Glycerol-3-phosphate Acyltransferase1 Is a Model-Agnostic Node in Nonalcoholic Fatty Liver Disease: Implications for Drug Development and Precision Medicine

Kimberly Liao, Anthony J. Pellicano, Kai Jiang, Natalia Prakash, Jingsong Li, Shraddha Bhutkar, Zhijian Hu, Quaisar Ali, Itzhak D. Goldberg, and Prakash Narayan

ABSTRACT: Left untreated nonalcoholic fatty liver disease (NAFLD) can progress to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma. The observed failure of clinical trials in NASH may suggest that current model systems do not fully recapitulate human disease, and/or hallmark pathological features of NASH may not be driven by the same pathway in every animal model let alone in each patient. Identification of a model-agnostic disease-associated node can spur the development of effective drugs for the treatment of liver disease. Glycerol-3-phosphate acyltransferase1 (GPAT1) plays a pivotal role in lipid accumulation by shunting fats away from oxidation. In the present study, hepatic GPAT1 expression was evaluated in three etiologically different models of NAFLD. Compared to the sham cohort, hepatic GPAT1 mRNA levels were elevated by ∼5-fold in steatosis and NASH with fibrosis with immunofluorescent staining revealing increased GPAT1 in the fatty liver. A significant and direct correlation (r = 0.88) was observed between hepatic GPAT1 mRNA expression and severity of the liver disease. Picrosirius red staining revealed a logarithmic relation between hepatic GPAT1 mRNA expression and scar. These data suggest that hepatic GPAT1 is an early disease-associated model-agnostic node in NAFLD and form the basis for the development of a potentially successful therapeutic against NASH.

1. INTRODUCTION

With the recent rise in diabetes, obesity, and metabolic syndrome, nonalcoholic fatty liver disease (NAFLD) has reached epidemic proportions. Within the United States, NAFLD-associated complications are predicted to become the primary cause for liver transplant over the next few decades.1,2 The threat associated with NAFLD lies in more than just its risk for progression to nonalcoholic steatohepatitis (NASH), wherein hepatic inflammation accompanies steatosis. Left untreated, the NAFLD continuum can encompass fibrosis or scarring, cirrhosis, and hepatic decompensation. While cirrhosis is a known risk factor for progression to hepatocellular carcinoma (HCC), emerging evidence indicates that NASH with or without fibrosis can progress to HCC, a phenomenon known as noncirrhotic HCC.3–9 The observed failure of clinical trials10–15 in NASH and lack of robust translational success suggest that current model systems do not fully recapitulate human disease, and/or hallmark pathological features of NASH may not be driven by the same pathway in every animal model let alone in each patient.16,17 Identification of a model-agnostic, disease-associated node can spur the development of effective drugs for the treatment of liver disease.

According to the multihit hypothesis,18 steatosis is one of the earliest factors contributing to NAFLD. Glycerol-3-phosphate acyltransferase (GPAT) incorporates newly synthesized fatty acids into triacylglycerol (TAG) and decreases fatty acid oxidation.19–22 While 3 isoenzymes of GPAT have been described, GPAT1 activity accounts for 30–50% of total liver GPAT activity. Overexpression of hepatic GPAT1 is associated with steatosis and hepatic insulin resistance in the absence of obesity or a high-fat diet.19 Hepatic GPAT1 activity is elevated in obese rodents, whereas mice deficient in GPAT1 have an improved lipid profile.21 Consistent with this notion, Gpat1−/− mice synthesize and store less hepatic TAG, shunt more fatty acid toward oxidation, are resistant to diet-induced steatosis, and have reduced susceptibility to HCC.22

In the present study, we tested the hypothesis that hepatic GPAT1 is a model-agnostic, disease-associated node in
etiologically distinct models of NAFLD with or without fibrosis.

2. RESULTS

Compared to the livers from the sham cohort, the livers from the FFD, FFD + TAA, or FFD + CCL4 + glucose cohorts exhibited hallmark signs of NAFLD viz. accumulation of lipid droplets, inflammation, and hepatocyte ballooning (Figure 1). While macrovesicular steatosis was the dominant presentation in the livers from the FFD cohort, FFD + TAA and FFD + CCL4 + glucose livers were characterized by microvesicular and macrovesicular steatosis with the dominating feature being inflammation. Occasional hepatocyte ballooning was observed in all three modified diet cohorts. Consistent with these observations, increased NAS (all modified diet groups, \( p \ll 0.01 \) vs sham) was also observed in these groups (Figure 1).

Picrosirius red staining of livers revealed bridging fibrosis in the FFD + TAA and FFD + CCL4 + glucose cohorts. Bridging fibrosis was not evident in the FFD cohort (Figure 2). Indeed, quantification of Picrosirius red staining revealed no increase in scarring in the FFD group but a significant increase in scarring in the HFD + TAA (\( p = 0.038 \) vs sham) and FFD + CCL4 + glucose cohorts (\( p = 0.009 \) vs sham).

Querying of hepatic homogenates for GPAT1 expression revealed (Figure 3) a robust, multifold elevation in GPAT1 mRNA vs the sham cohort (FFD, \( p = 0.01 \) vs sham; FFD + TAA, \( p \ll 0.01 \) vs sham; FFD + CCL4 + glucose, \( p \ll 0.01 \) vs sham). Interestingly, there was a significant (\( p < 0.01 \)) and direct (\( r = 0.88 \)) correlation between the NAS and hepatic GPAT1 mRNA expression level. The relation between hepatic Picrosirius red staining and hepatic GPAT1 mRNA expression level was logarithmic, an early and steep increase in the hepatic GPAT1 mRNA expression level followed by a plateauing of this variable with increased hepatic scar formation.

Immunofluorescent staining of livers from the sham and FFD cohorts showed increased and intense GPAT1 staining in the modified diet group (Figure 4).

Figure 1. NAFLD. Representative images (25×) of H–E-stained liver sections from mice randomized to standard diet (A, sham), FFD (B), FFD + TAA (C), or FFD + CCL4 + glucose (D). The white arrows show lipid accumulation in the livers, the green arrows show a ballooned hepatocyte, and the orange arrows show the areas of inflammation. (E) NAS was elevated in the modified diet cohorts compared to the sham cohort. *, \( p \ll 0.01 \) vs sham.
3. DISCUSSION

Data from the present study demonstrate that the hepatic GPAT1 expression level is elevated in three etiologically distinct models of NAFLD. The increased hepatic GPAT1 expression level was observed in steatotic livers without scarring and also in livers with steatosis, inflammation, and scarring. There was a direct and significant association between hepatic GPAT1 expression and disease severity or NAS. Hepatic GPAT1 expression increased steeply and then plateaued with increasing scar formation. These data suggest that hepatic GPAT1 is an early and model-agnostic node in NAFLD.

In the United States alone, it is estimated that 60−80 million people have some fat accumulation in the liver, 10−15 million people have NASH, and 2−5 million people have NASH with fibrosis. Left untreated, the NAFLD continuum culminates in cirrhosis and increased risk for HCC. In fact, emerging experimental and clinical evidence indicates that NASH with or without fibrosis can lead to HCC, a phenomenon termed noncirrhotic HCC.8−9 It is therefore abundantly clear that for NAFLD, an area of tremendous unmet medical need, effective treatment is urgently required to prevent its transition to liver scarring and HCC. To date, no drug has proven sufficiently effective in reversing or even halting NASH. Clinical trials10−15 have been punctuated with failures, marginal success accompanied by significant side effects, or merely limited success. The weak translational data may be explained, at least in part, due to the targeting of disease-associated pathways restricted to a specific model of liver pathology. Furthermore, the majority of the drug candidates are evaluated against endpoints including NAS and fibrosis. Although laudable, these very endpoints may be a function of different mechanisms based on disease etiology or a subject’s genetics.16,17 A drug

![Figure 2. Liver fibrosis. Representative images (25X) of Picrosirius red-stained liver sections from mice randomized to standard diet (A), FFD (B), FFD + TAA (C), or FFD + CCL4 + glucose (D). The white arrows show bridging fibrosis. (E) Quantitation of Picrosirius red staining shows elevated fibrosis in the FFD + TAA and FFD + CCL4 + glucose cohorts. #, p < 0.05 vs sham. *, p < 0.01 vs sham.](https://pubs.acs.org/doi/10.1021/acsomega.0c02350)
that effectively mitigates NAS and/or scarring secondary to a particular mechanism of action might be ineffective against NAS and/or scarring secondary to a different mechanism of action.

Hepatic steatosis occurs when lipid accumulation exceeds lipid disposal, i.e., the uptake of fatty acids and de novo lipogenesis exceed fatty acid oxidation and export. According to the multihit hypothesis of NASH,18 steatosis sensitizes the liver to additional hits, including oxidative stress, inflammation, and scar formation. Hepatic steatosis is also a risk factor HCC. Typically, HCC-resistant C57Bl/6 mice developed NASH and HCC when fed a high-fat diet.23,24 Hepatic steatosis induced by a choline-deficient diet plus methionine in the drinking water or induced by a choline-deficient 1-amino acid-defined diet was associated with HCC in 100% of mice within 1 year of beginning the diet.25 Together these data suggest that steatosis plays a role in triggering a sequelae of events that can culminate in liver scarring and HCC.

Acyl-CoA: GPAT is the committed step in the de novo synthesis of TAG and glycerophospholipids. GPAT esterifies fatty acids to glycerol-3-phosphate at the sn-1 position, forming lysophosphatidic acid.19−22 Three different isoenzymes of GPAT have been described, but GPAT1 activity accounts for 30−50% of total hepatic GPAT activity but is only 10% of total GPAT activity in other tissues. Recent studies in primary hepatocytes demonstrated that overexpression of GPAT1

Figure 3. Functional transcriptomic analysis. (A) Hepatic GPAT1 mRNA expression level in the different groups #, p < 0.05 vs sham; *, p ≪ 0.01 vs sham. (B) Hepatic GPAT1 mRNA expression exhibits a significant and direct correlation correlated with NAS and a logarithmic relation (C) with hepatic Picrosirius red staining.

Figure 4. GPAT1 expression in NAFLD. GPAT1 immunofluorescence (red) is virtually absent in the sham liver (A, representative, 40×) but intense in the FFD liver (B, representative, 40×). Hepatocyte nuclei (white arrow) immunofluorescence green.
Table 1. Primer Design. The Primer List of Validated Forward and Reverse Primers for Gene Targets of Interest*

| gene | forward | reverse |
|------|---------|---------|
| PPIA | GTGTTCTTCGACATCACG | AAGTTTTCTCTGCTGTCTTTG |
| Gpat1 | CAATGAAAGCGCACAAGGCG | AACACTGGTGGCAAACATGC |

"Primers were generated based on sequencing data and validated via qPCR for efficacy before sample analysis.

primarily directs exogenous fatty acids away from β-oxidation and toward TAG. Conversely, when GPAT1 is absent, mice are protected from high-fat diet-induced hepatic steatosis and insulin resistance, have an improved lipid profile, and are resistant to HCC. In mice, overexpressing GPAT1 in the liver caused hepatic steatosis and hepatic insulin resistance in the absence of obesity or high-fat feeding. Mice administered the GPAT inhibitor FSG67 exhibited decreased gene expression for lipogenic enzymes in the white adipose tissue and liver and decreased lipid accumulation in white adipose, brown adipose, and liver tissues without signs of toxicity. Nevertheless, the role of hepatic GPAT1 in the NAFLD continuum remained to be fully investigated.

We tested the hypothesis that hepatic GPAT1 expression is altered early across multiple, etiologically distinct models of NAFLD. Mice administered FFD alone exhibit the entire continuum of NAFLD culminating in HCC. A number of chemicals are known to induce and accelerate this program of liver fibrosis and cirrhosis in rodents. In many aspects, it mimics human chronic diseases associated with toxic damage. Hepatic biotransformation of CCl₄ relies on cytochrome (CYP) 2E1 and yields the trichloromethyl radical, which is involved in several free-radical reactions and lipid peroxidation processes that contribute to an acute phase reaction characterized by necrosis of centrilobular hepatocytes, the activation of Kupffer cells, and the induction of an inflammatory response. Like CCl₄, TAA requires metabolic activation to become toxic. This bioactivation process, which is catalyzed by CYP450 isoenzymes, results in the formation of TAA sulfur dioxide, responsible for the overall toxicity. The mechanisms underlying the induction of liver fibrosis through TAA sulfur dioxide are not fully understood, but may imply downregulation of enzymes involved in fatty acid β-oxidation, branched chain amino acids, and methionine breakdown and upregulation of proteins related to lipid peroxidation and oxidative stress. The livers from mice fed an FFD were characterized predominantly by macrovesicular steatosis. In mice fed an FFD and administered TAA, the livers were characterized by some steatosis but predominantly inflammation and scarring, i.e., NASH with fibrosis. The livers from FFD + CCl₄ + glucose comprised animals on FFD and administered CCl₄ (0.32 μg/g, IP × 1×/week) and 18.9 g/L d-glucose in the drinking water for 12 weeks.

4. METHODS

4.1. Animal Models. All in-life studies were conducted in adult male C57BL/6 mice (18–20 g, ~6 weeks old) after approval (#2019-014) from our Institutional Animal Care and Use Committee (IACUC). Food and drink were provided ad libitum.

Mice were randomized to 4 groups (n = 8 animals each). A sham cohort comprised animals on a standard rodent diet for 12 weeks. A fast-food diet (FFD) cohort comprised mice on rodent diet containing 40 kcal % fat, 20 kcal % fructose, and 2% cholesterol (D90100301, Research Diets, NJ) for 12 weeks. An FFD + thioacetamide (TAA) cohort was provided FFD and injected with TAA (100 mg/kg, intraperitoneal (IP) × 3/week) for 12 weeks. The FFD + CCl₄ + glucose cohort comprised animals on FFD and administered CCl₄ (0.32 μg/g, IP × 1×/week) and 18.9 g/L d-glucose in the drinking water for 12 weeks.

4.2. Histopathology. At sacrifice, a portion of the liver was fixed in formalin (10%) and stained with hematoxylin–eosin (H&E) and evaluated by a blinded observer and assigned a NAFLD activity score (NAS). This scoring system on the 0–8 scale (8 being most diseased) totals the individual component scores for steatosis (0–3), lobular inflammation (0–3), and hepatocyte ballooning (0–3). Picrosirius red-stained liver sections were semiquantified by a blinded observer for extracellular fibrillar collagen using Bioquant Image analysis. Several fields per liver were evaluated to ensure that data were representative of that liver.

4.3. GPAT1 Measurements. The GPAT1 expression level was measured by a two-step quantitative polymerase chain
reaction (qPCR). RNA was extracted from liver homogenates (stored at \(-80\, ^\circ C\)) using the Qiagen RNaseasy Mini Kit following the manufacturer’s protocol. cDNA was synthesized from extracted RNA using the ThermoFisher High-Capacity cDNA Reverse Transcription Kit and a BioRad S1000 Thermocycler following the manufacturer's protocol. The samples were diluted 1:5 with nuclease-free H2O and stored at \(-20\, ^\circ C\). SYBR-Green qPCR for gene targets was performed with ThermoFisher Power-Up SYBR-Green Master-Mix with the reference gene Peptidylprolyl isomerase A (PPIA) using gene-specific forward and reverse primers generated with sequencing data from NCBI and designed using Primer3Plus and NCBI primer-design tools (Table 1). The use of PPIA as a reference gene was based on the previous literature, suggesting that it is a more optimal reference gene than other common reference genes such as GAPDH. Designed primer sequences (Table 1) were produced using Oligo Sigma services (Millipore-Sigma; Massachusetts, U.S.). The primers were validated by qPCR with cDNA at four serial dilutions to test for the production of a standard curve with efficiency 0.90–1.10 and a consistent \( r > 0.9 \). qPCR was performed on a ThermoFisher Quant-Studio 3 Real-Time PCR system, each sample was diluted three-fold, and qPCR reaction was performed in triplicate for all tissue samples following Power-Up SYBR-Green manufacturer protocol for Fast qPCR for a total volume of 10 \( \mu L \).

Immunohistochemical analysis for GPAT1 was performed in murine livers from the sham and FFD cohort. The slides containing hepatic sections (blanks) were deparaffinized by a series of three xylene washes and rehydrated with washes of descending concentrations of ethanol (100, 100, 95, 70, and 50%) followed by two washes in \( dH_2O \). The slides were placed in a glass dish filled with 1X Citrate Antigen-Retrieval buffer (pH 6), put into a microwave, and run on high power until boiling. The slides were maintained at a sub-boiling temperature for 10 min and then cooled on a bench top at room temperature for 30 min. The slides were permeabilized twice with phosphate-buffered saline (PBS) with 0.3% Triton X-100 for 10 min each. Blocking was performed with Background Sniper reagent (BS966M, Biocare Medical Pacheco, CA) for 11 min. A rabbit anti-GPAT1 antibody (NBP1-76907, Novus Biologicals, Centennial, CO) was diluted with 1% bovine serum albumin (BSA) in PBS + 0.3% Triton X-100 to a final concentration of 20 \( \mu g/mL \) and applied to each slide. The slides were incubated overnight in a humidified chamber at 4 \( ^\circ C \). After overnight incubation, the slides were washed with PBS + 0.3% Triton X-100 twice and secondary antibody, a goat antirabbit immunoglobulin G antibody conjugated to Alexa Fluor 635 (A31576, Thermofisher Scientific, Waltham, MA), was diluted in PBS + 1% BSA and applied to each slide at a concentration of 20 \( \mu g/mL \). The slides were incubated with the secondary for 1 h in the dark. After incubation, the slides were rinsed three times with PBS. A Vector TrueVIEW Autofluorescence Quenching kit (SP-8400, VECTOR Laboratories, Burlingame, CA) was utilized following the manufacturer’s protocol to reduce background fluorescence of the slides. Before coverslip mounting with VECTASHIELD Vibrance Antifade Mounting Medium (Step 5 of the TrueVIEW quenching kit protocol), nuclear counterstaining was performed using BioTracker 488 Green Nuclear Dye (SCT120, Millipore-Sigma, Burlington, MA) following the manufacturer’s protocol. The slides were mounted and allowed to cure overnight. The slides were visualized with a Leica TCS SPE confocal system in the 488 (nuclear dye) and the 635 (GPAT1) channels.

4.4. Statistical Analysis. All data (NAS, scarring, mRNA) were recorded and organized on Microsoft Excel. NAS data were presented as mean scores in each group. Liver scarring (PSR) data were presented as fold change in relation to the sham cohort. mRNA levels were presented as fold change in relation to the sham cohort as well. A Student’s T-test (p-value) was used to compare data diseased cohorts to the sham cohort. Microsoft Excel was used for the NAS vs mRNA correlation and to calculate Pearson product moment (r) and the p-value.

### AUTHOR INFORMATION

##### Corresponding Author

Prakash Narayan — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States; orcid.org/0000-0002-7007-0245; Phone: 516 326 1200; Email: pnarayan@angion.com

##### Authors

Kimberly Liao — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Anthony J. Pellicano — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Kai Jiang — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Natalia Prakash — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Jingsong Li — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Shraddha Bhutkar — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Zhijian Hu — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Quaisar Ali — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Itzhak D. Goldberg — Department of Preclinical Research, Angion Biomedica Corp., Uniondale, New York 11553, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c02350

##### Author Contributions

K.L. and A.J.P. contributed equally to this work.

##### Author Contributions

K.L., A.J.P., K.J., N.P., S.B., and Z.H. performed the studies; Q.A. and L.D.G. oversaw the project; P.N. conceived the project, compiled the data, and oversaw the project.

##### Notes

The authors declare no competing financial interest.

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