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BRIEF COMMUNICATION

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Inhibition of lysophosphatidic acid receptor 6 upregulated by the choline-deficient L-amino acid-defined diet prevents hepatocarcinogenesis in mice

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Abstract

Hepatocellular carcinoma (HCC) is one of the most worrying tumors worldwide today, and its epidemiology is on the rise. Traditional pharmacological approaches have shown unfavorable results and exhibited many side effects. Hence, there is a need for new efficacious molecules with fewer side effects and improvements on traditional approaches. We previously showed that lysophosphatidic acid (LPA) supports hepatocarcinogenesis, and its effects are mainly mediated by LPA receptor 6 (LPAR6). We also reported that 9-xanthylacetic acid (XAA) acts as an antagonist of LPAR6 to inhibit the growth of HCC. Here, we report that LPAR6 is involved in the choline-deficient L-amino acid-defined (CDAA) diet-induced hepatocarcinogenesis in mice. Our data demonstrate that CDAA diet-induced metabolic imbalance stimulates LPAR6 expression in mice and that XAA counteracts diet-induced effects on hepatic lipid accumulation, fibrosis, inflammation, and HCC development. These conclusions are corroborated by results on LPAR6 gain and loss-of-function in HCC cells.

KEYWORDS
hepatic metabolism, hepatocellular carcinoma, LPAR6, lysophosphatidic acid, NAFLD, NASH

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the sixth-leading cause of cancer-related death worldwide and is projected to become the third-leading cause in Western countries by 2030, despite a decline in the incidence of chronic hepatitis infection.[1] This tendency can be explained by the increased occurrence of dysmetabolic syndromes. Recent evidence highlights a positive correlation between metabolic syndrome, diabetes, obesity, and HCC.[2] The pharmacological management of HCC is mainly based on tyrosine kinase inhibitors (i.e., sorafenib, regorafenib, and lenvatinib[2]), often in combination with immunotherapeutic

Abbreviations: ALT, alanine aminotransferase; CAF, carcinoma-associated fibroblasts; CDAA, choline-deficient L-amino acid-defined; CSAA, choline-sufficient amino acid-defined; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HOMA-IR, homeostatic model assessment insulin resistance; LPA, lysophosphatidic acid; LPAR6, lysophosphatidic acid receptor 6; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OA, oleic acid; OCR, oxygen consumption rate; ORO, Oil Red O; PBS, phosphate-buffered saline; PTFs, peritumoral tissue fibroblasts; q-RT-PCR, quantitative real-time PCR; SBB, Sudan Black B; TAG, triglycerides; TGFα, transforming growth factor alpha; XAA, 9-xanthylacetic acid.

drugs.[3] However, this approach has many adverse effects and cannot be tolerated by patients for long periods of time.[4,5] Hence, the need for efficacious and better-tolerated therapeutic options for HCC. The autotaxin-lysophosphatidic acid (ATX-LPA) axis signaling pathway is important for the development and progression of HCC.[6,7] We previously reported that LPA triggers the transdifferentiation of peritumoral tissue fibroblasts (PTFs) in carcinoma-associated fibroblasts (CAF)[8] and that LPA receptor 6 (LPAR6) expression promotes the tumorigenicity of HCC and exacerbates the clinical outcomes of patients.[9] We later demonstrated that 9-xanthenylacetic acid (XAA) acts as an LPAR6 antagonist and inhibits HCC growth without toxicity.[10] Here, we aimed to extend the knowledge about the role of LPAR6 in driving hepatocarcinogenesis as well as the action of XAA as a promising pharmacologic agent. For this study, we fed C57BL/6J male mice with a choline-deficient L-amino acid-defined (CDAA) diet, which is known to lead to an increase in body weight, plasma triglyceride (TAG), and total cholesterol levels as well as homeostatic model assessment insulin resistance (HOMA-IR), indicating insulin resistance development. Also, CDAA diet-fed mice develop a severe degree of nonalcoholic steatohepatitis (NASH), with an increase in ALT levels and fibrosis after 20–22 weeks.[11] Our results demonstrated that LPAR6 expression is significantly upregulated after 9 months of a CDAA diet regimen. This is paralleled by increased hepatic TAG content, collagen deposition, liver inflammation, and HCC development. XAA significantly prevents these pathogenic patterns, thus providing a valuable tool in the management of HCC and predisposing dysmetabolic and inflammatory conditions.

2 | METHODS

2.1 | Chemicals

The selective LPAR6 antagonist XAA was synthesized and characterized as previously reported.[10,12]

2.2 | Animal models and in vivo procedures

C57BL/6J male mice were purchased from Charles River Laboratories. Animals were housed in a pathogen-free animal facility and all the experiments were conducted under the National and International Guidelines for the Care and Use of Laboratory and were approved by the local Institutional Animal Care and Use Committee. Mice aged 6–8 weeks were divided into two groups, one fed a choline-sufficient amino acid-defined control diet (CSAA diet, $n = 22$) and the other group fed a choline-deficient amino acid-defined diet (CDAA diet, $n = 24$). The CDAA diet is a well-described feeding regimen known to lead to hepatic steatosis, metabolic abnormalities, fibrosis, and the development of HCC. Mice under CDAA dietary regimens were then treated with XAA (CDAA diet + XAA, $n = 16$) for the indicated times. XAA was administered at a dose of 5 mg/kg body weight by intraperitoneal injection twice weekly, starting at the same time as the dietary intervention. Mice were killed after 3, 6, 9, and 12 months, and samples were processed for histopathological, serological, and molecular analyses. After 9 months, the following parameters were assessed: hepatic triglyceride (TAG) content, collagen α1 mRNA expression, F4/80-positive liver parenchyma, and HCC development. Moreover, mice's body weight and serum transforming growth factor-alpha (TGFα) were measured every 3 months, until 12 months. At sacrifice, the development of HCC was assessed by histochemical analysis, hematoxylin and eosin staining (H&E). After mice were euthanized, necropsies were performed, and snap-frozen liver tumor samples were collected and sectioned at 20 mm for RNA extraction.

2.3 | Histochemical and immunohistochemical analyses

Histochemical analysis to detect HCC lesions was performed by H&E staining Immunohistochemical analysis for detection of LPAR6 expression ([Santa Cruz cat. #sc-20126) was performed by deparaffinizing and rehydrating sections, followed by endogenous peroxidase inactivation by citrate. Staining was obtained by diaminobenzidine (DAB) as previously described.[13]

2.4 | Hepatic TAG content

Hepatic TAG content was evaluated by using a commercial assay kit (Sigma-Aldrich, cat. #TR0100), after homogenization of liver tissue sample in a 1:2 (v/v) chloroform/methanol mixture, according to the Bligh and Dyer method.[14]

2.5 | Transmission electronic microscopy (TEM) pictures

Samples for TEM pictures were processed as previously described.[15]

2.6 | F40/80 positive hepatic parenchyma

After a neutral buffered formalin tissue fixation and an antigen unmasking procedure, F4/80 positive cells were detected by an anti-F4/80 antibody—Macrophage Marker (abcam cat.#ab6640).

2.7 | Measurement of serum TGFα

Determination of serum TGFα was performed by using a commercial ELISA kit (Cusabio cat.# CSB-E07290m).

2.8 | Cell culturing

The HepG2 cell line was purchased from JCRB Cell Bank. Knocked-down LPAR6 HepG2 cells (HepG2 LPAR6 shRNA) were obtained in our laboratory by using a lentiviral-based shRNA technology, following the procedure we used in the Huh7 cell line.[9] Additional details are reported in Supporting Information Material.

2.9 | Quantitative real-time polymerase chain reaction (q-RT-PCR)

This method is described in Supporting Information Material.

2.10 | Oil Red O (ORO) staining and Sudan Black B (SBB) staining

These methods are described in Supporting Information Material.

2.11 | Cell growth assays

This method is described in Supporting Information Material.

2.12 | Cell cycle analysis

Cell cycle analysis was performed as previously described.[16] Additional details are reported in Supporting Information Material.

2.13 | Immunoblotting analyses

Immunoblotting analyses were performed as previously described.[17] Additional details are reported in Supporting Information Material.

2.14 | Measurement of oxygen consumption rate (OCR)

OCR was measured as previously described.[18] Additional details are reported in the Supporting Information Material.

2.15 | Statistical analysis

Statistical analysis is reported in the Supporting Information Material.

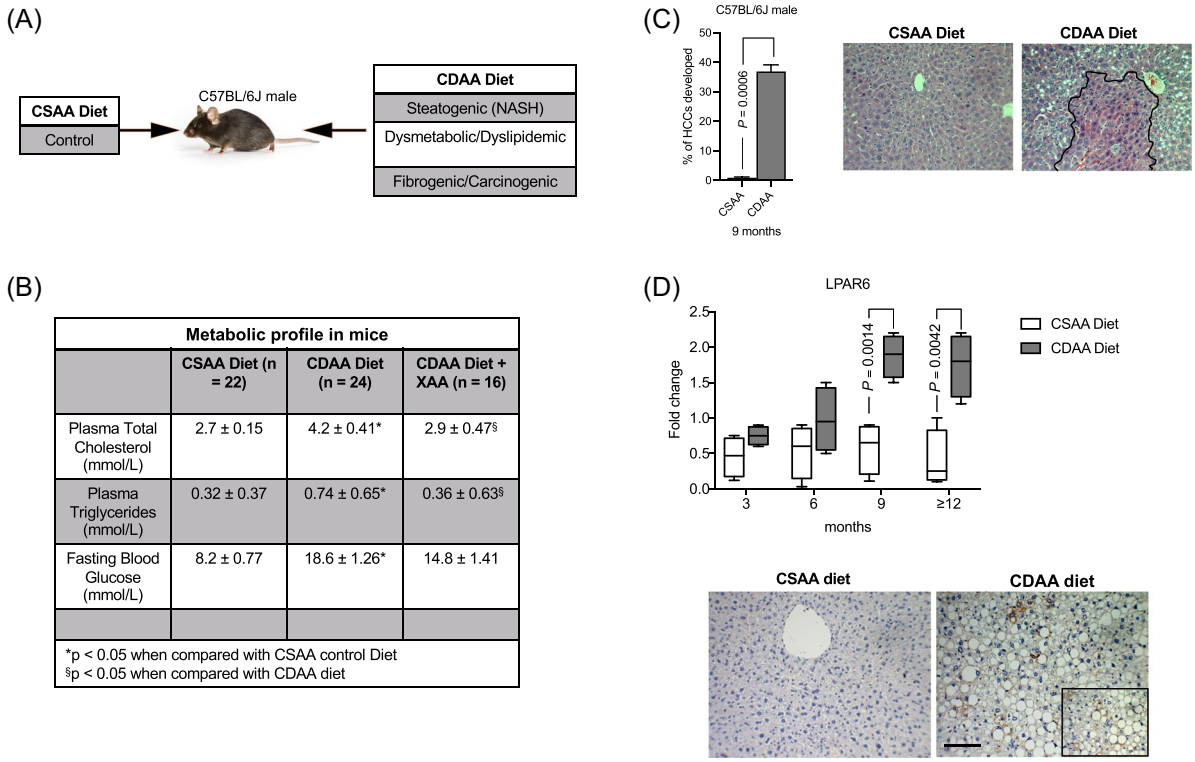
3 | RESULTS

3.1 | CDAA diet increases LPAR6 expression in mice

In this study, we employed C57BL/6J male mice fed with a CDAA diet (Figure 1A), which leads to a NASH-like liver, characterized by steatosis, fibrosis, weight gain, and dysmetabolic characteristics, such as increased plasma TAG and total cholesterol levels, as well as insulin resistance development.[11] As a first step, we assessed the effect of two different dietary regimens on the metabolic profile of the mice used in the study. Results confirm that the CDAA diet modifies mice's metabolic profile, with a significant increase in plasma cholesterol and TAG level and fasting blood glucose (Figure 1B). Interestingly, XAA treatment reduced plasma cholesterol and TAG levels (Figure 1B). CDAA diet-induced tumor lesions were demonstrated by histochemical analysis (H&E staining) (Figure 1C). We next analyzed the effect of the CDAA diet on LPAR6 expression. Results show that the CDAA diet upregulated the expression of LPAR6 in the liver of the mice after 3 months. The increase reached statistical significance after 9 months and remained significant after 12 months (Figure 1D—upper panel). These results were strengthened by immunohistochemical analysis (Figure 1D—lower panel).

3.2 | Inhibition of LPAR6 reduces CDAA diet-induced HCC development and improves nonalcoholic fatty liver disease (NAFLD)-related parameters

Next, we sought to investigate if LPAR6 inhibition by XAA reduced HCC development and ameliorated key parameters involved in NAFLD, namely hepatic TAGs accumulation, fibrosis, inflammation, and body weight. We found that the CDAA diet increased liver TAG, collagen α1 content, F4/80-positive liver parenchyma, and the development of HCC. Inhibition of LPAR6 by XAA significantly decreased hepatic TAG accumulation (Figure 2A), collagen α1 content (Figure 2B), F4/80-positive liver parenchyma (Figure 2C), and HCC development (Figure 2D). In addition, XAA reduced the body weight of mice after 9 months of treatment; the effect was still significant after 12 months (Figure 2E). Interestingly, XAA decreased serum TGFα, a trend that was already seen after 6 months of treatment, and achieved statistical significance after 9 months (Figure 2F). Overall, our data indicate that LPAR6 expression increases in response to diet-induced metabolic insults in mice. Moreover, LPAR6 upregulation contributes to supporting a pro-HCC environment, by increasing steatosis, fibrosis, and inflammation. A graphical sketch of the data reported above is shown in Figure 2G.



(B)

Metabolic profile in mice			
	CSAA Diet (n = 22)	CDAA Diet (n = 24)	CDAA Diet + XAA (n = 16)
Plasma Total Cholesterol (mmol/L)	2.7 ± 0.15	4.2 ± 0.41*	2.9 ± 0.47§
Plasma Triglycerides (mmol/L)	0.32 ± 0.37	0.74 ± 0.65*	0.36 ± 0.63§
Fasting Blood Glucose (mmol/L)	8.2 ± 0.77	18.6 ± 1.26*	14.8 ± 1.41

*p < 0.05 when compared with CSAA control Diet
§p < 0.05 when compared with CDAA diet

FIGURE 1 (A) Graphical summary of the dietary regimens employed in the study. (B) Table summarizing the metabolic lipid profiles of mice fed with two different dietary regimens (choline-sufficient amino acid-defined [CSAA] and choline-deficient l-amino acid-defined [CDAA]) used in the study, and that of CDAA diet-fed mice under 9-xanthylacetic acid (XAA treatment). (C) Quantitative and histochemical analysis (hematoxylin and eosin staining [H&E]) of tumor lesions in CDAA-fed mice versus CSAA-fed mice at 9 months (20×). (D) LPAR6 expression in livers of CSAA- and CDAA-fed mice (upper panel) and immunohistochemical analysis showing LPAR6 expression (lower panel) at month 9. The DAB brown staining indicates LPAR6 expression in steatotic hepatocytes (20× and inset 40×). Scale bar = 100 μm. [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Inhibition of LPAR6 reduces oleic acid (OA)-induced fat accumulation and cell growth in vitro

To confirm our findings in mice, we used an LPAR6-knockdown HepG2 cell line (HepG2 LPAR6 shRNA) obtained in our laboratory using shRNA technology. We treated cells with OA to obtain intracellular lipid accumulation, which was then assessed by two different lipid staining methods, ORO and SBB. Our results showed that OA-induced lipid accumulation was significantly reduced in LPAR6-knockdown HepG2 compared with control cells (Supporting Information: Figures S1 and S2). Furthermore, XAA decreased OA-induced lipid accumulation in parental HepG2 cells (Supporting Information: Figure S3). We also found that XAA significantly reduced OA-stimulated cell proliferation in parental HepG2 cells (Supporting Information: Figure S4A). These data were confirmed by cell cycle assessment (Supporting Information: Figure S4B) and gene expression analysis (Supporting Information: Figure S4C).

Interestingly, we observed downregulated expression and activity of the key lipogenic enzyme acetyl-CoA carboxylase (ACC) in HepG2 LPAR6 shRNA (Supporting Information: Figure S5A,B). Furthermore, XAA determined an increase in mitochondrial respiration, as observed by measuring the OCR (Supporting Information: Figure S6A,B), thus reverting the reduced mitochondrial respiration associated with the progression of fatty liver disease.[19,20] This provides important mechanistic insights into how LPAR6 is involved in NAFLD and ultimately in HCC development (Supporting Information: Figure S7).

4 | DISCUSSION AND CONCLUSIONS

HCC is today one of the most concerning tumors worldwide, and its pathogenesis is closely related to dysmetabolic conditions. NASH/NAFLD is considered a predisposing condition to HCC development. However, the link between NASH/NAFLD and the onset of HCC is largely unknown, and preventive and therapeutic options are likewise not available. We previously reported the implication of lysophosphatidic acid (LPA) in hepatocarcinogenesis and found that LPAR6 mostly conveys the LPA effect. We also identified a novel LPAR6 antagonist, XAA, which effectively blocks the growth of HCC. Here, we sought to further characterize the function of LPAR6 in hepatocarcinogenesis, as well as the role of XAA. Indeed, we found that LPAR6 expression in mice was significantly upregulated by CDAA diet-induced metabolic imbalance, which was paralleled by an

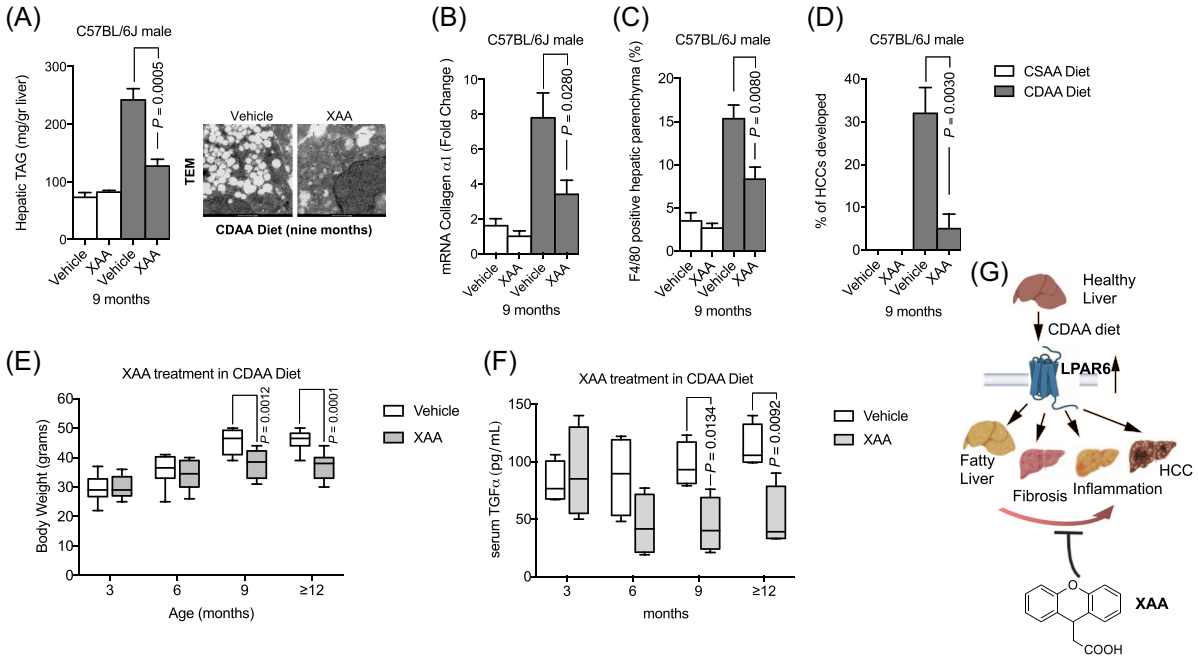


FIGURE 2 LPAR6 inhibition counteracts choline-deficient L-amino acid-defined (CDAA) diet-induced nonalcoholic steatohepatitis (NASH)-like parameters and the development of hepatocellular carcinoma (HCC) in mice. (A) LPAR6 inhibitor 9-xanthylacetic acid (XAA) significantly reduced CDAA diet-induced hepatic triglycerides (TAG) accumulation. (B) LPAR6 inhibitor XAA significantly decreased CDAA diet-induced expression of collagen α1. (C) The LPAR6 inhibitor XAA significantly reduces CDAA diet-induced F4/80 positive hepatic parenchyma. (D) LPAR6 inhibitor XAA significantly counteracts CDAA diet-induced HCC development. (E, F) LPAR6 inhibitor XAA significantly reduced CDAA diet-induced weight gain and serum TGFα increase. (G) A schematic diagram outlining the main findings of this article. Illustrations were created by using biorender.com. [Color figure can be viewed at wileyonlinelibrary.com]

increase in hepatic lipid accumulation, fibrosis, inflammation, and HCC development. Treatment with XAA is effective in preventing all these effects. In vivo results are supported and strengthened by data on LPAR6 gain and loss-of-function in HCC cells.

Our findings provide primary evidence linking increased expression of LPAR6 to diet-induced metabolic stress that leads to the development of HCC. Indeed, we identified for the first time LPAR6 as a "sensor" of metabolic distress and as the "missing link" between diet and hepatocarcinogenesis. Furthermore, we propose inhibition of LPAR6 as a pharmacological tool that can be used for preventive purposes in association with lifestyle medicine. Taken together, our results suggest that diet-induced metabolic distress stimulates hepatic LPAR6 expression, which is associated with the acquisition of the NASH/NAFLD phenotype in mice, ultimately leading to the development of HCC. In conclusion, our data shed new light on the role of LPAR6 in hepatocarcinogenesis and suggest new options for HCC prevention.

AUTHOR CONTRIBUTIONS

Davide Gnocchi: Collection of data, elaboration of data, writing of the manuscript. **Marta B. Afonso:** Collection of data. **Maria Maddalena Cavalluzzi** and **Giovanni Lentini:** Providing reagents. **Giuseppe Ingravallo:** Collection of data. **Carlo Sabbà:** Reviewing and editing. **Cecilia M. P. Rodrigues:** Reviewing and editing; **Antonio Mazzocca:** Conception of the work, supervising the study, writing of the manuscript, reviewing, and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available upon request from the corresponding author.

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SUPPORTING INFORMATION

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

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