Osteopontin deficiency protects against obesity-induced hepatic steatosis and attenuates glucose production in mice

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Abstract
Aims/hypothesis Obesity is strongly associated with the development of non-alcoholic fatty liver disease (NAFLD). The cytokine osteopontin (OPN) was recently shown to be involved in obesity-induced adipose tissue inflammation and reduced insulin response. Accumulating evidence links OPN to the pathogenesis of NAFLD. Here we aimed to identify the role of OPN in obesity-associated hepatic steatosis and impaired hepatic glucose metabolism.
Methods Wild-type (WT) and Opn (also known as Spp1) knockout (Opn−/−) mice were fed a high-fat or low-fat diet to study OPN effects in obesity-driven hepatic alterations.
Results We show that genetic OPN deficiency protected from obesity-induced hepatic steatosis, at least in part, by downregulating hepatic triacylglycerol synthesis. Conversely, absence of OPN promoted fat storage in adipose tissue thereby preventing the obesity-induced shift to ectopic fat accumulation in the liver. Euglycaemic–hyperinsulinaemic clamp studies revealed that insulin resistance and excess hepatic glucose production in obesity were significantly attenuated in Opn−/− mice. OPN deficiency markedly improved hepatic insulin signalling as shown by enhanced insulin receptor substrate-2 phosphorylation and prevented upregulation of the major hepatic transcription factor Forkhead box O1 and its gluconeogenic target genes. In addition, obesity-driven hepatic inflammation and macrophage accumulation was blocked by OPN deficiency.
Conclusions/interpretation Our data strongly emphasise OPN as mediator of obesity-associated hepatic alterations including steatosis, inflammation, insulin resistance and excess gluconeogenesis. Targeting OPN action could therefore provide a novel therapeutic strategy to prevent obesity-related complications such as NAFLD and type 2 diabetes.

Keywords Gluconeogenesis • High-fat diet • Inflammation • Insulin resistance • Non-alcoholic fatty liver disease

Abbreviations
2-[14C]DG 2-Deoxy-D-[1-14C]glucose
ALT Alanine aminotransferase
FOXO1 Forkhead box O1
GINF Glucose infusion rate
GWAT Gonadal white adipose tissue
HF High-fat diet
ITT Insulin tolerance test
LF Low-fat diet
MCP-1 | Monocyte chemoattractant protein-1  
NAFLD | Non-alcoholic fatty liver disease  
NASH | Non-alcoholic steatohepatitis  
NF-κB | Nuclear factor κB  
OPN | Osteopontin  
PAP | Peroxisome proliferator-activated receptor  
PGC | Peroxisome proliferator-activated receptor coactivator  
SAP | Serum amyloid P  
SREBP-1c | Sterol regulatory element-binding protein 1  
SWAT | Subcutaneous white adipose tissue  
WT | Wild-type

Introduction

The obesity epidemic is tightly linked to a spectrum of hepatic disorders collectively known as non-alcoholic fatty liver disease (NAFLD). NAFLD has become an important public health issue because of its high prevalence and association with serious cardiometabolic abnormalities, including metabolic syndrome, type 2 diabetes and coronary heart disease [1, 2]. NAFLD spans a spectrum from simple hepatic steatosis through non-alcoholic steatohepatitis (NASH) ultimately leading to liver fibrosis and cirrhosis [3].

Increased energy intake exceeding energy dissipation promotes ectopic triacylglycerol storage in non-fat organs such as skeletal muscle and liver [4]. Triacylglycerol homeostasis in the liver is regulated by a complex interplay between hepatic plasma NEFA uptake and de novo fatty acid and triacylglycerol synthesis (lipogenesis) on the one hand, as well as fatty acid oxidation and triacylglycerol export by VLDLs on the other hand. Hepatic steatosis develops when the rate of hepatic fatty acid input (uptake and synthesis) exceeds the rate of fatty acid output (oxidation and secretion) [5].

Steatosis is associated with hepatic insulin resistance, i.e. the reduced sensitivity of the liver to the suppressive effects of insulin on glucose and VLDL triacylglycerol production. Decreased ability of insulin to suppress the hepatic output of glucose and VLDL contributes to hyperglycaemia and hyperlipidaemia, intrinsic features of the metabolic syndrome. As such, hepatic steatosis is regarded as the hepatic component of the metabolic syndrome [4]. Insulin resistance in liver, adipose tissue, and skeletal muscle is directly related to intrahepatic triacylglycerol content. However, it still remains a matter of debate whether insulin resistance is a cause or consequence of NAFLD [6, 7].

In addition to lipid accumulation, another hallmark of NAFLD is hepatic inflammation resulting in NASH. Similar to its effects on adipose tissue, obesity induces inflammatory alterations in the liver as reflected by a higher abundance of immune cells such as macrophages [8], increased cytokine production [3] and by activation of the nuclear factor κB (NF-κB) pathway [9]. Adipose tissue inflammation and unfavourable adipokine secretion are enhanced in individuals with NAFLD compared with individuals with normal intrahepatic triacylglycerol content [10], indicating that inflammatory factors released from the adipose tissue could be involved in the pathogenesis of NAFLD and hepatic insulin resistance [11]. Only recently liver macrophages were reported to be increased in number and to promote hepatic steatosis and insulin resistance in obesity [12]. Chemokines such as monocyte chemoattractant protein-1 (MCP-1) contribute to obesity-induced recruitment of macrophages to the liver [12, 13].

Osteopontin (OPN) is a multifunctional protein produced in numerous cells including activated macrophages and T cells, osteoclasts, smooth muscle, endothelial cells, and also hepatocytes [14, 15]. OPN induces the production of a variety of proinflammatory cytokines and chemokines in peripheral blood mononuclear cells [16] and supports migration of monocytes/macrophages [15]. Systemic concentrations as well as adipose content of OPN are significantly elevated in obese patients and mice [17, 18]. It has recently been suggested that genetic OPN deficiency and antibody-mediated neutralisation improve obesity-associated adipose tissue inflammation and glucose tolerance [19, 20].

Notably, OPN content is also markedly upregulated in the liver in obesity, and hepatic OPN levels correlate with liver triacylglycerol content [18, 21–23]. Within the liver, OPN is predominantly produced in inflammatory cells but also hepatocytes [24]. A role for OPN in the pathogenesis of NAFLD was suggested in mice fed a methionine- and choline-deficient diet [22, 23]. Recently, antibody-mediated OPN neutralisation was shown to protect against high-fat diet-induced hepatic macrophage infiltration [19] and d-galactosamine-induced inflammatory liver injury [24]. However, a functional role of OPN in obesity-associated hepatic steatosis and liver insulin sensitivity still remains unclear.

Here we show that genetic OPN deficiency prevents high-fat diet-induced hepatic lipid accumulation and enhances hepatic insulin action to suppress gluconeogenesis. Obesity-driven liver inflammation, as well as deleterious signal transduction pathways related to hepatic insulin resistance, is attenuated in obese Opn (also known as Spp1−/−) mice. Hence, targeting OPN action in vivo could provide a novel therapeutic strategy to combat obesity-associated hepatic steatosis and related metabolic complications.

Methods

Animals and diet C57BL/6J wild-type (WT) and B6.Cg-Spp1tm1Hib/J (here referred to as Opn knockout [Opn−−])
mice were purchased from Charles River Laboratories (Sulzfeld, Germany). At 7 weeks of age, male littermates were placed for 24 weeks on a high-fat (HF, 60% of energy from fat, D12492, Research Diets, New Brunswick, NJ, USA) and a low-fat diet (LF, 10% of energy from fat, D12450B, Research Diets) to induce obesity or to serve as lean controls, respectively. All mice were housed in a specific pathogen-free facility that maintained a 12 h light/dark cycle. Mice had free access to food and water and food intake was monitored. The protocol was approved by the local ethics committee for animal studies and the Federal Ministry for Science and Research and followed the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

**Insulin treatment in mice** WT and Opn−/− mice were kept on HF for 24 weeks. After 6 h fasting, mice were intraperitoneally injected with 1.5 U/kg insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) and saline, respectively, 15 min prior to being killed (n=5 per group). The liver was removed and immediately snap frozen for later immunoblot analyses.

**Metabolic measurements** Plasma glucose, triacylglycerol, and NEFA concentrations were measured in EDTA plasma using an automated analyser (Falcor 350, A. Menarini Diagnostics, Florence, Italy). We used commercially available ELISA kits to determine plasma insulin (Mercodia AB, Uppsala, Sweden) and serum amyloid P (SAP) (Alpco Diagnostics, Windham, NH, USA). Plasma concentrations of alanine aminotransferase (ALT) were measured using the Reflotron analysis system (Roche, Mannheim, Germany). Insulin tolerance tests (ITTs) were performed after a 3 h fasting period. Blood glucose concentrations were measured before and 30, 60, 90 and 120 min after an intraperitoneal injection of recombinant human insulin (Actrapid, Novo Nordisk, 0.75 U/kg body weight for the HF group and 0.25 U/kg for the LF group, respectively).

**Euglycaemic–hyperinsulinaemic clamp studies** WT and Opn−/− mice were fed a HF diet for 24 weeks in order to induce obesity. One week prior to clamp experiments an intravenous silicone catheter was inserted into the right jugular vein of weight-matched mice (n=10 per group). Mice were overnight fasted prior to the clamp procedure. In vivo experiments lasted for 240 min and consisted of a 120 min basal period followed by a 120 min euglycaemic–hyperinsulinaemic clamp as previously described [25, 26]. Briefly, the clamp period was initiated by a 600 pmol/kg, 3 min prime, followed by a 36 pmol kg⁻¹ min⁻¹ continuous infusion of insulin (Humulin Normal, Lilly Deutschland, Bad Homburg, Germany) raising plasma insulin concentrations within a physiological range. By means of a variable 20% glucose infusion 'steady state' (minute 90–120) conditions for plasma glucose concentration and specific activity were achieved. At minute 70, a single intravenous 2-deoxy-D-[1-¹⁴C]glucose (2-[¹⁴C]DG, 370 kBq) injection was administered. Plasma samples for determination of insulin-stimulated plasma [¹³H₃]glucose, [³H₂O] and 2-[¹⁴C]DG concentrations were collected at t= 77.5, 80, 85, 90, 100, 110, and 120 min of the glucose clamp, and basal [³H₃]glucose concentrations in the final 10 min of the basal period. At the end of each experiment, organs (liver, gonadal white adipose tissue [GWAT], and M. gastrocnemius) were immediately dissected, and snap frozen.

**Tissue 2-[¹⁴C]DG uptake** 2-[¹⁴C]DG injected during the steady state conditions of the euglycaemic–hyperinsulinaemic clamp experiments resulted in intracellular accumulation of 2-[¹⁴C]DG-6-phosphate, which was separated from 2-[¹⁴C]DG using ion-exchange columns (Bio-Rad, Hercules, CA, USA). Tissue 2-[¹⁴C]DG uptake was calculated from the plasma 2-[¹⁴C]DG AUC and tissue 2-[¹⁴C]DG-6-phosphate content as previously described [26].

**Determination of liver triacylglycerol content** Liver triacylglycerol was determined following lipid extraction as described previously [27], modified by using a commercially available enzymatic reagent (A. Menarini Diagnostics).

**Histology and immunohistochemistry** Liver samples were fixed with neutral buffered 4% paraformaldehyde and subsequently paraffin embedded. Haematoxylin–eosin staining was performed in liver as described [22]. NAFLD activity scores were determined by a certified pathologist blinded to mouse genotype and dietary intervention. After dewaxing and rehydration, immunohistochemical staining for MAC-2 (Serotec, Oxford, UK) was performed using the ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s recommendations. As a negative control, staining was performed on selected sections with isotype control. Samples were analysed with standard light microscopy.

**Immunoprecipitation and immunoblotting** Standard immunoblotting techniques were applied as described previously [28] using specific antibodies against forkhead box O1 (FOXO1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), thymoma viral proto-oncogene 1 (AKT) and pAKT (ser473) (Cell Signaling, Danvers, MA, USA). IRS-2 was immunoprecipitated from liver tissue extracts (anti-IRS-2 polyclonal antibody, Cell Signaling). IRS-2 tyrosine phosphorylation was determined using anti-phosphotyrosine polyclonal antibody, Cell Signaling). IRS-2 tyrosine phosphorylation was determined using anti-phosphotyrosine...
**Statistics** All data are given as means±SE. All data were normally distributed by visual inspection. Comparisons between genotypes on the same diet were assessed by unpaired two-tail Student’s *t* tests. Multiple regression analysis was performed to evaluate association between liver and GWAT weight in WT and *Opn*−/− mice. A *p* value of 0.05 or less was considered to be statistically significant.

**Results**

**OPN deficiency protects from obesity-induced hepatic steatosis** To assess the role of OPN in obesity-associated NAFLD and hepatic insulin resistance, WT and *Opn*−/− mice were fed either the HF or LF diet for 24 weeks to induce obesity or to serve as lean controls, respectively. Whereas OPN deficiency did not affect body weight gain or feed efficiency, liver weight was significantly lower in obese *Opn*−/− mice compared with obese WT mice (Table 1). Systemic concentrations of glucose, triacylglycerols and NEFA did not differ between the two genotypes, while the obesity-induced rise in circulating plasma insulin was drastically blunted in *Opn*−/− mice to one third of the levels measured in WT mice. ALT, a marker of hepatocyte damage, was similarly reduced in obese *Opn*−/− mice (Table 1). HF feeding induced severe lipid accumulation in livers of WT mice as determined by liver histology and quantification of hepatic triacylglycerol content. Strikingly, *Opn*−/− mice on HF were markedly protected from diet-induced hepatic steatosis (Fig. 1).

**OPN deficiency counteracts hepatic insulin resistance and excessive glucose production** We examined whether lack of OPN affects obesity-associated hepatic insulin resistance and gluconeogenesis. First we performed ITTs in WT and *Opn*−/− mice showing that the insulin effect on plasma glucose was comparable in lean mice irrespective of the genotype. Notably, glucose clearance following insulin injection in obese HF-fed animals was significantly accelerated in *Opn*−/− mice compared with WT controls, indicating improved whole-body insulin sensitivity (Fig. 2a, b). In order to directly evaluate insulin sensitivity and the relative contribution of the liver to this metabolic phenotype we performed euglycaemic–hyperinsulinaemic clamp tests in obese WT and *Opn*−/− mice. An increased glucose infusion rate (GINF) was required in obese OPN deficient mice to maintain euglycaemia during the clamp, confirming improved whole-body insulin sensitivity compared with WT controls (Fig. 2c). Basal glucose production was unchanged between both genotypes but, importantly, insulin-mediated suppression of hepatic glucose production was significantly enhanced in obese *Opn*−/− mice compared with WT animals (Fig. 2d, e). Whole-body glycolytic activity under insulin-stimulated conditions was unaffected by OPN deficiency (Fig. 2f). To determine if organs other than liver contributed to the

| Table 1  | Body characteristics and plasma variables |
|----------|------------------------------------------|
| Variable | WT LF | *Opn*−/− LF | WT HF | *Opn*−/− HF |
| Body mass (g) | 32.5±1.0 | 30.7±0.7 | 50.8±0.6 | 50.6±0.9 |
| Liver mass (g) | 1.34±0.07 | 1.41±0.06 | 2.08±0.11 | 1.76±0.10* |
| Feed efficiency | 2.08±0.41 | 1.42±0.45 | 8.35±1.24 | 8.22±1.92 |
| Glucose (mmol/l) | 7.50±0.06 | 6.92±0.69 | 15.15±0.88 | 14.77±0.76 |
| Insulin (pmol/l) | 55.8±8.0 | 40.5±4.3 | 448.2±65.9 | 134.7±24.9*** |
| Triacylglycerol (mmol/l) | 0.41±0.03 | 0.38±0.01 | 0.64±0.06 | 0.67±0.09 |
| NEFA (μmol/l) | 224.5±13.2 | 271.3±27.0 | 328.7±26.2 | 286.3±31.9 |
| ALT (U/l) | 15.1±1.6 | 11.0±2.1 | 53.±6.9 | 31.6±4.3** |
| Serum amyloid P (ng/ml) | 32.7±5.8 | 34.6±6.1 | 72.9±10.9 | 40.6±8.7* |

Data are expressed as mean±SEM.

WT and *Opn*−/− mice were fed LF or HF for 24 weeks (*n* = 10 per group). Body and liver mass were measured. Feed efficiency was determined as weight gain per food consumption. Blood samples were obtained after 3 h fasting period and analysed for depicted plasma variables.

Significant differences between genotypes on the same diet are indicated as *p*≤0.05, **p**≤0.01, ***p**≤0.001.
Reduced hepatic triacylglycerol synthesis may contribute to the absence of hepatic steatosis in OPN deficiency. In order to investigate possible mechanisms underlying the prevention of hepatic steatosis in OPN deficient mice, we analysed the expression of genes relevant for fatty acid and triacylglycerol homeostasis in the liver. Markers of fatty acid synthesis such as Fasn and Acaca did not differ between obese WT and Opn−/− mice (Fig. 3a). Neither was enhanced fatty acid oxidation likely to contribute to reduced hepatic steatosis since important transcription factors driving β-oxidation were unchanged or even down-regulated as shown for Ppara and Ppargc1a, respectively (Fig. 3a). However, gene expression of the major transcriptional regulator of hepatic lipogenesis Srebf1 was markedly decreased in Opn−/− compared with WT animals (Fig. 3b). This was similarly found to be the case for Pparg (Fig. 3c), a hepatic sterol regulatory element-binding protein 1 (SREBP-1c) target gene [29]. Given the downregulation of hepatic Pparg mRNA expression, we next examined its transcriptional targets Dgat1 and -2, which catalyse the formation of triacylglycerols from diacylglycerol and acylcoenzyme A [30]. Notably, Dgat1 expression was significantly downregulated in obese Opn−/− livers while Dgat2 followed the same trend, although the change was not significant (Fig. 3d, e). These results strongly emphasise that reduced hepatic triacylglycerol synthesis by down-regulation of Dgat expression could contribute to the absence of hepatic steatosis in obese OPN-deficient mice.

Lack of OPN favours lipid accumulation in visceral fat instead of liver. Decreased hepatic triacylglycerol content in obese Opn−/− mice (Fig. 1) indicates the prevention of ectopic fat accumulation, suggesting orthotopic fat storage in adipose tissue. Subcutaneous white adipose tissue (SWAT) weight was similar in both genotypes on the same diet, whereas GWAT weight was strikingly increased in Opn−/− compared with WT animals, both on HF diets (Fig. 4a, b). Since liver weight and steatosis were significantly reduced in obese Opn−/− vs WT mice (Table 1 and Fig. 1), we next investigated a possible relationship between liver and GWAT mass. Multiple regression analyses revealed no association between liver and GWAT mass in lean animals (r²=0.05, p=0.28, Fig. 4c). Notably, liver mass was strongly negatively associated with GWAT weight in obese WT and Opn−/− mice (r²=0.42, p=0.008, Fig. 4d), indicating that storage of excess lipids in gonadal adipose tissue prevents hepatic steatosis in the absence of OPN. Taken together, OPN deficiency inhibits ectopic fat deposition in liver thereby protecting from deleterious effects on hepatic metabolism.

Genetic OPN deletion improves hepatic insulin signalling in obesity and regulates gluconeogenic enzyme expression via FOXO1. HF-induced obesity impairs insulin-stimulated tyrosine phosphorylation of IRSs, thereby causing insulin resistance [31]. Since IRS-2 is the predominant IRS in liver, we studied insulin-stimulated hepatic IRS-2 tyrosine phosphorylation in obese WT and Opn−/− mice, 15 min following intraperitoneal injection of 1.5 U/kg insulin or saline. Probably as a consequence of markedly elevated basal plasma insulin levels in WT mice, hepatic IRS-2 phosphorylation was pronounced under unstimulated conditions, but exogenous insulin failed to further stimulate phosphorylation.
IRS-2 phosphorylation (Fig. 5a). Conversely, tyrosine-phosphorylated IRS-2 was barely detectable at baseline in HF-fed OPN-deficient mice but significantly increased following insulin stimulation (Fig. 5a). In addition, insulin-stimulated serine phosphorylation of AKT, which mediates resistance and glucose production. WT and OPN deficiency reduces obesity-induced hepatic insulin signaling in obesity is improved in the absence of OPN. Intact insulin signalling is required for the inactivation of the transcription factor Foxo1 [32], a potent regulator of hepatic gluconeogenesis. Improved insulin signalling in obese OPN−/− livers, together with a down-regulation of peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α (Fig. 3a), a transcriptional coactivator of FOXO1 [33], prompted us to examine hepatic abundance of FOXO1 in WT and OPN-deficient mice. Foxo1 mRNA and protein levels were significantly enhanced in obese WT livers, whereas absence of OPN protected from HF-induced hepatic FOXO1 upregulation (Fig. 5c, d). In addition, expression of the FOXO1 targets G6pc and Mttp was upregulated in obese WT livers but remained close to lean levels in OPN-deficient mice (Fig. 5e, f). Thus, downregulation of hepatic FOXP1 and gluconeogenic enzyme production probably contributes to the beneficial effects of OPN deficiency on glucose and lipid homeostasis in obesity.

**OPN deficiency prevents obesity-mediated hepatic inflammation and macrophage accumulation** In order to assess NAFLD disease stage, liver histology was performed and the NAFLD activity score was determined by a certified pathologist. Mice on a normal chow diet did not show any alterations in liver histology irrespective of the genotype (electronic supplementary material [ESM] Fig. 1). However, 24 weeks of HF feeding significantly enhanced hepatic steatosis (Fig. 1), lobular inflammation and hepatocyte ballooning in WT but not in OPN−/− mice (Fig. 6a, b). In addition, circulating concentrations of the liver-derived inflammatory marker serum amyloid P (SAP) were highly elevated in the plasma of obese WT, compared with OPN-deficient, animals (Table 1). Increasing evidence links macrophage accumulation in the liver to hepatic steatosis and insulin resistance [12, 34]. OPN was recently shown to mediate obesity-induced adipose tissue macrophage accumulation (19; 20). HF feeding raised gene expression of the macrophage marker CD68 and the pro-inflammatory cytokine TNFα in GWAT.
expression of the macrophage marker *Emr1* significantly less in OPN-deficient than in WT livers (Fig. 6c). Hepatic gene expression of *Ccl2* was markedly increased in obesity in WT but not in *Opn−/−* animals (Fig. 6d). Immunohistochemical analysis of liver sections revealed a sparse number of macrophages in lean mice without any genotypic difference (not shown). In contrast, hepatic macrophages were highly abundant in obese WT mice and were clustered in close vicinity to lipid droplets (Fig. 6e) resembling the so-called crown-like structures described in obese adipose tissue [35]. However, only a few such structures were found in livers of obese *Opn−/−* mice, where macrophages were less abundant and located rather individually throughout the tissue (Fig. 6e). Macrophages are the major source of obesity-driven cytokine production not only in adipose tissue but also in liver [36]. Hepatic mRNA expression of inflammatory *Tnf* and *Tgfb1*, known to be crucially involved in pathogenesis and progression of NAFLD [37, 38] was increased with HF in WT mice but significantly less in *Opn−/−* mice (Fig. 6f, g). Taken together, these results reveal that OPN is not only a mediator of hepatic steatosis but also of obesity-driven inflammatory alterations including macrophage accumulation in the liver.

**Discussion**

The growing prevalence of obesity and related inflammatory and metabolic disorders such as NAFLD and type 2 diabetes demands novel therapeutic approaches. Osteopontin is an inflammatory cytokine, hepatic content of which is upregulated in the course of human and murine hepatic steatosis and various models of liver injury [18, 21, 39]. Indirect evidence suggests that OPN is involved in hepatic steatosis, but a functional role has not been demonstrated. Here we show that genetic OPN deficiency prevents high-
Fig. 5 OPN deficiency ameliorates hepatic insulin signalling and determines FOXO1 gluconeogenic enzyme levels in obesity. WT and Opn−/− mice were kept on HF for 24 weeks. 15 min prior to sacrifice mice were intraperitoneally injected either with insulin (1.5 U/kg) or saline (n=5 per group). IRS-2 tyrosine phosphorylation (pY) was analysed in liver tissue extracts following immunoprecipitation (IP) of IRS-2. Representative immunoblots (IB) are given (a). Phosphorylation (ser473) of AKT was determined in livers of obese WT and Opn−/− mice (Fig. 3a, b). Interestingly, HF feeding upregulated hepatic production of the lipogenic factor Pparg in WT but not in Opn−/−, which could be the result of decreased SREBP-1c production and/or the lack of OPN (Fig. 3e). Consequently, the expression of Dgat1, which is regulated by peroxisome proliferator-activated receptor (PPAR)-γ [29] and catalyses triacylglycerol formation [30], was significantly decreased (Fig. 3d). Hence, our data point toward unaltered transcriptional regulation of fatty acid metabolism, but reduced hepatic triacylglycerol synthesis, underlying the lack of steatosis in OPN deficiency. Given that NEFA concentrations as well as feed efficiency (Table 1), respiratory quotient and energy expenditure (data not shown and [20]) were unchanged between both genotypes, increased fatty acid dissipation was unlikely to occur in Opn−/− mice. Thus, we hypothesised that, in the absence of OPN, plasma NEFA were preferentially distributed toward non-hepatic tissue. Indeed, GWAT weight was strikingly increased in obese Opn−/− mice (Fig. 4b) indicating that OPN deficiency facilitates orthotopic fat storage in adipose tissue thereby preventing its ectopic accumulation in the liver. This conclusion was corroborated by multiple regression analyses revealing a strong negative association between liver and GWAT weight in obese mice from both genotypes (Fig. 4d).

Visceral fat is metabolically highly active, in contrast to SWAT. The enlargement of GWAT, which is regarded as the visceral fat depot in mice, is frequently associated with insulin resistance [40]. We assume that the reduction of hepatic lipid content occurring in OPN deficient animals (Fig. 1) is more relevant for insulin sensitivity than the accompanying increase in GWAT mass (Fig 4b). Observations of decreased inflammatory alterations in GWAT of obese Opn−/− mice [20] could contribute to the mitigation of metabolic perturbation occurring with GWAT enlargement in the absence of OPN.

Notably, decreased steatosis in obese Opn−/− mice was paralleled by improved whole-body insulin sensitivity, which was mainly due to reduction in hepatic insulin resistance and glucose production as revealed by euglycaemic–hyperinsulinaemic clamp studies (Fig. 2). Enhanced...
insulin-stimulated phosphorylation of IRS-2 and AKT in OPN deficient, compared with WT, livers provided additional evidence for ameliorated hepatic insulin signalling (Fig. 5). A hallmark of hepatic insulin action is reduction of gluconeogenesis, which is downstream regulated by the transcription factor FOXO1 and its coactivator PGC-1α. Both FOXO1 and PGC-1α were downregulated in livers from obese $\text{Opn}^{-/-}$ compared with WT mice, as were the FOXO1 target genes $\text{G6pc}$ and $\text{Mttp}$ (Fig. 3a and Fig. 5) indicating abated gluconeogenesis. These results are in line with our previous observation that antibody-mediated OPN neutralisation reduces hepatic gluconeogenic enzyme production in HF-fed WT mice [19].

An obvious parallel between obesity-related pathologies of adipose tissue and liver pertains to the emerging role of inflammation. Evidence is growing that hepatic macrophages such as Kupffer cells are critically implicated in the pathogenesis and progression of NAFLD [34]. Only recently, chemical depletion of Kupffer cells was shown to prevent HF-induced hepatic steatosis and insulin resistance [12, 41, 42]. Here we demonstrate that OPN deficiency markedly counteracts obesity-induced hepatic inflammation and macrophage accumulation as assessed by NAFLD activity score, SAP concentrations and expression of macrophage markers, respectively (Fig. 6, Table 1). These data concur with recent reports showing that genetic OPN deletion and antibody-mediated OPN neutralisation antagonise macrophage recruitment in obese adipose tissue [19, 20]. Macrophages are the major source of obesity-associated inflammatory cytokine production in liver as shown for adipose tissue [36, 40]. OPN deficiency inhibited HF-induced upregulation of hepatic $\text{Tnf}$ and $\text{Tgfb1}$ gene expression (Fig. 6f, g), both of which are predominantly derived from hepatic macrophages and were repeatedly reported to underlie NAFLD progression [37, 38]. Lack of OPN also downregulated hepatic gene expression of MCP-1 (Fig. 6d), a chemokine that promotes hepatic macrophage infiltration and steatosis [13, 43]. Since OPN and MCP-1 cooperate in monocyte chemotaxis [15, 44], abrogation of inflammatory cell migration is likely to account for reduced abundance of macrophages in livers from HF-fed $\text{Opn}^{-/-}$ mice. Moreover, deficiency and neutralisation of OPN improved liver injury and fibrosis elicited by methionine- and choline-deficient diet, concanavalin A, and d-galactosamine [22, 24, 45, 46] indicating a general implication of OPN in inflammatory liver damage.

In conclusion, at least two mechanisms are likely to contribute to the improvement of obesity-related hepatic steatosis and metabolic perturbation during OPN deficiency. On the one hand lack of OPN reduces hepatic triacylglycerol accumulation and fosters lipid storage in adipose tissue. On the other hand, hepatic inflammation is attenuated in the absence of OPN. Hence, OPN is shown here as a novel mediator of obesity-associated hepatic inflammation, steatosis and insulin resistance. Blocking OPN effects in vivo could normalise altered hepatic lipids.
and glucose homeostasis in obesity and therefore prevent progression of NAFLD and development of type 2 diabetes.

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**Duality of interest** The authors declare that there is no duality of interest associated with this manuscript.

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