I, chronic serum TG concentrations <6.0 mmol/L are among the biomedical targets.[4] The more recent American College of Medical Genetics and Genomics GSD I guidelines state that "elevated triglycerides and cholesterol above the normal ranges may persist in some patients with GSD I, despite appropriate dietary treatment".[13] In GSD Ia patients, serum TG levels can go up to 100 mmol/L,[17] posing major health risk given that TG concentrations >11.3 mmol/L are associated with acute pancreatitis.[71] Adenoma progression is significantly increased in GSD Ia subjects with a 5-year mean TG concentration >5.6 mmol/L.[14] How to translate these preclinical insights on the link between hypoglycemia and hypetriglyceridemia into management and monitoring of GSD Ia patients in clinical practice and future trial design? Our finding that hypoglycemia and hypertriglyceridemia are physiologically linked emphasizes the importance of diagnosing (asymptomatic) hypoglycemia. In this respect, better monitoring of glycemic control, for example by means of continuous glucose monitoring, will likely result in better control of hypertriglyceridemia. In addition, dedicated research on the role of factors that control VLDL-TG catabolism, such as NEFA, hormones, apolipoproteins, and angiopoietin-like proteins, will potentially result in identification of novel biomarkers that will aid better clinical management and monitoring of GSD Ia. With respect to monitoring, our results in L-*G6pc*$^{-/-}$ mice illustrate that the timing of sampling vs meals is crucial to adequately interpret blood biomarkers. Many preclinical studies in GSD models are performed under fasting conditions, and time to hypoglycemia is considered an important outcome during clinical trials aiming at therapeutic interventions. On the other hand, for GSD Ia patients, self-management and self-monitoring of a strict dietary management aims to maintain glucose concentrations in a relatively narrow normal range, reflecting a small therapeutic window between undertreatment and overtreatment. The use of

biomarkers in different stages of GSD Ia should be more clearly defined to (a) identify the risk of developing an illness, (b) screen for subclinical disease, (c) diagnose disease, (d) categorize disease severity, and (e) predict prognosis or complications. The thoughtful design of the current study with regard to physiological status, and the use of CRISPR-cas9 mediated gene editing to generate a spectrum of GSD Ia disease phenotypes in mice (Rutten et al, in revision), will allow for identification of novel, clinically-translatable biomarkers in GSD Ia.

In conclusion, our study identifies the physiological mechanism via which acute hypoglycemia is linked to hypertriglyceridemia in a liver-specific mouse model for GSD Ia. Moreover, our work reveals a marked difference in the origin of hepatic steatosis in normoglycemic vs hypoglycemic GSD Ia mice. We propose that increased adipocyte lipolysis and the resulting elevated NEFA levels in GSD Ia impairs catabolism of TG-rich lipoproteins, leading to hypertriglyceridemia, and that these changes are most marked in patients with poor metabolic control. Our findings highlight the contribution of hypoglycemia to GSD Ia pathophysiology. We therefore propose that preclinical studies in normo- vs hypoglycemic GSD Ia models will provide clinically relevant insights into the underlying mechanisms contributing to disease symptoms and complications in GSD Ia patients.

## 4 | METHODS

### 4.1 | Study approval

The study with human materials was performed in accordance with the Declaration of Helsinki and the institutional rules for studying biological rest materials and thereby approved for waived consent as it concerned retrospective, anonymous data. The Medical Ethical Committee of the University Medical Center Groningen stated that the Medical Research Involving Human Subjects Act was not applicable and that official study approval by the Medical Ethical Committee was not required (METc 2019/119).

All animal procedures were approved by the Dutch Central Committee Animal Experiments (Centrale Commissie Dierproeven) under permit number AVD105002015245 and DEC 6246 and adhered to guidelines set out in the 2010/63/EU directive.

### 4.2 | Human subjects

For FPLC profiles, plasma samples from three GSD Ia patients (two female, one male; age range 32-79 years)

**TABLE 1**  Plasma and liver characteristics in male L-*G6pc*$^{-/-}$ mice and wild-type littermates in fed state or after an overnight fast

| Parameter | L-*G6pc*$^{+/+}$ fed<br>Median (range) | L-*G6pc*$^{-/-}$ fed<br>Median (range) | P value | L-*G6pc*$^{+/+}$ fasted<br>Median (range) | L-*G6pc*$^{-/-}$ fasted<br>Median (range) | P value |
|---|---|---|---|---|---|---|
| Plasma | | | | | | |
| Glucose (mmol/L) | 8.6 (6.8-9.5) | 6.5 (5.6-9.9) | .126 | 5.0 (3.7-8.4) | 2.1 (1.1-2.4) | **<.001** |
| Insulin (ng/mL) | 0.4 (0.3-0.6) | 0.2 (0.1-0.4) | **.009** | 0.3 (0.1-0.6) | 0.2 (0.1-0.4) | .328 |
| Glucagon (pg/mL) | 113 (55-163) | 148 (73-320) | .429 | 113 (86-132) | 236 (137-582) | **<.001** |
| Insulin/glucagon ratio | 3.5 (2.6-5.8) | 1.1 (0.5-3.2) | **.017** | 3.0 (1.1-4.7) | 0.9 (0.3-1.8) | **.003** |
| Lactate (mmol/L) | 3.2 (2.5-4.0) | 4.4 (3.7-4.7) | .052 | 1.8 (1.2-3.3) | 2.1 (0.9-3.9) | .328 |
| Triglycerides (mmol/L) | 0.6 (0.4-0.7) | 1.7 (0.3-2.7) | .082 | 0.5 (0.4-0.6) | 3.5 (2.3-8.3) | **<.001** |
| Cholesterol (mmol/L) | 2.1 (1.6-2.8) | 3.7 (1.7-4.3) | **.030** | 2.3 (2.0-3.8) | 2.9 (1.6-3.8) | .195 |
| Liver | | | | | | |
| G6P (nmol/g) | 406 (339-505) | 1675 (819-2306) | **.004** | 422 (265-483) | 2586 (1981-3457) | **<.001** |
| Glycogen (mg/g) | 51 (44-57) | 66 (53-77) | **.017** | 18 (12-30) | 54 (46-62) | **<.001** |

*Note:* Bold values indicate that *p*-values <.05.

and one control subject were collected in fed state. For plasma NEFA profiles, plasma from seven GSD Ia patients (three female, four male; age range 22-51 years) and age- and sex-matched controls were randomly collected during the day in fed state. For patient demographics, see Table S1. Plasma samples were stored at −20°C until further analysis.

## 4.3 | Animals

Male B6.*G6pc*$^{lox/lox}$ and B6.*G6pc*$^{lox/lox}$.SA$^{creERT2/w}$ mice[39] (10-16 weeks old) were housed in a light (lights on: 7:00 AM-7:00 PM) and temperature (21°C)-controlled facility and fed a standard laboratory chow diet (RMH-B, ABdiets, Woerden, The Netherlands). Animals received i.p. injections of tamoxifen for five consecutive days to generate hepatocyte-specific *G6pc*-deficient mice (L-*G6pc*$^{-/-}$) and wild-type littermates (L-*G6pc*$^{+/+}$) by deletion of exon 3 of the *G6pc* gene, as described previously.[39] Ten days after the last tamoxifen injection, mice were sacrificed by heart puncture (8:00 AM) in either a fed or a 9 hour fasted (11:00 PM-8:00 AM) state. Tissues were quickly excised and stored at −80°C. Blood was centrifuged (4.000 rpm for 10 minutes at 4°C), and plasma was stored at −20°C.

Additional experimental and analytical procedures are described in the Supporting Information.

## 4.4 | Statistics

Statistical analysis was performed using BrightStat software. Differences between two or multiple groups were tested by Mann-Whitney *U* test or Kruskal-Wallis *H* test followed by post hoc Conover pairwise comparisons, respectively. Data was represented by Tukey boxplots or means ± SEM. \*\*\*$P < .001$, \*\*$P < .01$, \*$P < .05$ indicates significance compared to wild-type littermates. ###$P < .001$, ##$P < .01$, #$P < .05$ indicates significance compared to fed condition. Correlations were analyzed by Spearman's correlations coefficient using SPSS24.0 for Windows software (SPSS, Chicago, Illinois).

## CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

## AUTHOR CONTRIBUTIONS

Designing research studies: Joanne A. Hoogerland, Brenda S. Hijmans, Sander Kooijman, Folkert Kuipers, Fabienne Rajas, Gilles Mithieux, Dirk-Jan Reijngoud, Terry G. J. Derks, and Maaike H. Oosterveer; Conducting experiments: Joanne A. Hoogerland, Brenda S. Hijmans, Fabian Peeks, Justina C. Wolters, Sander Kooijman, Trijnie Bos, Aycha Bleeker, Henk Wolters, Albert Gerding, Karen van Eunen, Rick Havinga, and Amanda C. M. Pronk; Analyzing data: Joanne A. Hoogerland, Brenda S. Hijmans, Fabian Peeks, Justina C. Wolters, Theo H. van Dijk, Folkert Kuipers, Terry G. J. Derks, and Maaike H. Oosterveer; Writing the first draft of the manuscript: Joanne A. Hoogerland, Fabian Peeks, Folkert Kuipers, and Maaike H. Oosterveer; Critical revisions of the manuscript: Fabian Peeks, Sander Kooijman, Patrick C. N. Rensen, Theo H. van Dijk, Henk Wolters, Karen van Eunen, Fabienne Rajas, Gilles Mithieux, Folkert Kuipers, Terry G. J. Derks, and Maaike H. Oosterveer; Material support: Fabienne Rajas and Gilles Mithieux.

## DATA AVAILABILITY STATEMENT

Raw data will be available when requested.

## ETHICS STATEMENT

The Medical Ethical Committee of the University Medical Center Groningen stated that the Medical Research Involving Human Subjects Act was not applicable and that official study approval by the Medical Ethical Committee was not required (METc 2019/119).

## PATIENT CONSENT STATEMENT

The study with human materials was performed in accordance with the Declaration of Helsinki and the institutional rules for studying biological rest materials and thereby approved for waived consent as it concerned retrospective, anonymous data.

## DOCUMENTATION OF APPROVAL OF ANIMAL EXPERIMENTATION

All animal procedures were approved by the Dutch Central Committee Animal Experiments (Centrale Commissie Dierproeven) under permit number AVD105002015245 and DEC 6246 and adhered to guidelines set out in the 2010/63/EU directive.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.