ORIGINAL RESEARCH

Intestinal gluconeogenesis prevents obesity-linked liver steatosis and non-alcoholic fatty liver disease

Justine Vily-Petit,[1,2] Maud Soty-Roca,[1,2] Marine Silva,[1,2] Margaux Raffin,[1,2] Amandine Gautier-Stein,[1,2] Fabienne Rajas,[1,2] Gilles Mithieux [1,2]

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[1]U1213 Nutrition, Diabetes and the Brain, Institut national de la santé et de la recherche médicale, Lyon, France
[2]U1213 Nutrition, Diabetes and the Brain, Université Lyon 1 Faculté de Médecine Lyon-Est, Lyon, France

Correspondence to
Dr Gilles Mithieux, U1213, Institut national de la santé et de la recherche médicale, Lyon 69372, France;
gilles.mithieux@inserm.fr

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ABSTRACT

Objective Hepatic steatosis accompanying obesity is a major health concern, since it may initiate non-alcoholic fatty liver disease (NAFLD) and associated complications like cirrhosis or cancer. Intestinal gluconeogenesis (IGN) is a recently described function that contributes to the metabolic benefits of specific macronutrients as protein or soluble fibre, *via* the initiation of a gut-brain nervous signal triggering brain-dependent regulations of peripheral metabolism. Here, we investigate the effects of IGN on liver metabolism, independently of its induction by the aforementioned macronutrients.

Design To study the specific effects of IGN on hepatic metabolism, we used two transgenic mouse lines: one is knocked down for and the other overexpresses glucose-6-phosphatase, the key enzyme of endogenous glucose production, specifically in the intestine.

Results We report that mice with a genetic overexpression of IGN are notably protected from the development of hepatic steatosis and the initiation of NAFLD on a hypercaloric diet. The protection relates to a diminution of de novo lipogenesis and lipid import, associated with benefits at the level of inflammation and fibrosis and linked to autonomous nervous system. Conversely, mice with genetic suppression of IGN spontaneously exhibit increased hepatic triglyceride storage associated with activated lipogenesis pathway, in the context of standard starch-enriched diet. The latter is corrected by portal glucose infusion mimicking IGN.

Conclusion We conclude that IGN per se has the capacity of preventing hepatic steatosis and its eventual evolution toward NAFLD.

Significance of this study

What is already known on this subject?

- The accumulation of lipid in the liver is a major complication of obesity, since it can lead to serious health problems as hepatic cirrhosis or even liver cancer.
- A recently described function, intestinal gluconeogenesis (IGN), positively regulates glucose and energy homoeostasis and exerts anti-diabetes and anti-obesity effects.
- Induced IGN minors body weight gain, reduces hepatic glucose production and improves insulin sensitivity and overall glucose control in the context of nutrition-based manipulations as protein-enriched or fibre-enriched diets or in response to gastric bypass surgery.
- These nutrition-induced metabolic benefits are substantially blunted in mice with an intestine-specific knockout of gluconeogenesis.

What are the new findings?

- We assess here the proper effect of IGN, that is, independently of any nutritional manipulation, in metabolic control and more specifically on hepatic steatosis, a question not previously addressed.
- Increased IGN improves glucose control and prevents the set up of hyperglycaemia under high-fat/high-sucrose (HF-HS) diet.
- Increased IGN markedly moderates hepatic steatosis and its eventual evolution toward non-alcoholic fatty liver disease (NAFLD) under HF-HS diet.
- IGN preventive actions are associated with increased innervation of the liver by tyrosine hydroxylase-expressing neurons.
- Conversely, the suppression of IGN promotes the accumulation of lipid in the liver even in the context of standard starch diet.

INTRODUCTION

The last decades have seen an alarming increase worldwide in the prevalence of obesity, which is now qualified of epidemic by the WHO.[1] This metabolic state is associated with many complications including, type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD).[2 3] Moreover, it is an important risk factor for many serious health problems as retinopathy, nephropathy, neuropathy, cardiovascular diseases and various cancer types.[2 4 5]

The different metabolic complications of obesity or T2D can occur sequentially or simultaneously depending on the patient. During obesity development, food excess can lead to ectopic fat storage in the liver, which causes a defect in hepatic insulin sensitivity that can lead to the development of T2D.[6] In parallel, even in the insulin resistance state featuring T2D, the stimulation of lipogenesis remains preserved in the liver, which permits the maintaining of insulin-dependent lipid storage.[7] Therefore, the set-up of T2D can also promote lipid accumulation in the liver.[3]

On the ground of hepatic steatosis, a continuum of liver abnormalities, the so-called NAFLD, may take place. The liver can remain fatty without disturbed cellular function or progressively evolve

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Significance of this study

How might it impact on clinical practice in the foreseeable future?

- In humans, the positive metabolic outcomes of gastric bypass surgery have been associated with both increased IGN and correction of hepatic steatosis and NAFLD features.
- That IGN controls hepatic lipid metabolism pave the way to novel therapeutic approaches (prevention or correction) based on IGN (*via* its manipulation by nutrients or drugs) against this serious metabolic consequence of obesity that is hepatic steatosis and NAFLD.

by various mechanisms from lipid accumulation to non-alcoholic steatohepatitis (NASH), a state in which steatosis may then combine with inflammation and/or fibrosis. In some cases, further worsening can lead to cirrhosis and even liver cancer (for review see reference 8). Furthermore, it is more and more frequent to observe the development of liver cancers in the context of steatosis without fibrosis or cirrhosis.[9–11] So, there is currently a huge interest to decipher the mechanisms underlying the development of hepatic steatosis and NAFLD and to identify any means to prevent it.

In this context, it is noteworthy that a recently described function, intestinal gluconeogenesis (IGN), positively interferes with the control of glucose and energy homoeostasis to exert anti-diabetes and anti-obesity effects. Glucose released in the portal vein is sensed by a glucose receptor present in the neural system (sodium-glucose co-transporter 3, SGLT3), which initiates a nervous signal to the hypothalamus.[12–14] The induction of IGN minors body weight gain, reduces hepatic glucose production and improves insulin sensitivity and overall glucose control.[14–17] This was previously observed in response to various nutritional manipulations such as on protein-enriched or fiber-enriched diets, or after gastric bypass surgery of obesity.[15–20] While the nutrient-induced benefits were substantially blunted in mice with an intestinal-specific knockout of the glucose-6 phosphatase (G6Pase) catalytic subunit (G6PC), this did not allow us to unequivocally evaluate the role of IGN per se in these benefits.[18 21] To assess the proper effect of IGN, that is, independently of nutritional manipulation, in metabolic control has been a major preoccupation in our ongoing works.

In particular, the eventual interference of IGN with the development of hepatic steatosis and eventually NAFLD induced by a deleterious diet remained an outstanding question, not addressed in our previous works. Therefore, we combined here studies using mice with an intestine-targeted knockout of IGN and mice with an intestine-targeted overexpression of IGN to document this important question.

METHODS

Animals and diets

We used two murine models related to IGN: a knockout model (I.G6pc$^{-/-}$ mice), previously described,[22] and a novel model of activated IGN (I.G6pc^{overexp} mice), based on the overexpression of G6PC, that is, the catalytic subunit of G6Pase, the crucial enzyme of gluconeogenesis, specifically in the intestine (see below). Wild-type C57Bl6/J (WT, Charles River Laboratories, France) mice were housed in parallel and used as control mice. All the mice were housed in the animal facility of Lyon 1 University ('Animalerie Lyon Est Conventionnelle and Specific Pathogen Free') under controlled temperature (22°C) conditions, with a 12 hours light/dark cycle, and *ad libitum* access to water and food. I.G6pc$^{-/-}$ mice were fed a standard chow diet and I.G6pc^{overexp} mice were fed a high-fat/high-sucrose diet (HF-HS) (see online supplementary methods for diet composition).

All procedures were performed in accordance with the principles and guidelines established by the European Convention for the protection of Laboratory Animals. The regional animal care committee (CEEA-55, University Lyon I, France) approved all the experiments herein.

Glucose/insulin tolerance tests

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed, respectively, in 16 hour-fasted and 6 hour-fasted in I.G6pc^{overexp} and WT mice after 3 months on HF-HS diet. After intraperitoneal injection of glucose (0.75 g/kg bw (body weight)) or insulin (0.75 U/kg bw), blood glucose was monitored for 120 or 150 min using a glucometer from blood samples collected from the tail vein.

Portal glucose infusion

A catheter was inserted into the portal vein of I.G6pc$^{-/-}$ mice under isoflurane anaesthesia (2%). After 7 days, a 0.9% saline or 20% glucose was infused at 40 μmol/kg/min for 24 hours in conscious mice having free access to water and food. Then, they were killed, and the livers were collected and frozen by freeze-clamping at the temperature of liquid nitrogen and stored at −80°C (see online supplementary methods for more information).

Plasma parameters

Blood was withdrawn from the submandibular vein and collected in ethylene diamine tetraacetic acid (EDTA) and conserved at −20°C. Plasma triglycerides (TG), cholesterol and non-esterified fatty acids (NEFA) concentrations were determined with colorimetric kits. Plasma insulin and glucagon concentrations were quantified using Mouse ELISA kits.

Histological and immunofluorescence analysis

For livers histological analyses, formalin-fixed and paraffin-embedded tissues were cut in 4 µm thick sections and stained with haematoxylin and eosin staining or Masson's Trichrome staining. The sections were examined under an inverted microscope.

For livers tyrosine hydroxylase (TH) labelling, mice were perfused transcardially with ice-cold 4% paraformaldehyde, and the livers were removed in cryopreservation solution. Then, livers were cut into 40 µm thick section, using a cryostat. Immunostaining with TH primary antibody and Alexa 546 goat anti-rabbit IgG conjugated secondary antibodies was performed. Cell nuclei were stained with Hoechst. Confocal images of randomly selected portal areas of the liver (three to five per liver) were acquired on a confocal laser scanning microscope. For each image, we quantified TH labelling area in relation to the total area (see online supplementary methods for more information).

Glycogen assay

Hepatic glycogen was extracted by the Keppler and Decker method, as previously described.[23] Briefly, glycogen was hydrolysed, and the glucose released was measured by colorimetric assay (see online supplementary methods for more information).

Triglyceride assay

Liver triglycerides were extracted using Folch extraction procedure, as previously described.[24] Triglycerides were measured

with a colorimetric kit (see online supplementary methods for more information).

Western blot analysis

Cell extracts from livers were lysed, and aliquots of 30 µg proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride (PVDF) Immobilon membranes. After saturation, the membranes were probed with primary antibodies and then with goat secondary anti-rabbit IgG linked to peroxidase. The intensity of the spots was determined by densitometry (see online supplementary methods for more information).

Gene expression analysis

Cell extracts from livers were lysed and total RNAs were isolated according to the Trizol protocol. A reverse transcription and real-time qPCRs were performed using sequence-specific primers (see online supplementary methods for more information).

Glucose-6-phosphatase activity analysis

The intestine, liver and kidney were sampled as previously described.[25] G6Pase activity was assayed in homogenates in the presence of a saturating glucose-6-phosphate concentration[25 26] (see online supplementary methods for more information).

Statistical analysis

All data are presented as mean±SEM. Two-group comparisons were analysed using unpaired t-test, and multiple comparisons were analysed using two-way analysis of variance followed by a Bonferroni post-hoc test. Values were considered significant at *p<0.05 and **p<0.01. Statistical details and exact value of 'n' can be found in the figure legends. Statistical analyses were performed with GraphPad Prism 6 software.

RESULTS

The induction of intestinal gluconeogenesis prevents the development of obesity and the deterioration of glucose control in mice fed a high-fat/high-sucrose diet

To assess whether IGN per se could prevent the development of obesity and its associated complications, that is, independently of its activation on specific nutritional manipulations (see above), we generated a murine model of intestine-targeted G6Pase overexpression, based on the insertion of the complementaryDNA (cDNA) of its catalytic subunit (*G6pc*) in the Rosa26 locus. The latter is capable of exhibiting strong transcriptional activity, but is blocked here by a stop cassette of the *Rosa26* transcription initiation flanked with lox sequences (figure 1A). The excision of lox sequences to remove the stop cassette was made possible by crossing these mice with mice expressing the CRE recombinase under the control of the Villin promoter, allowing us to generate mice overexpressing *G6pc* in the intestine (I.G6pc^{overexp}). These mice were backcrossed on a C57Bl6/J genetic background. Western blot analysis revealed that the G6PC protein was four-fold-overexpressed in the intestine (proximal part of the jejunum), while no change was observed in the liver and kidneys (figure 1B). Moreover, the enzymatic activity of G6Pase was approximately three-fold-increased in the intestine, while it was unchanged in the liver and kidneys (figure 1C). This demonstrated the specificity of the overexpression of *G6pc* in the intestine in this new murine model.

Then, I.G6pc^{overexp} and WT mice were fed a HF-HS diet for 12 weeks. It is noteworthy that, despite food intake was comparable in I.G6pc^{overexp} and WT mice, the increase in body weight was markedly attenuated in I.G6pc^{overexp} compared with WT mice (figure 1D,E). Furthermore, mice overexpressing G6Pase in the intestine showed a better glucose tolerance (figure 2A,C). This might be explained by an improvement in insulin tolerance in I.G6pc^{overexp} compared with WT mice (figure 2B,D). Consistently, the plasma insulin concentration of I.G6pc^{overexp} mice 6 hours after food removal was lower than that of WT mice (figure 2E), which was associated with a greater basal phosphorylation level of AKT in the mice (figure 2F). These data were in agreement with a higher insulin sensitivity of I.G6pc^{overexp} compared with WT mice. It is of note that plasma glucagon concentration was not modified in I.G6pc^{overexp} compared with WT mice (figure 2E). Finally, we measured blood glucose after overnight fasting along the 3 months preceding the glucose and insulin tolerance tests. Overnight-fasted plasma glucose was progressively increased in both WT and I.G6pc^{overexp} during the two first months on HF-HS feeding (figure 2G). It is noteworthy that I.G6pc^{overexp} showed a clear moderation of the development of fasting hyperglycaemia compared with WT mice after 3 months under HF-HS diet (figure 2G). Together, these results suggest that an activation of IGN per se protects mice from the development of obesity and the deterioration of glucose control promoted by a HF-HS diet.

Intestinal gluconeogenesis prevents the development of hepatic steatosis promoted by a HF-HS diet

Then, we assessed whether IGN could interfere with the development of hepatic steatosis on prolonged deleterious feeding. After 5 months under HF-HS diet, compared with WT mice, I.G6pc^{overexp} mice exhibited a lower liver weight associated with a two-fold-decrease in hepatic triglyceride content, in absence of a change in hepatic glycogen content (figure 3A). No change was observed in plasma lipid and lipoprotein parameters (see online supplementary figure 1). To decipher the mechanisms leading to the attenuation of liver steatosis in I.G6pc^{overexp} mice, we studied de novo lipogenesis and β-oxidation pathways. The diminution in hepatic steatosis in I.G6pc^{overexp} mice was associated with a decrease in the expression of several genes involved in lipogenesis, including: *Fasn* (*fatty acid synthase*), *Acaca* (*acetyl-CoA carboxylase 1*) and the transcription factor *Srebpc1c* (*sterol regulatory element-binding protein*) (figure 3B). In addition I.G6pc^{overexp} mice showed a significant decrease in expression of *Cd36* (*cluster of differentiation 36*) in liver (figure 3C), suggesting a decrease in hepatic lipid uptake in addition to the decrease in lipogenesis. In keeping with the decreased hepatic triglyceride content, the histological analysis of the livers showed a dramatic diminution in the amount of lipid droplets in the liver of I.G6pc^{overexp} compared with WT mice (figure 3D). Unexpectedly, the messengerRNA (mRNA) expression level of carnitine-palmitoyltransferase-1, a key enzyme in intra-mitochondrial fatty acyl-CoA transport, and some proteins regulating fatty acid oxidation, or directly involved in this pathway, was also decreased in the liver of I.G6pc^{overexp} mice compared with WT mice (figure 3E). We previously reported that IGN mobilises key peripheral functions involved in energy homoeostasis via the mean of the autonomous nervous system after a hypothalamic relay.[27] We thus visualised liver TH-expressing neurons to evaluate the involvement of a neural pathway in the hepatic lipid-related events observed here. It is noteworthy that the number of TH-expressing neurons was increased 2.7 times in the liver of I.G6pc^{overexp} mice (figure 3F).

To sum up, the activation of IGN is associated with the development of local sympathetic innervation and prevents hepatic lipid accumulation promoted by a hypercaloric diet.

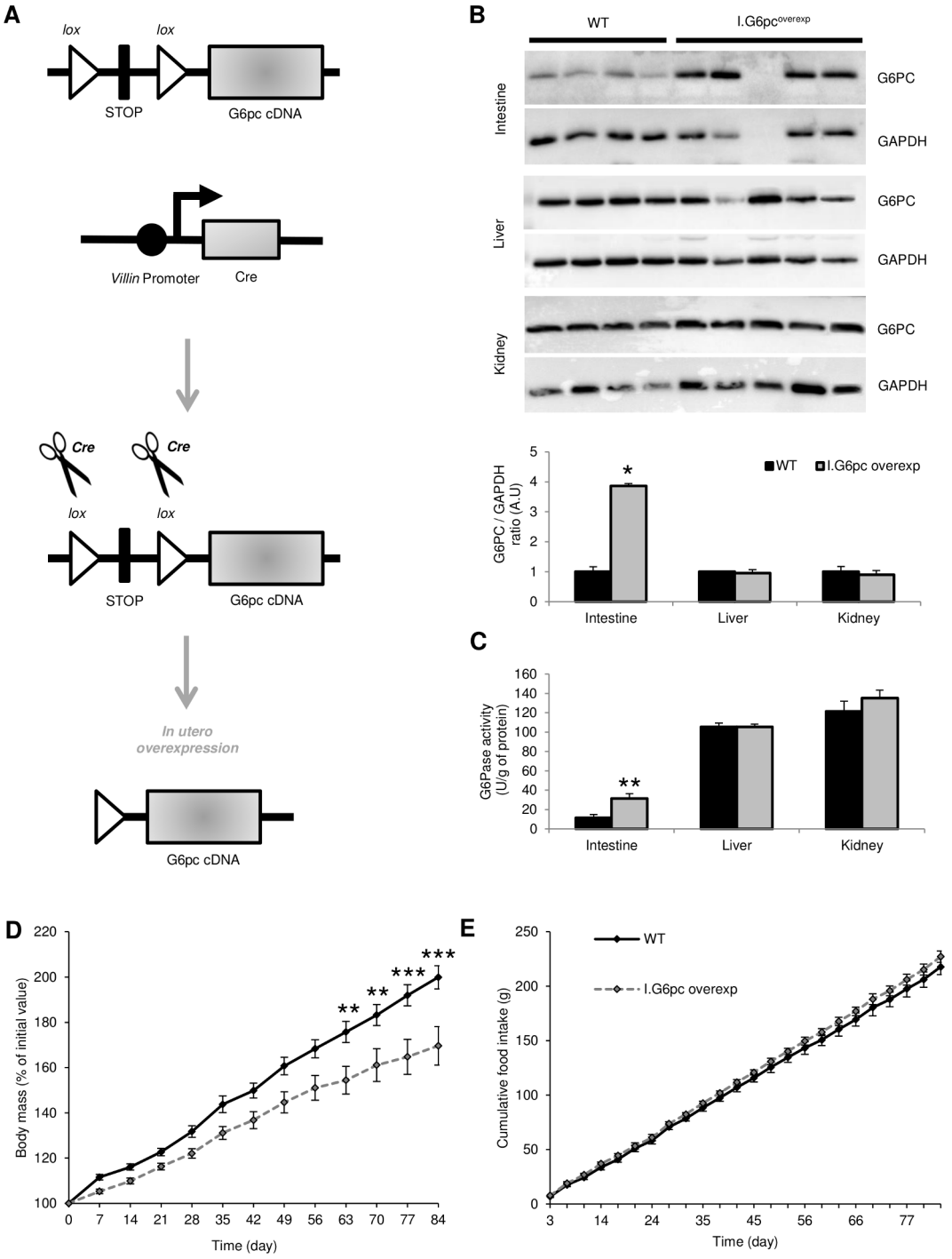

Figure 1 Murine model of constitutive induction of intestinal gluconeogenesis. (A) Schematic representation of obtaining mice overexpressing catalytic subunit of glucose-6-phosphatase (G6PC) specifically intestine according to CRE-Lox strategy. (B) Representative western blot analysis of catalytic subunit of G6PC expression in the three gluconeogenesis organs: the liver, kidney and intestine. The graph shows the quantification of protein expression in I.G6pc^{overexp} and WT mice (mean±SEM n=4–5). (C) Glucose-6-phosphatase (G6Pase) activity in the three gluconeogenic organs: the liver, kidney and intestine. The graph shows the G6Pase activity relative to protein concentration (mean±SEM n=4–5). (D) Weight gain expressed as a percentage of the initial weight (means±SEM, n=12–13). (E) Food intake (means±SEM, n=12–13). (B–C) Student's t-test was performed as a statistical analysis (D–E) two-way analysis of variance followed by a Bonferroni post-hoc test was performed as a statistical analysis. *p<0.05; **p<0.01; ***p<0.001 versus WT. cDNA,complementary DNA; WT, wild type.

Intestinal gluconeogenesis prevents the development of non-alcoholic fatty liver disease

In the context of obesity, NAFLD may develop and progress towards the installation of a pro-inflammatory state and then a pro-fibrotic state eventually preceding cirrhosis and liver cancer. We assessed whether the induction of IGN could be able to modulate the first stages of NAFLD progression to NASH in the context of a HF-HS diet.

It is noteworthy that I.G6pc^{overexp} mice showed a reduction in gene expression of several pro-inflammatory markers as

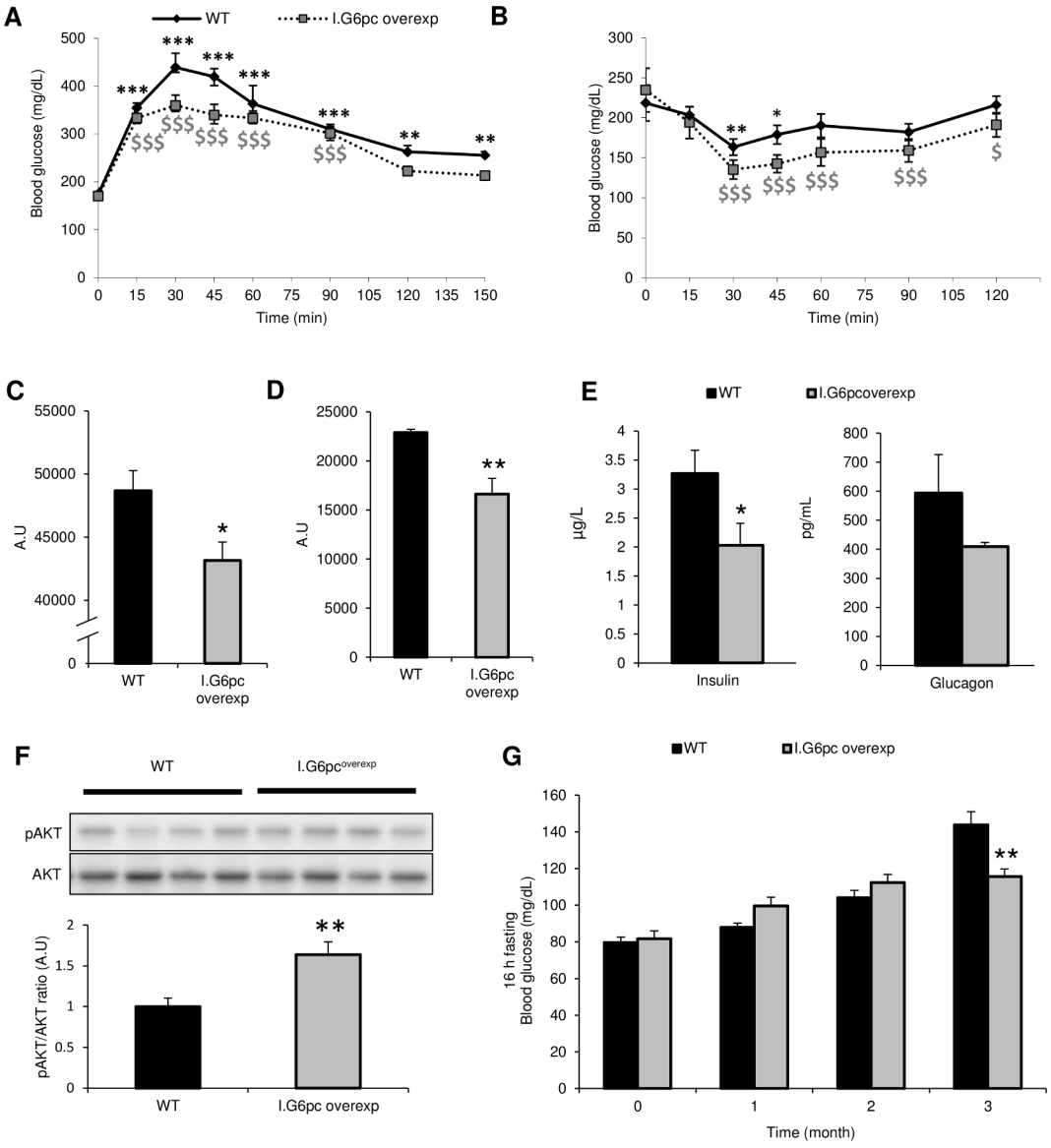

Figure 2 Activation of intestinal gluconeogenesis prevents development of diabetes on a high-fat/high-sucrose diet (A). Glucose tolerance test (means±SEM, n=4) in I.G6pc^{overexp} and WT mice (B) Insulin sensitivity test (means±SEM, n=7). (C) Area under the curve of glucose tolerance test (panel C). The results are expressed in arbitrary units (means±SEM, n=4). (D) Area under the curve of insulin tolerance test (panel D). The data are expressed as in panel E (means±SEM, n=7). (E) Plasma insulin and glucagon levels after 6 hour-fasting (means±SEM, n=4–6). (F) Phosphorylation state of AKT studied at 6 hours of fasting in the liver. The graph shows the quantification of protein expression (means±SEM, n=5–6). (G) Plasma glucose concentration after 16 hour-fasting throughout HF-HS diet (means±SEM, n=10–11). (A–B) We compared each point using two-way analysis of variance followed by a Bonferroni post-hoc test as a statistical analysis, with * represents WT mice and $ represents I.G6pc^{overexp} mice. (C–G) Student's t-test was performed as a statistical analysis. *p<0.05; **p<0.01; ***p<0.001 versus WT mice. WT,wild type.

monocyte chemoatractant protein 1 (*Mcp1*), *tumour necrosis factor* (*Tnfa*) and *interleukin 6* (*Il6*) compared with WT mice (figure 4A). However, the protein expression of these three pro-inflammatory markers remains unchanged (see online supplementary figure 2). In addition, the induction of IGN had an effect on pro-fibrosis processes induced by HF-HS diet. Indeed, a decrease in the expression of genes encoding for key fibrotic markers, like *vimentin* and *alpha-smooth muscle actin* (*Acta2*), was highlighted in the liver of I.G6pc^{overexp} mice compared with WT mice (figure 4B). Consistently, collagen fibre staining revealed the presence of fibrosis around the blood vessels that spread into the hepatic parenchyma of WT mice, whereas this was not viewable in the liver of I.G6pc^{overexp} mice (figure 4C). Finally, a decrease in the expression of collagen-type-1-α1 (Col1a1) and collagen-type-3-α1 (Col3a1)isoforms confirmed the beneficial effects of IGN on fibrosis processes that are promoted on HF-HS diet (figure 4D).

Taken together, these results may suggest that IGN may exert preventive effects against the eventual development of NASH induced by HF-HS diet.

Activation of intestinal gluconeogenesis modulates hepatic metabolism in the context of a standard diet

We then wished to know whether IGN could exert metabolic benefits only on a challenge with a hypercaloric diet or could also modulate hepatic metabolism in a standard nutritional condition. To this aim, 16 weeks old I.G6pc^{overexp} and WT mice

A

	Liver weight and triglyceride and glycogen content		
	Liver weight (%)	Hepatic triglyceride (mg /g)	Hepatic glycogen (mg /g)
WT	6.5 ± 0.4	227 ± 7	14.9 ± 2.5
I.G6pc^{overexp}	4.4 ± 0.5*	133 ± 11**	15.1 ± 3.5

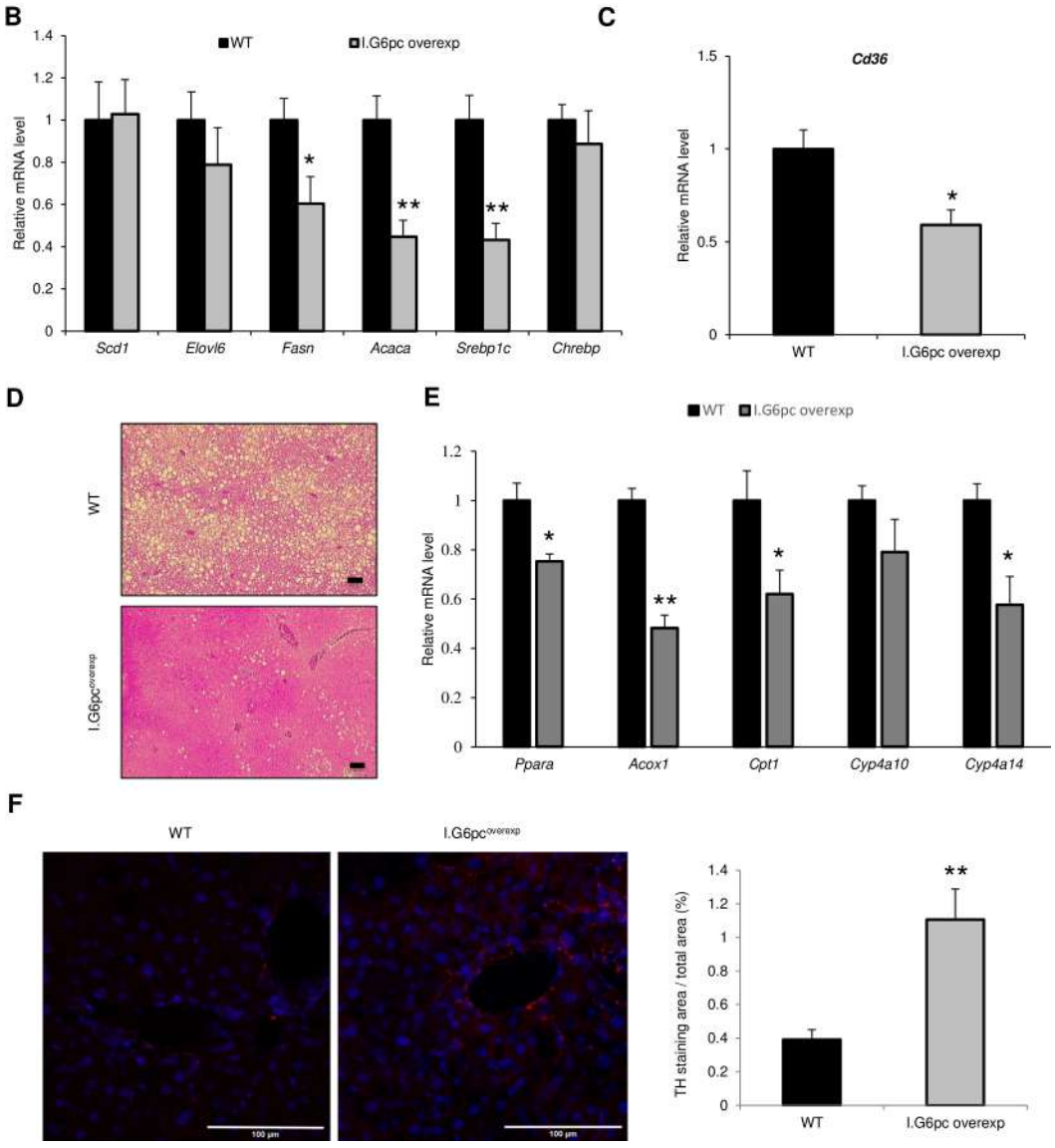

Figure 3 Activated intestinal gluconeogenesis prevents the development of non-alcoholic fatty liver disease on a high fat-high sucrose diet (A). Liver weight, triglyceride and glycogen content, and lipid plasma parameters of I.G6pc^{overexp} and WT mice. Liver triglyceride and glycogen content are expressed relative to liver weight (means±SEM, n=6). (B) mRNA levels of proteins involved in fatty acid synthesis (means±SEM, n=5–9). (C) mRNA levels of *Cd36* (means±SEM, n=5). (D) Haematoxylin and eosin staining of liver histological sections. Scale bar represents 100 µm. (E) mRNA levels of proteins involved in β-oxidation (means±SEM, n=8–12). (F) Representative pictures of tyrosine hydroxylase staining (in red) of liver histological sections and quantification (n=3–4; three to five pictures per mice). (A–C, E–F) Data are expressed relative to WT mice. Student's t-test was performed as a statistical analysis. *p<0.05; **p<0.01; versus WT. mRNA,messenger RNA; WT, wild type.

fed a standard diet were compared in relation with food intake, body weight gain and some metabolic parameters related to hepatic steatosis development. It is noteworthy that I.G6pc^{overexp} mice gained less weight than WT mice, despite comparable food intake (see online supplementary figure 3A,B). This was not associated with a change in hepatic triglyceride or glycogen content (see online supplementary figure 3C). However, a significant decrease in plasma triglyceride concentration was noted in I.G6pc^{overexp} compared with WT mice, without modification of the cholesterol and non-esterified fatty acids levels (see online supplementary figure 3C).

Then, we studied the expression of genes implicated in hepatic lipogenesis and β-oxidation. As in I.*G6pc*^{overexp} mice fed a HF-HS diet, we highlighted that the induction of IGN decreased the expression of the main genes involved in lipogenesis and β-oxidation pathways in the context of standard diet compared with

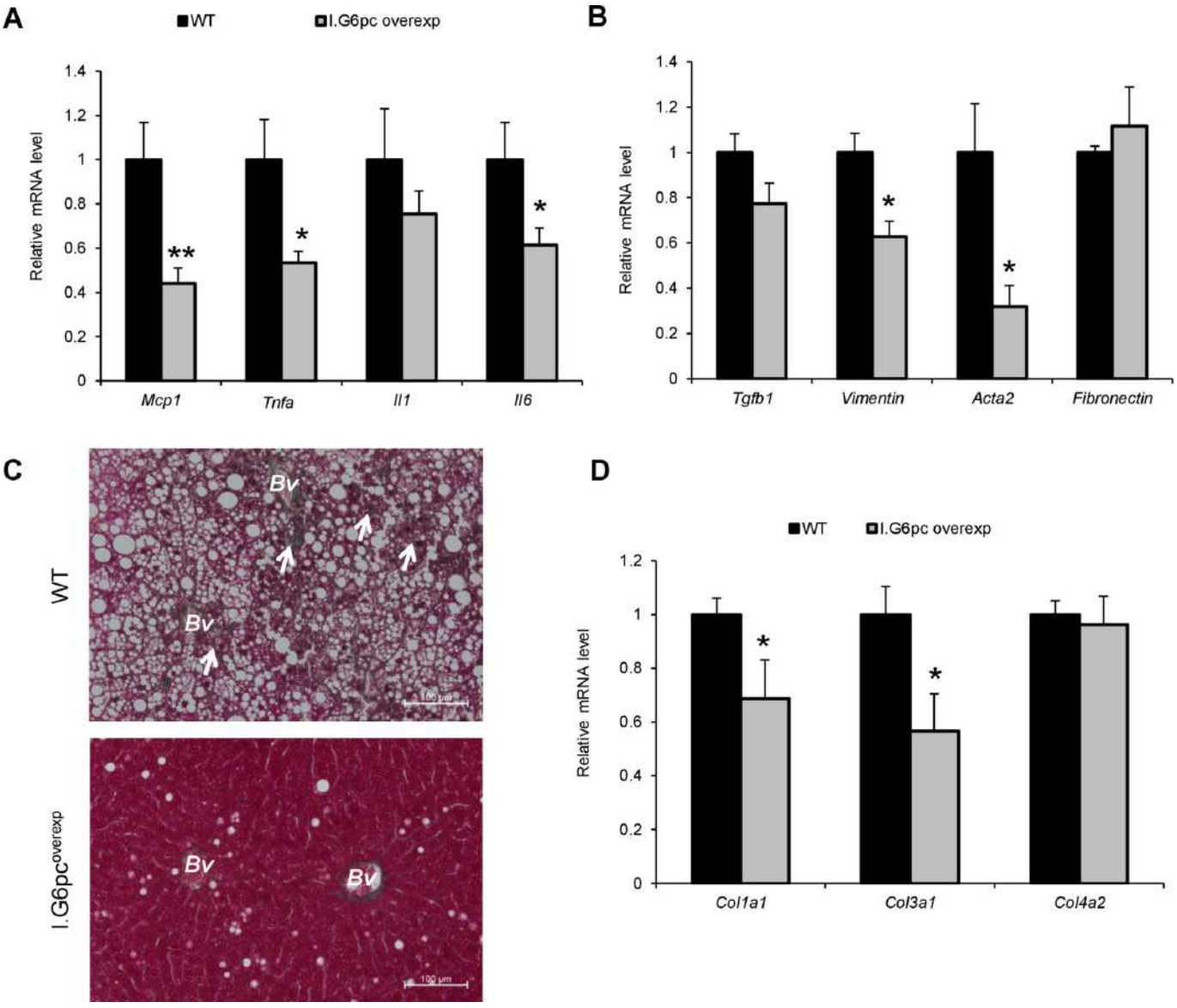

Figure 4 Intestinal gluconeogenesis protects against non-alcoholic fatty liver disease progress toward inflammation and fibrosis. (A) mRNA levels of proteins involved in pro-inflammatory processes in I.G6pc^{overexp} and WT mice (means±SEM, n=7–8). (B) Gene expression of proteins involved in pro-fibrotic processes (means±SEM, n=4–5). (C) Masson trichrome staining of liver histological sections. The arrows show the labelled collagen fibres, indicating the presence of liver fibrosis (n=3–4). Bv: blood vessels. (D) mRNA levels of collagen isoforms (means±SEM, n=8–12). (A–B, D) Data are expressed relative to WT mice. Student's t-test was performed as a statistical analysis. *p<0.05; **p<0.01 versus WT. mRNA,messenger RNA; WT, wild type.

WT mice (see online supplementary figure 3D,E). This was also associated with a decrease in some inflammation and fibrosis markers, as *Tnfa*, *interleukin 1* (*Il-1*) and *fibronectin* (see online supplementary figure 3F,G).

These data suggest that IGN is able to modulate the hepatic parameters of NAFLD and NASH even in the absence of a nutritional challenge.

The deficiency of intestinal gluconeogenesis is sufficient to promote initiation of hepatic steatosis under standard diet conditions

In previous studies, we showed that the absence of IGN has negative impacts on glucose metabolism under standard feeding conditions.[27] Indeed, we showed that I.G6pc$^{-/-}$ mice exhibit the metabolic disorders featuring a pre-diabetic state, even though they do not develop obesity. In this study, we wished to know whether the absence of IGN could result per se in the initiation of NAFLD in absence of any nutritional challenge by a HF-HS diet.

Interestingly, 13 weeks old I.G6pc$^{-/-}$ mice fed a standard diet exhibited a substantially higher hepatic triglyceride content (about +70%) than WT mice at the same age, despite no change in liver weight and glycogen content (figure 5A). In line with the liver data, the plasma triglyceride level was increased in I.G6pc$^{-/-}$ mice, without modification of the cholesterol and NEFA levels (figure 5A). Furthermore, the increase in hepatic TG storage in I.G6pc$^{-/-}$ mice was associated with an increase in mRNA levels of key genes involved in fatty acid synthesis, such as *stearoyl-CoA desaturase 1 (Scd1)*, *elongation of long-chain fatty acids family member 6 (Elovl6)*, *Fasn* and *Srebp1c* (figure 5B). The lipogenesis activation was confirmed by the increase in FAS protein expression in the liver of I.G6pc$^{-/-}$ mice (figure 5C)

A

	Liver weight and triglyceride and glycogen content			Lipid plasma parameters		
	Liver weight (%)	Hepatic triglyceride (mg /g)	Hepatic glycogen (mg /g)	Triglyceridemia (mg/dL)	Cholesterolemia (mg/dL)	NEFA (mg/dL)
WT	3.9 ± 0.1	9.1 ± 0.4	19.4 ± 1.8	0.23 ± 0.04	65.6 ± 1.8	16.7 ± 2.4
I.G6pc$^{-/-}$	3.9 ± 0.2	15.7 ± 0.8 **	17.7 ± 2.8	0.39 ± 0.02 **	67.4 ± 3.6	13.1 ± 1.5

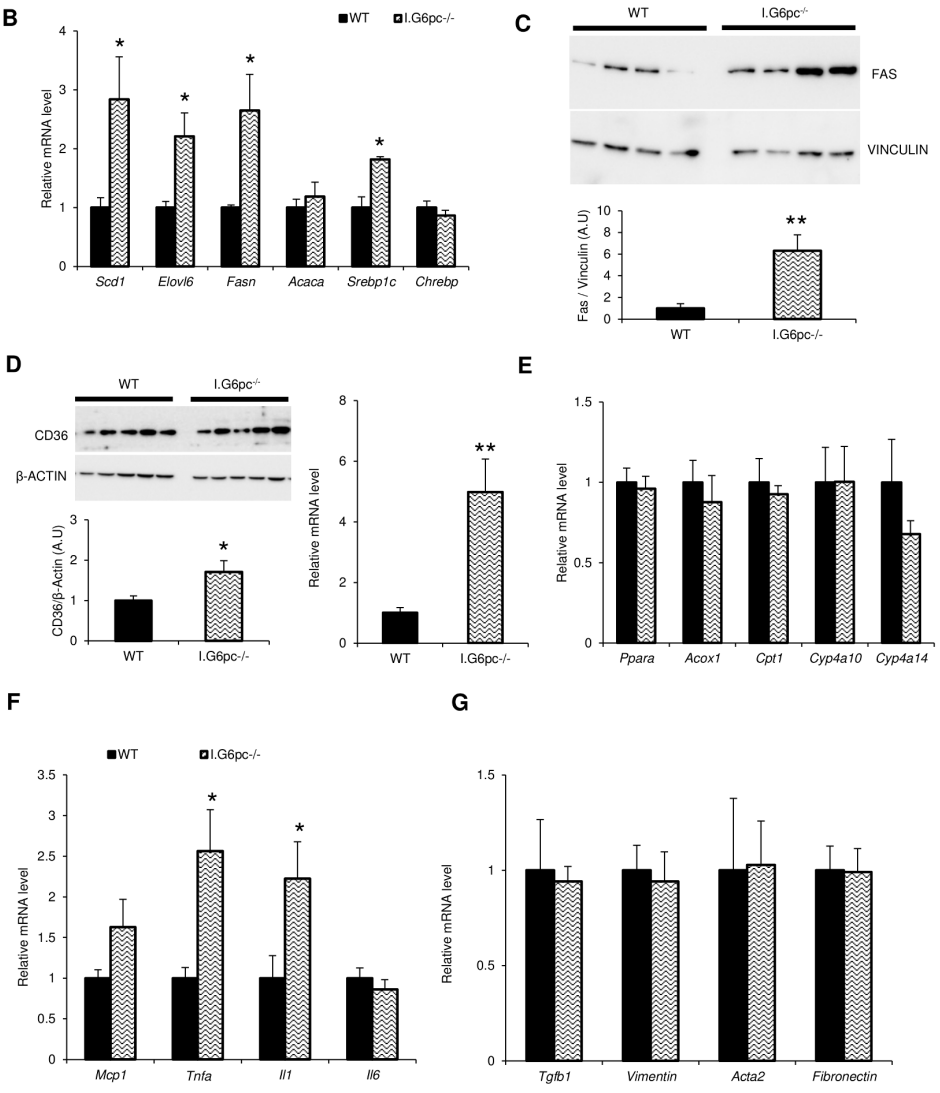

Figure 5 Deficiency of intestinal gluconeogenesis induces non-alcoholic fatty liver disease that progresses toward inflammation and fibrosis. (A) Liver weight, triglyceride and glycogen content, and lipids plasma parameters of I.G6pc$^{-/-}$ and WT mice. Liver triglyceride and glycogen content are expressed relative to liver weight (means±SEM, n=8). (B) Gene expression of proteins involved in fatty acid synthesis (means±SEM, n=5). (C) Fatty acid synthase protein expression level (means±SEM, n=6). (D) Gene and protein expression of CD36. The graphs show the quantification of CD36 expression (means±SEM, n=5–6). (E) Gene expression of proteins involved in β-oxidation (means±SEM, n=5). (F) Gene expression of proteins involved in pro-inflammatory processes (means±SEM, n=5). (G) Gene expression of proteins involved in pro-fibrotic processes (means±SEM, n=4–5). (A–F) Data are expressed relative to WT mice. Student's t-test was performed as a statistical analysis. *p<0.05; **p<0.01 versus WT. mRNA,messenger RNA; NEFA, non-esterified fatty acids;WT, wild type.

compared with WT mice. In addition, I.G6pc$^{-/-}$ mice showed a significant increase in *Cd36* gene and protein expression in the liver (figure 5D), suggesting an increase in lipid uptake besides the increase in the lipogenesis pathway. However, the expression of genes encoding some key proteins regulating fatty acid oxidation or directly involved in this pathway were not altered in the liver of I.G6pc$^{-/-}$ compared with WT mice (figure 5E).

All these results suggest that the absence of IGN is sufficient to induce the initiation of hepatic steatosis by increasing fatty acid uptake and synthesis in mice fed a standard diet. To ascertain whether the alteration of hepatic lipid metabolism in I.G6pc$^{-/-}$ mice was caused by the absence of IGN and glucose release in the portal vein or not, we rescued the function by infusing glucose into the portal vein at a rate mimicking IGN and measured the mRNA expression of genes encoding proteins involved in fatty acid synthesis. After 24 hours of glucose infusion in the portal vein, there was a marked reduction in the hepatic expression of *Elovl6* and *Fasn* genes compared with saline infusion (see online supplementary figure 4). This highlighted the causal relationship between IGN and hepatic lipogenesis.

Finally, we studied whether the disturbances in hepatic lipid metabolism induced by the deficiency of IGN could be associated with the initiation of NAFLD or NASH in a context of standard feeding. In agreement with this hypothesis, IGN deficiency led to

an increase in the expression of pro-inflammatory markers, such as *Tnfa* and *Il-1* (figure 5F). However, it must be noted that the expression of major liver fibrosis markers was not modified by the absence of IGN at 13 weeks of age (figure 5G).

Therefore, these data suggest that the deficiency of IGN is sufficient to induce a hepatic proinflammatory state.

DISCUSSION

The metabolic benefits associated with IGN were previously suggested from nutritional manipulations.[17 20 21] The benefits associated with protein-enriched or fiber-enriched diets, indeed, were suppressed in I.G6pc$^{-/-}$ mice.[17 21 22] Whereas these previous studies strongly suggest an inhibitory action of IGN on hepatic glucose production,[14] they did not raise the question of the impact of IGN on other intrahepatic mechanisms generally associated with metabolic control, such as the crucial question of steatosis and related hepatic deregulations. We used two mouse models of deficient-IGN and increased-IGN to address this question here. This approach allowed us to evaluate the proper role of IGN, independently of any exogenous influence of relevant macronutrients. It is noteworthy that intestinal G6Pase activity was increased by about three times in I.G6pc^{overexp} mice, which is comparable to the induction promoted by protein-enriched or fiber-enriched diets.[17 18 20 22]

We report that, in addition to a global improvement of glucose control providing a protection against the set up of hyperglycaemia, the increase in IGN suppresses by about 50% the hepatic lipid storage promoted by a HF-HS diet. The general decrease in gene expression of the main regulatory proteins involved in lipogenesis and of lipid import could represent the underlying mechanisms of such an improvement. Interestingly, the regulation of lipogenesis in the liver of I.G6pc^{overexp} and I.G6pc$^{-/-}$ mice is associated with modulation of *Srebpc1c* expression (decreased in I.G6pc^{overexp} and increased in I.G6pc$^{-/-}$ mice) and not of *Chrebp* expression (carbohydrate-responsive element-binding protein). Hepatic lipogenesis is controlled by insulin (via SREBP1c) and by glucose (via ChREBP) (for a review see reference 28). This suggests that the effect of IGN could involve a specific inhibitory action via SREBP1c of the insulin effect on the lipogenic pathway, and not a regulatory action on the glucose effect via ChREBP.

It is of note that a decrease in the lipid oxidation pathway was also observed, which could appear inconsistent with a decreased steatosis. However, this could be an adaptation to the decreased lipid content, which has been already reported in situations of decreased hepatic lipid accumulation caused independently of an activation of lipid oxidation.[29 30] Interestingly, the expression of several markers of inflammation and of fibrosis were decreased in I.G6pc^{overexp} mice fed a HF-HS diet, suggesting that the benefits arising from increased IGN might be extended to the complete chain of possible deleterious events accompanying HF-HS-initiated NAFLD. It is noteworthy that the benefit conferred by IGN on fibrosis takes place at the level of collagen protein deposits (figure 4C). On the contrary, the protein expression of the proinflammatory markers studied was unchanged. This suggests that the benefits of IGN could depend on processes downstream of proinflammatory factors. It is noteworthy that these IGN preventive actions were associated with the increased innervation of the liver by TH-expressing neurons. This is in agreement with the previous reports of the key role of the sympathetic nervous system in the control of hepatic lipogenesis.[31 32] Further investigations to specify the mechanism of this IGN-derived brain to liver neural signalling deserve consideration, for example, from mice with selective peripheral inactivation of the sympathetic nervous system.[33] Altogether, these data are in agreement with the previous indications that gut-brain-periphery nervous circuits may underlay the beneficial effects of IGN in energy homeostasis and glucose control.[14 27]

It is noteworthy that a diminution of body weight, liver weight and hepatic lipid storage takes also place in I.G6pc^{overexp} mice fed a normal starch-based diet, that is, independently of a nutritional challenge by a HF-HS diet, although the effects were smaller. This was accompanied by molecular events comparable to those taking place under a HF-HS diet. This strongly suggests that IGN and its nervous-relayed hepatic effects could also positively modulate the metabolic pathways involved in hepatic TG storage or NAFLD independently of deleterious nutritional habits.

In agreement with the data obtained from I.G6pc^{overexp} mice, I.G6pc$^{-/-}$ mice appeared prone to develop hepatic TG storage (although weakly) and the molecular events underlying NAFLD, including the activation of de novo lipogenesis, lipid transport and inflammation. That infusing glucose in the portal vein for 24 hours to mimic IGN corrected the markers of lipogenesis strongly suggests the causal role of this function in the modulation of NAFLD-related parameters. Interestingly, the suppression of basal IGN did not appear sufficient to initiate an increase in the expression of the markers of fibrosis. It must be noted that the experiments herein were performed in rather young animals (13 weeks old). This age could not be sufficient to the initiation of fibrosis. Alternatively, this could also suggest that only an increased IGN is susceptible to modulate the fibrosis pathway, or that the modulation of fibrosis by basal IGN could not be evidenced in a situation where fibrosis is not initiated. It is of note that, when they were fed a HF-HS diet, I.G6pc$^{-/-}$ mice developed hepatic steatosis and the molecular events featuring NAFLD. However, this was qualitatively and quantitatively similar to what was taking place in WT mice (Vily-Petit, Soty and Mithieux, unpublished results). One could speculate that the absence of IGN might accelerate the development of steatosis and NAFLD at the beginning of the nutritional challenge and that a plateau might be reached, comparable in both mice genotypes. A matching observation was previously done relating to the development of hyperglycaemia induced by a HF-HS diet. It was more rapid in I.G6pc$^{-/-}$ than in WT mice, while a comparable plateau was reached.[27] Alternatively, basal IGN may be blunted under conditions of HF diet, due to disturbances at the level of the pyruvate pool.[14 34 35] Therefore, the situation might be comparable in WT and I.G6pc$^{-/-}$ mice on HF-HS diet in terms of intestinal gluconeogenic flux (lowered), which might explain the similarity of the effects produced. It is noteworthy that when IGN is activated, as in I.G6pc^{overexp} mice, it is able to counterbalance the eventual impairments at the level of the pyruvate pool.

In conclusion, we show here that, harmoniously fitting within the previously reported processes of its beneficial action in energy homoeostasis, IGN is capable through a gut-brain-liver neural circuit of exerting a specific modulation of the set-up of hepatic steatosis and associated molecular events concurring to the development of NAFLD. Moreover, this modulation takes place independently of any supplementation with exogenous beneficial macronutrients, such as protein or fibre, which could exert an action independent of IGN. Obesity-linked liver steatosis represents the early stage preceding the development of NAFLD, which may eventually lead to NASH, cirrhosis and even liver cancer.[8] However, liver cancers may also arise in the context of steatosis independently of concomitant fibrosis and cirrhosis, while the mechanisms by which this takes place are less

known.[9–11] In humans, intestinal gluconeogenesis is induced in specific situations as during the anhepatic phase of liver transplantation and after gastric bypass surgery.[14 36–38] Interestingly, positive metabolic outcomes of gastric bypass surgery in humans have been associated with both increased IGN[19] and with major correction of hepatic steatosis and NAFLD features.[39 40] Therefore, the data here pave the way to therapeutic approaches of prevention or correction based on IGN against this serious metabolic consequence of obesity that is NAFLD.

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ORCID iD
Gilles Mithieux http://orcid.org/0000-0003-3579-8529

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