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ORIGINAL ARTICLE

Hepatic-specific Pgc-1α ablation drives fibrosis in a MASH model

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Abstract

Background & Aims: Metabolic dysfunction-associated steatohepatitis (MASH) is a growing cause of chronic liver disease, characterized by fat accumulation, inflammation and fibrosis, which development depends on mitochondrial dysfunction and oxidative stress. Highly expressed in the liver during fasting, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) regulates mitochondrial and oxidative metabolism. Given the relevant role of mitochondrial function in MASH, we investigated the relationship between PGC-1α and steatohepatitis.

Methods: We measured the hepatic expression of Pgc-1α in both MASH patients and wild-type mice fed a western diet (WD) inducing steatosis and fibrosis. We then generated a pure C57BL6/J strain loss of function mouse model in which Pgc-1α is selectively deleted in the liver and we fed these mice with a WD supplemented with sugar water that accurately mimics human MASH.

Results: We observed that the hepatic expression of Pgc-1α is strongly reduced in MASH, in both humans and mice. Moreover, the hepatic ablation of Pgc-1α promotes a considerable reduction of the hepatic mitochondrial respiratory capacity, setting up a bioenergetic harmful environment for liver diseases. Indeed, the lack of Pgc-1α decreases mitochondrial function and increases inflammation, fibrosis and oxidative stress in the scenario of MASH. Intriguingly, this profibrotic phenotype is not linked with obesity, insulin resistance and lipid disbalance.

Conclusions: In a MASH model the hepatic ablation of Pgc-1α drives fibrosis independently from lipid and glucose metabolism. These results add a novel mechanistic piece to the puzzle of the specific and crucial role of mitochondrial function in MASH development.

Abbreviations: α-SMA, alpha-smooth muscle actin; βHB, β-hydroxybutyrate; 8-oxo-dg, 8-Oxo-2′-deoxyguanosine; Acta2, actin alpha 2; ALT, alanine aminotransaminase; Atp5b, ATP synthase F1 subunit beta; BW, body weight; Ccl2, C-C motif chemokine ligand 2; CD, Chow Diet; Col1a1, collagen type I alpha 1 chain; Cox1, cytochrome C oxidase subunit I; Cyt-C, cytochrome C; Gpx4, glutathione peroxidase 4; Gr, glutathione reductase; hCLS, hepatic crown-like structure; HE, haematoxylin–eosin; HFD, high fat diet; IL-1b, interleukin 1b; ITT, insulin tolerance test; LW, liver weight; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; MCD, methionine-choline deficient; MMP13, matrix metallopeptidase 13; MMP2, matrix metallopeptidase 2; NAS, NAFLD activity score; NITT, nitrotyrosine; oGTT, oral glucose tolerance test; Pgc-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; Pgc-1β, peroxisome proliferator-activated receptor-γ coactivator-1β; Prdx3, peroxiredoxin 3; ROS, reactive oxygen species; Sod1, superoxide dismutase 1; SR, Sirius Red; SW, sugar water; Tbp, TATA-binding protein; TGF-β, transforming growth factor-β; TIMP1, metallopeptidase inhibitor 1; TNF-α, tumour necrosis factor-α; Txn2, thioredoxin 2; WD, western diet; WW, white adipose tissue weight.

KEYWORDS
inflammation, metabolic dysfunction-associated steatohepatitis, metabolic dysfunction-associated steatotic liver disease, mitochondria, non-alcoholic steatohepatitis, peroxisome proliferator-activated receptor-gamma coactivator 1alpha

1 | INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD), previously termed non-alcoholic fatty liver disease, is a growing cause of chronic liver disease with high incidence worldwide.[1] This burden is estimated to rise as widespread obesity and metabolic syndrome continue to expand.[2] Indeed, obesity-associated insulin resistance represents a key cause of MASLD onset.[3] MASLD is not a single phenomenon, but a spectrum of conditions fluctuating from simple fat accumulation, a reversible and relatively benign condition, to the inflammatory and fibrotic condition of metabolic dysfunction-associated steatohepatitis (MASH), formerly termed non-alcoholic steatohepatitis, which may evolve into severe forms of cirrhosis and associated hepatocellular carcinoma.[4] The diagnosis of MASH crops up in the presence of hepatic fat infiltration associated with necroinflammation and hepatocyte ballooning.[5] The oversimplified 'two-hit' hypothesis of MASH pathogenesis defined by Day and James 25 years ago[6] has been superseded by a multifactorial 'multiple-hit' theory in which several pathogenic events such as mitochondrial dysfunction and oxidative stress concur synergistically to MASH development.[7] Even though insulin resistance is a major contributing factor, insulin sensitizers have been ineffective in certain MASH treatments,[8] underscoring the implication of other mechanisms. Mitochondria are the workforce of the liver and the primary sites both of oxygen consumption and reactive oxygen species (ROS) production. Several studies pinpointed mitochondria dysfunctions and oxidative stress as distinctive features of hepatocellular injury in MASH pathogenesis,[9–12] thus depicting MASH as a mitochondrial disease.[13,14]

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a powerful transcriptional coactivator involved in the regulation of expression of a series of genes appointed to mitochondrial biogenesis and antioxidant defence.[15] Highly expressed in tissues marked by intense aerobic energy demand, in the liver PGC-1α is active during food deprivation,[15] promoting mitochondrial metabolism, antioxidant response, gluconeogenesis and fatty acids β-oxidation.[16] It was demonstrated that the increased susceptibility to western diet-induced liver damage is related to PGC-1α-dependent regulation of antioxidant defences linked to oestrogen signalling.[17] Additionally, PGC-1α has a protective role in tetrachloride (CCl_4)-induced hepatocyte epithelial to mesenchymal transition and liver fibrosis in mice.[18] Currently, several studies were conducted using methionine-choline deficient (MCD) diet or CCl_4 treatment to induce MASH. These approaches exhibit important study limitations. Indeed, CCl_4-induced fibrosis promotes a severe reduction of mitochondrial respiratory chain complex IV activity, mitochondrial depletion and damage,[19] altering the bioenergetic environment characterizing MASH while MCD diet treatment causes significant body weight loss and the absence of key metabolic features of human MASH, such as insulin resistance and obesity,[20] differing from human MASH systemic phenotype.

Key points

- Hepatic expression of Pgc-1α is reduced in MASH, in both humans and mice.
- Hepatic ablation of Pgc-1α decreases mitochondrial respiratory capacity and function.
- Lack of Pgc-1α drives liver fibrosis independently of lipid and glucose metabolism.

Here, by feeding mice lacking Pgc-1α in the hepatocytes with a diet that accurately recapitulates the metabolic, histological, transcriptomic and clinical endpoints of human MASH,[21] we investigate the contribution to steatohepatitis of the master regulator of mitochondrial biogenesis and antioxidant response, PGC-1α.

2 | METHODS

2.1 | Animal studies and dietary interventions

All animals were housed in a controlled pathogen-free facility, at 21 ± 2°C with a 12 h light/dark cycle and had free access to food and water. All the animals were allocated in the same rack, in the same room to minimize potential confounders. All the murine strains were in C57BL6/J background. Mice with liver-specific Pgc-1α ablation (LivPgc-1αKO) were obtained by crossing Pgc-1αflox/flox (Pgc-1αflox) mice[22] with Alb-CRE (Jackson Laboratory, Bar Harbor, ME), generating liver-specific Pgc-1α heterozygous mice. These mice were then backcrossed with Pgc-1αflox/flox to restore homozygosity for the Pgc-1α floxed allele. Homozygous male LivPgc-1αKO mice and their corresponding control Pgc-1αflox littermates were used throughout this study. For diet-induced steatosis and fibrosis, 8- to 10-week-old wild-type male mice were fed ad libitum for 8 weeks with a 42% Kcal/fat diet enriched with saturated fats (0.2% total cholesterol, milk fat) (Teklad, TD.180342). Chow diet (CD) (Global Diet 2018, Teklad) was used as control. For diet-induced MASH studies, 8- to 10-week-old male mice were fed ad libitum for 16 weeks with a 42% Kcal/fat WD enriched with saturated fats (0.2% total cholesterol, milk fat) (Teklad, TD.180342),

supplemented with a high fructose-glucose solution (23.1 g/L d-fructose + 18.9 g/L d-glucose, Sugar Water, SW).[21] CD and normal water were used as control. Body weight and food intake were monitored weekly. All mice were sacrificed randomly fasted at ZT3. Mice were casually assigned to treatment groups for in vivo studies. Group size calculation was decided *a priori* based on an ANCOVA model as approved by the ethical study. At the time of sacrifice, blood was collected by cardiac puncture using heparin-coated syringes and subsequently centrifuged to obtain plasma (1000 × g, 10 min at 4°C) and kept at −80°C until use. Tissues were either snap-frozen in liquid nitrogen and stored at −80°C or formalin-fixed and paraffin-embedded for subsequent histological analysis. All animals used in this study received human care and monitoring according to ethical principles and guidelines as specified in the directive 2010/63/EU and all the experiments were reviewed and received ethical approval by the Italian Ministry of Health (authorization n. 1208/2020-PR).

3 | RESULTS

3.1 | Hepatic Pgc1-α expression is reduced in human and mouse liver disorders

To evaluate the potential role of PGC-1α in liver diseases, we first investigated its expression in the liver of MASH patients using the public-available microarray data derived from the gene expression omnibus (GEO) database. Analysing three public datasets (GSE89632, GSE164760, GSE83452),[23–25] we detected a significant decrease in PGC1-α hepatic expression in MASH patients compared to healthy subjects, suggesting a possible role of the coactivator in the context of MASH (Figure 1A). In line with this evidence, we decided to feed C57BL/6J wild-type mice a western diet (WD) inducing steatosis and fibrosis. After 2 months, the hepatic expression of Pgc1-α in WD-fed mice was significantly reduced compared to mice fed a standard chow diet (CD) (Figure 1B). These data confirm the

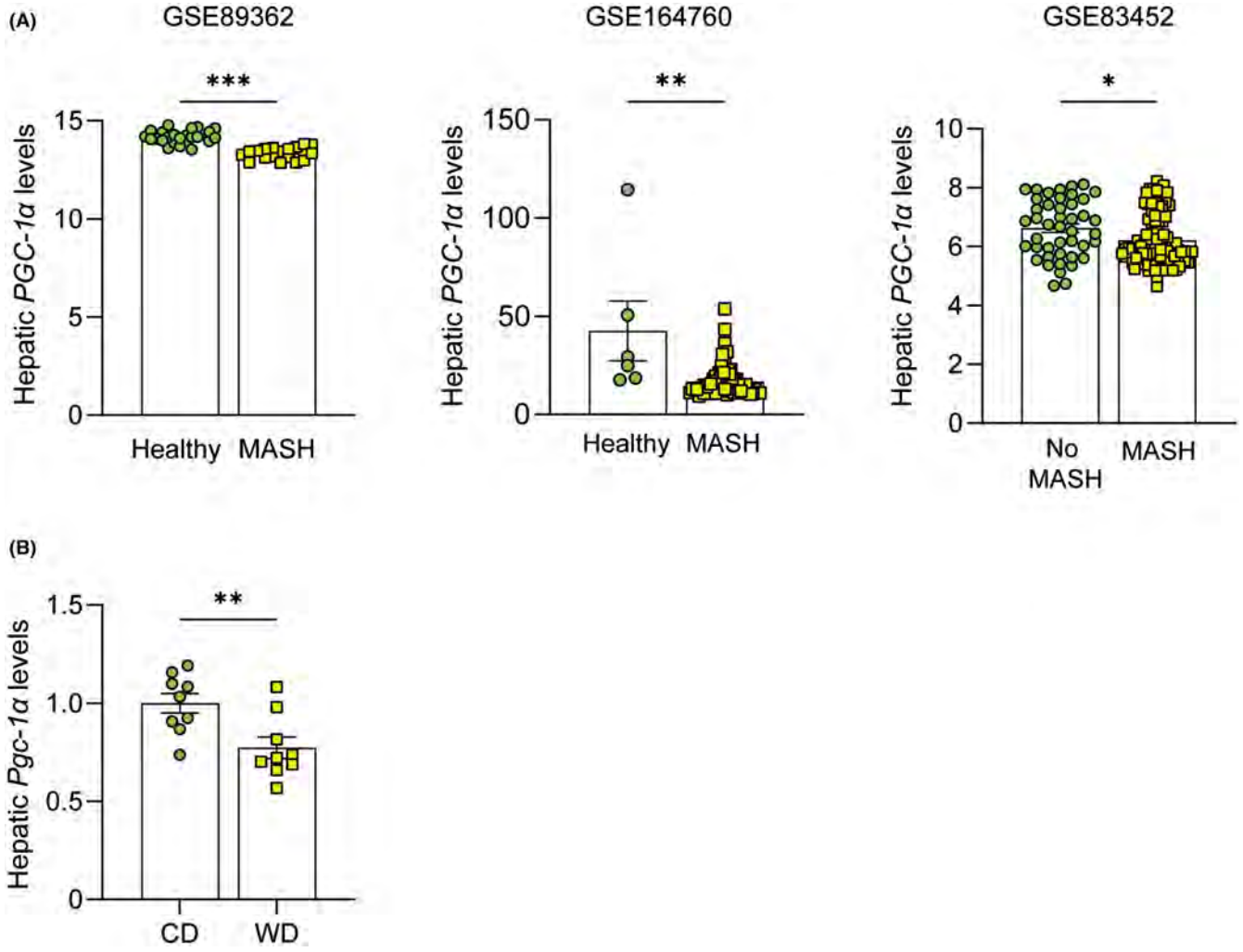


FIGURE 1 Hepatic Pgc1-α expression is reduced in human and mouse liver disorders. (A) Pgc-1α hepatic expression in MASH patients and healthy subjects (GSE89632, GSE164760, GSE83452) and (B) Pgc1-α hepatic expression in C57BL/6J wild-type mice fed with WD and standard CD ($n = 9$ mice/group). Data are expressed as mean ± SEM. Comparison between the two groups was performed using Mann–Whitney *U* test (*$p < .05$, **$p < .01$, ***$p < .001$).

decrease in Pgc1-α during liver steatosis and fibrosis, thus pointing to a reduction in mitochondrial function.

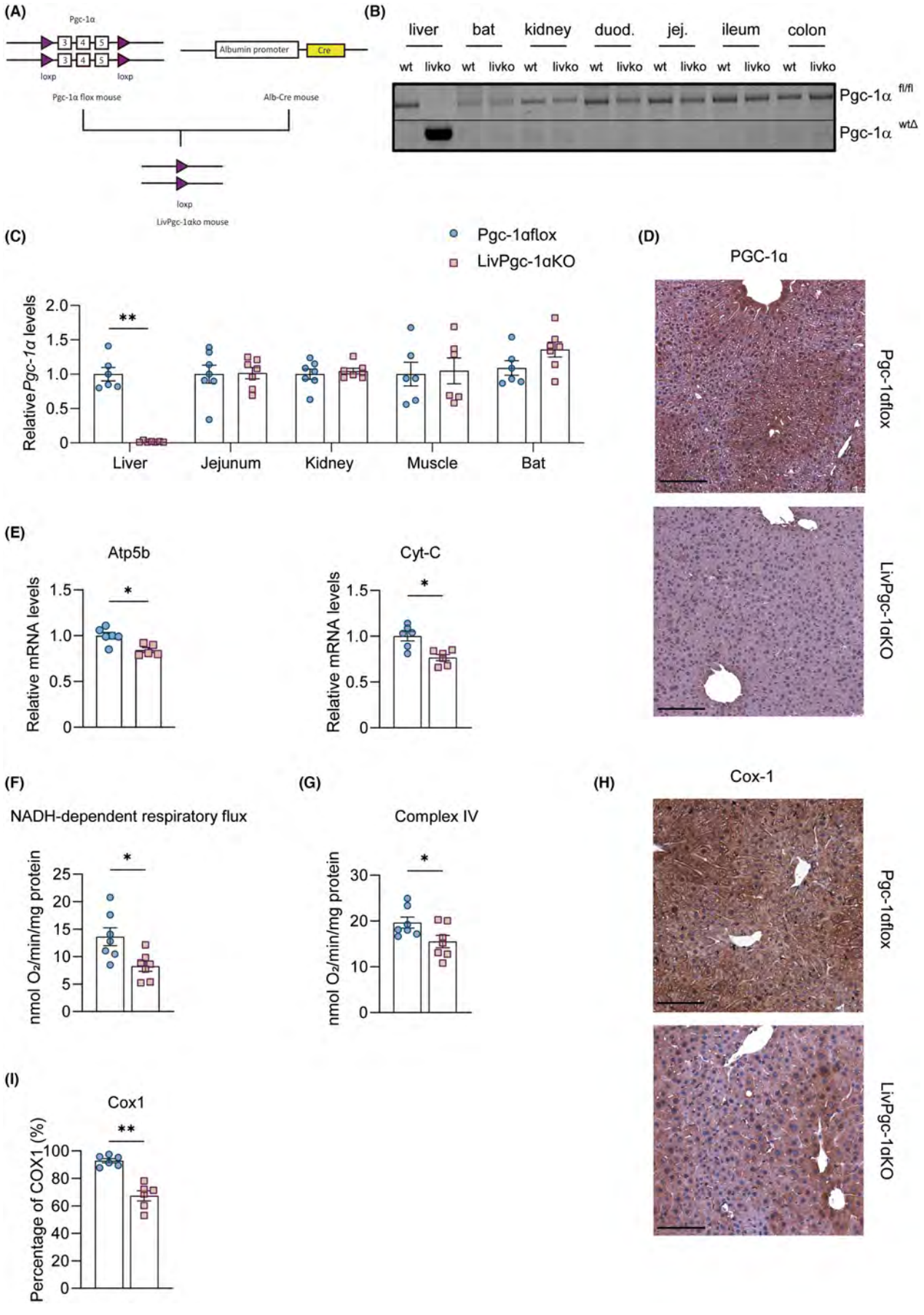
3.2 | The hepatic ablation of Pgc1-α decreases mitochondrial respiratory capacity

To further investigate the relationship between PGC-1α and MASH, we generated a mouse model in which Pgc1-α is selectively deleted in the liver. Specifically, we crossed mice that harbour loxP sites flanking exons 3 and 5 of the Pgc1-α gene with transgenic mice overexpressing the CRE-recombinase under the control of the albumin promoter (Alb-Cre) (Figure 2A). We then verified the hepatic specificity of our engineered line by genomic DNA analysis of liver and other different tissues (Figure 2B). As expected, the deletion of the exons 3, 4 and 5 occurred exclusively in the liver of LivPgc-1αKO mice (Pgc-$1^{wt\Delta}$), while the liver of Pgc-1αflox mice and all the other organs of LivPgc-1αKO showed the floxed amplicon (Pgc-$1\alpha^{fl/fl}$). The hepatic-specific ablation of Pgc1-α was confirmed by real-time qPCR, while no difference in the expression of Pgc-1α was observed in the jejunum, kidney, muscle and bat (Figure 2C). Next, we analysed the expression of Pgc-1α at the protein level by immunohistochemical staining of the liver and we observed no signal in the liver section of LivPgc-1αKO mice compared to Pgc-1αflox (Figure 2D). We then investigated the expression of PGC-1α target genes involved in mitochondrial metabolism and we detected a significant reduction of the hepatic expression of ATP synthase F1 subunit beta (Atp5b) and cytochrome C (Cyt-C) in mice lacking Pgc-1α in the liver compared to controls (Figure 2E). This led us to explore the hepatic mitochondrial respiratory function by measuring the NADH-dependent oxygen consumption rates in total liver lysates (Figure 2F). We observed a significant reduction of the NADH-dependent respiratory flux related to complexes I, III and IV in mice lacking Pgc-1α in the liver. Considering that the three catalytic subunits of complex IV are codified by the mitochondrial DNA and in the light of the strong control exerted by cytochrome C oxidase on respiratory fluxes, particularly in liver cells,[26] we measured the oxygen consumption rates exclusively related to complex IV activity (Figure 2G) and observed a significant reduction in LivPgc-1αKO mice compared to Pgc-1αflox littermates. In line with this result, we observed that the mitochondrial DNA-encoded cytochrome C oxidase subunit I (Cox1) of respiratory complex IV was significantly decreased in animals lacking Pgc-1α as shown by hepatic immunohistochemical staining (Figure 2H, I). All these data indicate that the hepatic deletion of Pgc-1α significantly impaired mitochondrial respiratory capacity, setting up a bioenergetic harmful environment for liver diseases (Tables 1 and 2).

3.3 | Hepatic Pgc-1α ablation does not influence body weight and insulin resistance

To mimic the onset of MASH, 8- to 10-week-old LivPgc-1αKO mice and Pgc-1αflox littermates were fed with a dietary regimen based on WD administration concomitant with supplementation of SW for 16 weeks to accurately recapitulate all the features of human MASH.[21] Standard CD with normal water was used as a control. We first investigated the hepatic gene expression of PGC-1s family members, Pgc-1α and peroxisome proliferator-activated receptor-γ coactivator-1β (Pgc-1β), in our mice (Figure 3A). As expected, we confirmed the total absence of Pgc-1α in LivPgc-1αKO. Intriguingly, the mRNA levels of Pgc-1α are significantly reduced in Pgc-1αflox mice fed with WD+SW compared to Pgc-1αflox littermates fed with a standard diet, underling the solid reduction in the expression of Pgc1-α also during liver fibrosis. Moreover, no changes in the hepatic expression of Pgc-1β were evident between the two genotypes, thus indicating the absence of a possible compensatory mechanism induced by the lack of Pgc-1α. Then, we investigated the hepatic expression of PGC-1α target genes encoding mitochondrial proteins and we observed a significant lower expression of Atp5b and a trend to decrease in Cyt-C in LivPgc-1α KO mice fed with WD+SW, suggesting impaired mitochondrial biogenesis in mice lacking Pgc-1α in the liver (Figure 3B). During these 4 months of diet, the body weight growth curve was only influenced by WD+SW consumption, but not by genotype (Figure 3C). Additionally, the liver-to-body weight ratio and the WAT-to-body weight ratio were also influenced only by WD+SW consumption (Figure 3D). Since insulin resistance is frequently associated with MASH, we assessed the glucose response in our mice. We observed that the hepatic lack of Pgc-1α in mice fed either with WD+SW or standard diet did not affect insulin sensitivity compared to Pgc-1αflox mice, as shown by the oral glucose tolerance test (oGTT) and insulin tolerance test (ITT) (Figure 3E, F). Moreover, no differences were detected in the hepatic expression of genes involved in insulin pathway and gluconeogenesis (Figure S1). Despite we did not

FIGURE 2 The hepatic ablation of Pgc-1α decreases mitochondrial respiratory capacity. (A) LivPgc-1αKO mice generated crossing mice that harbour loxP sites flanking exons 3 and 5 of the Pgc1-α gene with transgenic mice overexpressing CRE recombinase under the control of the albumin promoter (Alb-Cre) to drive a specific hepatic deletion of exons 3-4-5 of Pgc-1α gene. (B) PCR analysis of Pgc-1α floxed (Pgc-$1\alpha^{fl/fl}$) and Pgc-1α after recombination (Pgc-$1\alpha^{wt\Delta}$) from mice that are liver wild-type (WT) or liver knockout (livko) for Pgc-1α using DNA extracted from different organs. (C) Relative gene expression of Pgc-1α in the liver and other organs by real-time qPCR. (D) Representative immunohistochemical staining of Pgc-1α in liver sections (scale bar 100 μm). (E) Relative mRNA expression of Pgc-1α target genes cytochrome C (Cyt-C) and ATP synthase F1 subunit beta (Atp5b) in the liver by real-time qPCR. (F) Rotenone-sensitive NADH-dependent respiratory fluxes (complexes I-III-IV) and (G) KCN-sensitive Asc-TMPD-dependent oxygen consumption rates (antimycin-isolated complex IV) measured in liver homogenates. (H) Hepatic immunohistochemical staining (scale bar 100 μm) and (I) relative quantification of staining-positive areas of C oxidase subunit I (Cox1) of respiratory complex IV. mRNA gene expression levels were performed via real-time qPCR using TATA-binding protein (Tbp) as endogenous control. All the experiments were performed on seven mice/group. Data are expressed as mean ± SEM. Comparison between LivPgc-1αKO and Pgc-1αflox was performed using Mann–Whitney *U* test or Welch's t Test (*$p < .05$, **$p < .01$).



observe major modifications in the macroscopic analysis between the two genotypes, the plasma alanine aminotransaminase (ALT) level, a specific marker of liver damage, was increased in the plasma of mice lacking Pgc-1α in the liver compared to controls in WD+SW condition, suggesting specific liver damage (Figure 3G). These data suggest that the MASH condition is not linked with obesity and insulin resistance in our mice, but that other mechanisms are intervening in the disease's development.

3.4 | The hepatic deletion of Pgc-1α does not affect lipid and ketogenesis pathways

The hepatic lipid accumulation is the trigger factor promoting MASH. To evaluate whether the hepatic absence of Pgc-1α may affect the lipid content and composition, we investigated the expression of genes involved in both fatty acid synthesis and oxidation pathway, detecting no differences between the two genotypes fed

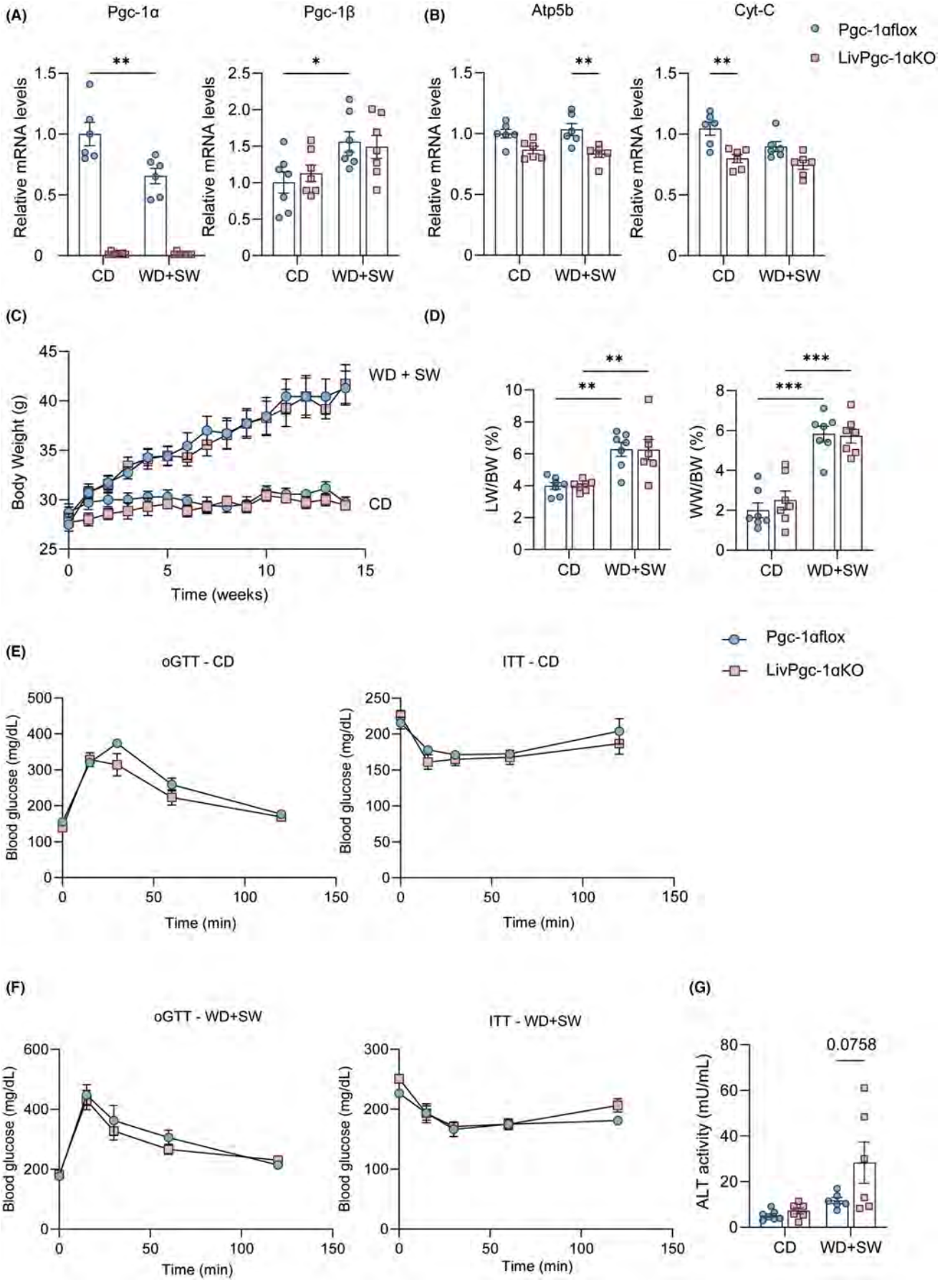
TABLE 1 List of qPCR primers used in this study.

Gene	Sequence primer forward	Sequence primer reverse
Atp5b	CACCAAGAAGGGATCGATCAC	GCAGGGTCAGTCAGGTCATCA
Ccl2	CCTTTTCCACAACCACCTCAAG	TAATTAAGGCATCACAGTCCGAGTC
Cd68	CTTCCCACAGGCAGCACAG	TGTAGCCTTAGAGAGAGCAGGTCA
Col1a1	TAGGCCATTGTGTATGCAGC	ACATGTTCAGCTTTGTGGACC
Col4a4	GTTGTACATGGAAGGACAGGAG	ACTTGGTGGATGTTGCAGTAG
Cyt-C	GGTGATGTTGAAAAAGGCAAGAA	TTGCCTCCCTTTTCCACAGT
Gpx4	GCTGGGAAATGCCATCAAA	CACCACGCAGCCGTTCTT
Gr	GCAAGTGGAAACCTGCTATGC	CATACATGCAGGGTAGAGTCATTCTT
Il-1b	CCTCAATGGACAGAATATCAACCAA	TCTCCTTGTACAAAGCTCATGGAG
Prdx3	GGTCCATCAGTAGGCCATCCTA	CTTCAAGGCATTGGAAGGATTC
Sod1	CTTCCGTCCGTCGGCTT	GGTTCACCGCTTGCCTTCT
Sod2	GAGAAGTACCACGAGGCTCTGG	ATTGAACTTCAGTGCAGGCTGA
Mmp2	ACTGACACTGGTACTGGCCC	GTCACGTGGTGTCACTGTCC
Mmp13	AGGCCTTCAGAAAAGCCTTC	TCCTTGGAGTGATCCAGACC
Pgc-1α	TTGATGCACTGACAGATGGAG	GCTGAGTGTTGGCTGGT
Pgc-1β	GCTTATGCAGTTCCGTACAAG	CCAGACCGCCTCAGTTC
Tbp	ACTTCGTGCAAGAAATGCTGAA	GCAGTTGTCCGTGGCTCTCT
Tgfb	GCAGTGGCTGAACCAAGGA	AGAGCAGTGAGCGCTGAATC
Tnfa	CTGAGGTCAATCTGCCCAAGTAC	CTTCACACAGCAATGACTCCAAAG
Timp1	AGGTGGTCTCGTTGATTTCT	GTAAGGCCTGTAGCTGTGCC
Txn2	GAATATGAGGTGTCAGCTGTGCC	CCACGTCCCCGTTCTTGAT

TABLE 2 List of antibodies used in this study.

Antibody	Host species	Incubation
Pgc-1α (N-Terminal)-ab191838, Abcam, UK	Rabbit polyclonal	At 1:2000 for 24 h (4°C)
F4/80–D2S9R, Cell Signaling Technology, USA	Rabbit monoclonal	At 1:200 for 24 h (4°C)
Cox-1–1-D6E1A8, Thermo Fisher Scientific, USA	Mouse monoclonal	At 1:200 for 24 h (4°C)
8-Oxo-dG—LSBio, USA	Rabbit polyclonal	At 1:200 for 48 h (4°C)
NITT–ab42789, Abcam, UK	Rabbit polyclonal	At 1:200 for 48 h (4°C)
α-SMA—ab5694, Abcam, UK	Rabbit polyclonal	At 1:2000 for 24 h (4°C)

FIGURE 3 Hepatic Pgc-1α ablation does not influence body weight and insulin resistance. To mimic MASH onset, 8- to 10-week-old LivPgc-1αKO and littermates Pgc-1αflox controls were fed ad libitum with WD+SW or CD for 4 months. (A) Hepatic relative mRNA expression of Pgc-1 family members, Pgc-1α and Pgc-1β, by real-time qPCR. (B) Relative mRNA expression of Pgc-1α target genes Cyt C and Atp5b in the liver by real-time qPCR. (C) Body weights were measured weekly in mice under WD+SW diet or CD. (D) Relative liver weight (LW) and white adipose tissue (WW) weight after 4 months of diet treatment showed as a percentage of LW or WW to body weight (BW) ratio. (E) Oral glucose tolerance test and insulin tolerance test performed in fasted chow diet-fed animals. (F) Oral glucose tolerance test and insulin tolerance test performed in fasted WD+SW-fed animals. (G) Alanine aminotransferase level in plasma. mRNA gene expression levels were performed via real-time qPCR using TATA-binding protein (Tbp) as endogenous control. All the experiments were performed on seven mice/group. Data are expressed as mean ± SEM. Comparison between LivPgc-1αKO and Pgc-1αflox was performed using Mann–Whitney *U* test. Comparison between different groups was performed using two-way ANOVA followed by Tukey test (*$p < .05$, **$p < .01$, ***$p < .001$).



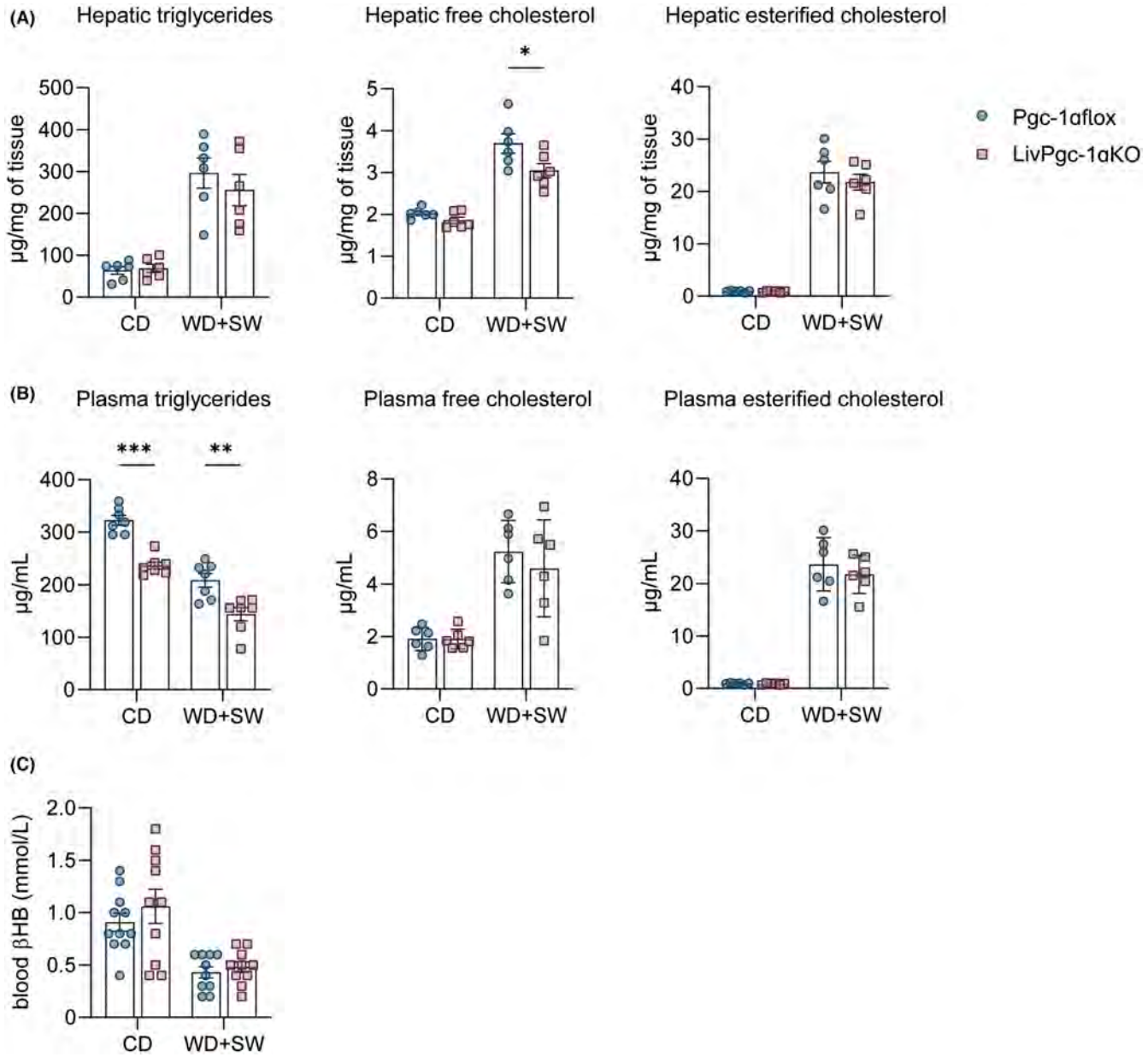


FIGURE 4 The hepatic deletion of Pgc-1α does not affect lipid and ketogenesis pathways. Neutral lipids (triglycerides, free and esterified cholesterol) extracted from (A) liver tissues and (B) plasma. After extraction, lipids were analysed by liquid gas chromatography. Plasma levels of triglycerides were measured using a colorimetric kit. (C) Blood β-hydroxybutyrate (βHB) levels were performed in fasted mice ($n = 12$). All the experiments were performed on seven mice/group. Data are expressed as mean ± SEM. Comparison between different groups was performed using two-way ANOVA followed by Tukey test (*$p < .05$, **$p < .01$, ***$p < .001$).

FIGURE 5 The hepatic ablation of Pgc-1α promotes inflammation and fibrosis. (A) Relative mRNA expression of genes related to inflammatory processes in the liver, tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), C-C motif chemokine ligand 2 (Ccl2), interleukin 1-β (IL1-β), CD16 and CD68. (B) Relative mRNA expression of genes involved in fibrosis processes in the liver, collagen type I alpha 1 chain (Col1a1), actin alpha 2 (Acta2), metallopeptidase inhibitor 1 (TIMP1), matrix metallopeptidase 2 (MMP2) and MMP13. mRNA gene expression levels were performed via real-time qPCR using TATA-binding protein (Tbp) as endogenous control. (C) Representative histological section of liver stained with haematoxylin–eosin (HE) (first lane, scale bar 200 µm), Sirius Red (SR) (third lane, scale bar 100 µm), alpha-smooth muscle actin (α-SMA) (fourth lane, scale bar 100 µm) and F4/80 (second lane, scale bar 100 µm). Yellow arrows indicate hepatic crown-like structures. (D) Quantification of F4/80 immunostaining. (E) Fibrosis quantified as percent surface area occupied by Sirius Red-stained collagen by image analysis. (F) Quantification of α-SMA immunostaining. (G) Quantification of hepatic crown-like structure (hCLS) as a number per mm^2. (H) Steatosis and inflammation evaluated adopting NAFLD activity score (NAS). (I) Relative miR-150 expression in the liver. The data were normalized on the geometrical mean ('best-keeper' gene) of the endogenous controls sno202, sno234 and U6 snRNA. All the experiments were performed on seven mice/group. Data are expressed as mean ± SEM. Comparison between different groups was performed using two-way ANOVA followed by Tukey test. (*$p < .05$, **$p < .01$, ***$p < .001$).

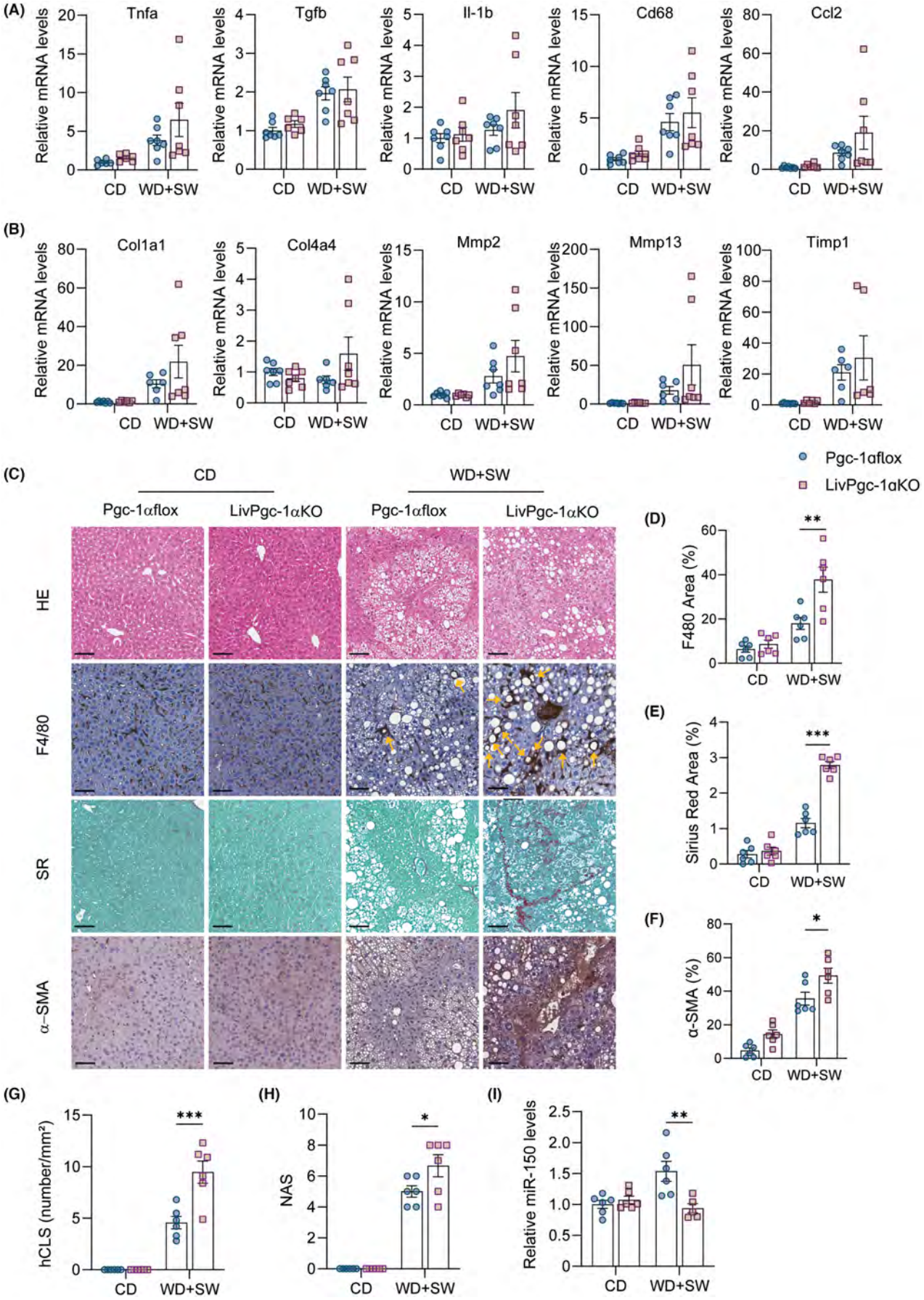


FIGURE 6 The hepatic lack of Pgc-1α fosters oxidative damage in the MASH scenario. (A) Relative mRNA expression of genes involved in antioxidant response in the liver, peroxiredoxin 3 (Prdx3), superoxide dismutase 1 (Sod1), superoxide dismutase 2 (Sod2), glutathione reductase (Gr), glutathione peroxidase 4 (Gpx4) and thioredoxin 2 (Txn2). (B) Representative histological section of liver stained with 8-oxo-2′-deoxyguanosine (8-Oxo-dG) (upper panel, scale bar 100 μm) and nitrotyrosine (NITT) (lower panel, scale bar 100 μm). Relative quantification of 8-Oxo-dG (C) staining-positive areas and NITT (D). mRNA gene expression levels were performed via real-time qPCR using TATA-binding protein (Tbp) as endogenous control. All the experiments were performed on seven mice/group. Data are expressed as mean ± SEM. Comparison between different groups was performed using two-way ANOVA followed by Tukey test (*$p < .05$). (E) Rotenone-sensitive NADH-dependent respiratory fluxes (complexes I-III-IV) and (F) KCN-sensitive Asc-TMPD-dependent oxygen consumption rates (antimycin-isolated complex IV) were measured in liver homogenates of MASH samples. Comparison between LivPgc-1αKO and Pgc-1αflox was performed using Welch's t Test (**$p < .01$).

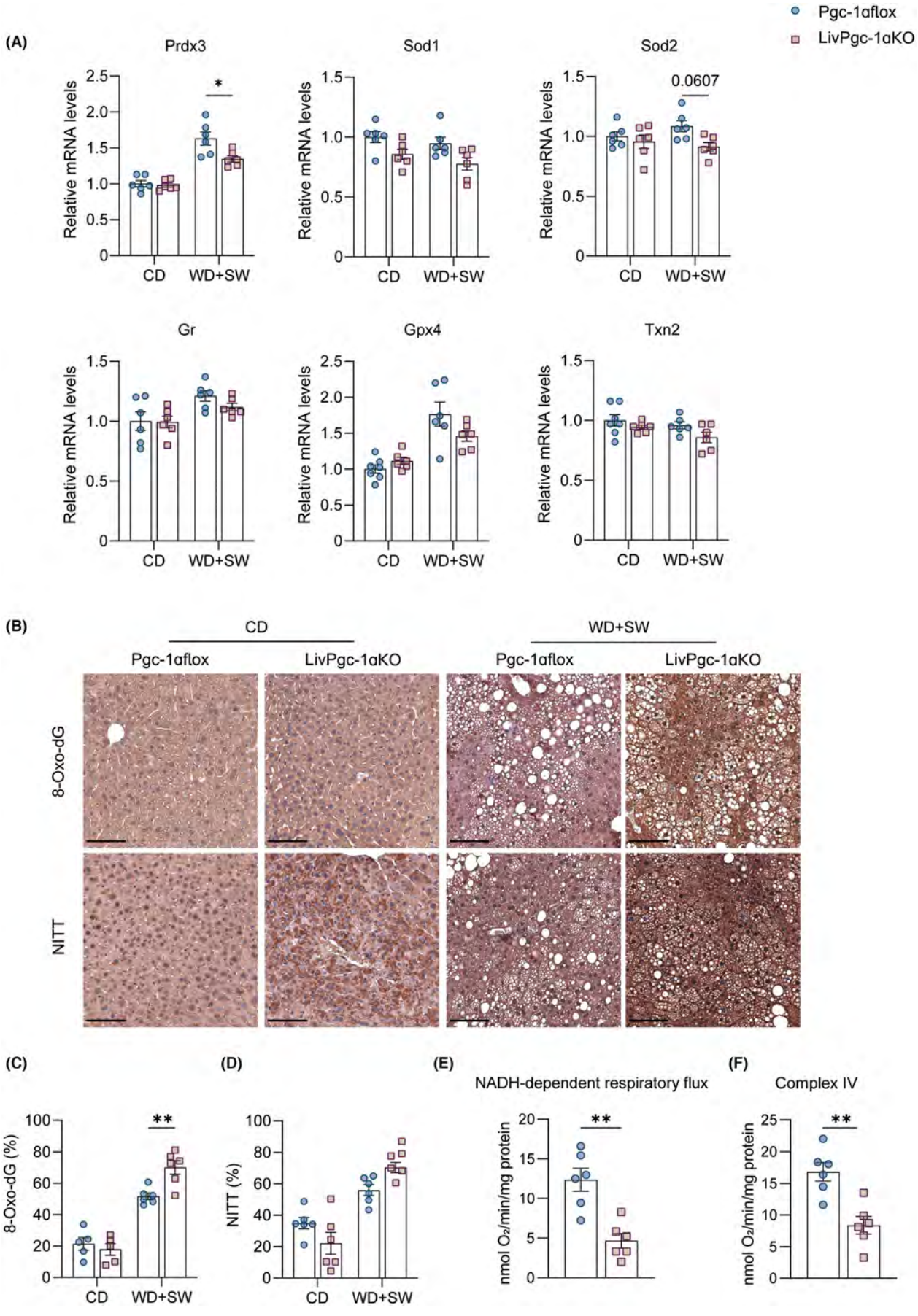
with the same diet (Figure S2). Moreover, we measured and analysed the hepatic neutral lipids, observing no difference in LivPgc-1αKO mice compared to controls, except for the hepatic cholesterol level that was significantly reduced in mice lacking Pgc-1α in the liver (Figure 4A). Intriguingly, when we measured the circulating triglycerides in our mice, we observed that mice lacking Pgc-1α in the liver fed either with WD+SW or standard diet showed significantly reduced plasma triglycerides compared to controls (Figure 4B). Considering that impaired ketogenesis could potentially contribute to MASH progression,[27] we measured plasma β-hydroxybutyrate (βHB) level in our mice, and we found no difference in ketone body production between LivPgc-1αKO mice fed with WD+SW and Pgc-1αflox control mice (Figure 4C), indicating that the lack of Pgc-1α in the liver did not affect the ketogenesis pathway.

3.5 | The hepatic ablation of Pgc-1α promotes inflammation and fibrosis

Although we did not observe differences in the hepatic lipid content between the two genotypes, LivPgc-1αKo mice fed with WD+SW showed microvesicular steatosis which is not limited to the perivascular zone as in Pgc-1αflox controls but involved all the hepatic parenchyma (Figure 5C—first lane), thus correlating with a severe form of MASH.[28] In line with this evidence, we observed a trend to increase in the hepatic gene expression profile of the inflammatory cytokines tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), C-C motif chemokine ligand 2 (Ccl2), interleukin 1-β (IL1-β), Cd16 and Cd68 in LivPgc-1αKO mice fed with WD+SW compared to controls (Figure 5A), suggesting that the hepatic ablation of Pgc-1α impacted inflammation. To further corroborate our observations, we examined the distribution of macrophages in the liver with F4/80 immunostaining (Figure 5C—second lane), a representative macrophage marker. No considerable staining was detected in the liver of mice fed a standard chow diet. In contrast, liver sections of mice fed with WD+SW showed significant F4/80 staining, with higher prevalence in LivPgc-1αKO mice compared to Pgc-1αflox ones (Figure 5D). The presence of hepatic crown-like structures (hCLS) is an important histological feature of the pro-inflammatory process in the MASH microenvironment. Specifically, in hCLS the macrophages aggregate to surround hepatocytes with lipid droplets, promoting fibrosis. Considering that the number of hCLS positively correlates with the extent of liver fibrosis,[29] we measured the histological number of these structures in our mice. We observed a significant increased number of hCLS in LivPgc-1αKO mice fed with WD+SW compared to control littermates (Figure 5G), suggesting a relevant activation of inflammatory and fibrotic processes in these mice. Then, we evaluated the hepatic gene expression of markers of fibrosis, including collagen type I alpha 1 chain (Col1a1), actin alpha 2 (Acta2), metallopeptidase inhibitor 1 (TIMP1), matrix metallopeptidase 2 (MMP2) and MMP13 (Figure 5B), finding a trend to increase in LivPgc-1αKO mice fed with WD+SW compared to Pgc-1αflox ones. To further corroborate our observations, we performed a Sirius Red/Fast Green staining, which allows to observe and quantify red-stained collagen fibers in contrast to green-stained non-collagen components in the liver of our mice. Intriguingly, we observed a significant major deposition of collagen in the liver of LivPgc-1αKO mice fed with WD+SW compared to Pgc-1αflox controls, thus indicating increased fibrosis in the absence of Pgc-1α in the liver (Figure 5C – third lane, Figure 5E). Additionally, we performed and quantified the immunostaining of α-SMA (alpha-smooth muscle actin), which is a well-established marker of myofibroblast activation, a key event in the pathogenesis of fibrosis.[30] Intriguingly, we observed a significant extent of myofibroblast activation in the liver of LivPgc-1αKO mice fed with WD+SW compared to controls, thus confirming increased fibrosis in the absence of Pgc-1α in the liver (Figure 5C—fourth lane, Figure 5F). NAFLD activity score (NAS), a well-established histological scoring system widely recognized for its reliability in assessing inflammation, steatosis and hepatocytes ballooning,[31] was increased in mice lacking Pgc-1α in the liver compared to controls (Figure 5H). Moreover, the absence of PGC-1α reduces the hepatic expression of the antifibrotic miR-150, especially when mice are challenged with MASH protocol (Figure 5I). Overall, these data demonstrated that the hepatic-specific Pgc-1α deletion exacerbates inflammation and fibrosis in MASH scenario.

3.6 | The hepatic lack of Pgc-1α fosters oxidative damage in the MASH scenario

Oxidative stress is a detrimental state characterizing MASH. Oxidative stress can cause direct damage to lipid, protein and DNA molecules, triggering the inflammatory and fibrogenic signalling pathways, which promote the progression from steatosis to MASH.[32] Since PGC-1α is a crucial modulator of ROS scavenger, to assess whether the reduced hepatic Pgc-1α expression impairs ROS detoxification, we



evaluated the gene expression profile of enzymes involved in antioxidant response (Figure 6A). Both genotypes showed a significant increase in the antioxidant peroxiredoxin 3 (Prdx3) mRNA levels when treated with WD+SW, suggesting that a chronic diet-induced MASH insult fosters an oxidative stress status which boosts the antioxidant defence gene expression. However, LivPgc-1αKO mice fed WD+SW displayed a significant decrease in the expression of this enzyme compared to Pgc-1αflox controls. A trend to decrease was also observed for the other antioxidant enzymes superoxide dismutase 2 (Sod2), superoxide dismutase 1 (Sod1), glutathione reductase (Gr), glutathione peroxidase 4 (Gpx4) and thioredoxin 2 (Txn2), suggesting a limited ROS scavenging capacity in LivPgc-1αKO mice compared to control littermates. As a consequence, the staining for 8-Oxo-2′-deoxyguanosine (8-Oxo-dG) and Nitrotyrosine (NITT), two peculiar markers of oxidative damage to DNA and proteins, respectively, were strongly increased in the mice lacking Pgc-1α compared to controls (Figure 6B–D). Moreover, these mice showed a considerable reduction of respiratory fluxes (Figure 6E, F), promoting a bioenergetic hepatic harmful environment for MASH that intensively affects oxidative stress, thus exacerbating liver damage.

4 | DISCUSSION

In the current study, we demonstrate in both humans and mice that hepatic ablation of Pgc-1α correlates with severe inflammation and fibrosis, fostering the development of MASH. A recent study reported that PRMT1 was required to stimulate Pgc-1α mRNA expression via recruitment of HNF-4α to the promoter of Pgc-1α and hence mitigated HFD-induced hepatic steatosis by enhancing PGC-1α-mediated fatty acid oxidation.[33] Additionally, in liver fibroblasts of MASH patients, lipid excess inhibits PGC-1α by CHOP, the downstream sensor of endoplasmic reticulum (ER) stress.[34] Recently, Wan *et al.* proved that the specific overexpression of Pgc-1α in the liver protects from hepatic steatosis and insulin resistance via improving IL10-mediated anti-inflammatory response.[35] Moreover, Estall *et al.* illustrated that even a moderate decrease in Pgc-1α function was sufficient to cause dysfunction in fasting lipid oxidation promoting liver disease.[36] We have previously shown that the specific hepatic overexpression of Pgc-1α induces the expression of the microRNA miR-150 in the liver,[37] an antifibrotic miRNA.[38–42] miR-150 is a representative antifibrotic miRNA that inhibits the activation of hepatic stellate cells (HSCs).[41] Cells overexpressing miR-150 showed a reduction of extracellular matrix proteins and α-SMA.[42] Intriguingly, this microRNA exhibits not only an anti-fibrotic action in the liver,[40–42] but it also plays a protective role in early-stage inflammation during MASH.[43] Additionally, the miR-150 secretome has been shown to ameliorate liver fibrosis, reduce systemic inflammatory responses and enhance antioxidant enzyme expression.[40] Specifically, intravenous infusion of the miR-150 secretome in mice with liver fibrosis markedly decreased profibrogenic markers, such as α-SMA, mitigated systemic inflammatory cytokines and increased the expression of markers of antioxidant activity,[40] aligning with our observations. Furthermore, higher levels of miR-150 in the serum of AMA-negative PBC patients suggest its potential role in the induction of the disease and progression to fibrosis.[44] An inverse correlation between circulating miR-150 levels and the severity of schistosome-induced hepatic fibrosis has also been noted, offering the possibility to distinguish patients with mild versus severe hepatic fibrosis.[45] In contrast to this hepatic protective role of miR-150 in liver disease Chen W *et al.*[46] demonstrated that miR-150 deficiency protects against Fas-induced hepatocyte apoptosis and liver injury by upregulating the Akt pathway. Additionally, it was reported that miR-150 is decreased in alcoholic fatty liver and promotes hepatic lipid accumulation by upregulating PLIN2 expression, promoting a compensatory mechanism that attempts to regulate hepatic lipid homeostasis in response to alcohol-induced damage.[47] Here, we showed that mice expressing Pgc-1α in the liver present a high level of miR-150 when challenged with the MASH diet, while the lack of PGC-1α fails to induce this microRNA during MASH. While our study indicates a correlation between hepatic Pgc-1α ablation and changes in miR-150 levels, and subsequently inflammation and fibrosis, it is important to note that these findings are correlative. Future further studies involving mechanistic rescue experiments are required to establish a causal relationship. Although several studies examined the hepatic role of PGC-1α in the context of MASH, few of these focused on the PGC-1α-mediated regulation of mitochondrial energetic capacity. Here, we described a scenario in which the hepatic ablation of Pgc-1α severely impairs oxidative phosphorylation and antioxidant response, thus exacerbating inflammation and fibrosis. Mitochondria are the powerhouse of cells decisive for the maintenance of energy homeostasis.[48] Mitochondria abnormalities were observed in MASH patients[49,50] and MASH-related animal models.[51] It is well-documented that reduced hepatic respiratory capacity can create a detrimental bioenergetic environment, contributing to the exacerbation of fibrosis in MASH. Indeed, mitochondrial dysfunction in MASH leads to decreased ATP production, resulting in a bioenergetic crisis and subsequent fibrosis.[52] Additionally, impaired mitochondrial function exacerbates oxidative stress and inflammation, key drivers of fibrosis in MASH.[53] However, it is important to recognize that certain therapeutic agents, such as metformin and mitochondrial uncouplers, have shown benefits by modulating mitochondrial function in liver disease under specific conditions.[54] Metformin activates AMPK, which mediates the inhibition of the expression of genes involved in glucose and lipid synthesis while promotes fatty acid oxidation and glucose uptake.[54] The activation of AMPK by metformin in the liver and other tissues results from a transient reduction in cellular energy status caused by the mild and specific inhibition of respiratory-chain complex 1 by the drug.[54] It is also worth mentioning that metformin probably exerts some non-mitochondrial effects, as it has been shown to influence the metabolism in erythrocytes, i.e. mitochondria-lacking cells, by modulating membrane fluidity.[55] In agreement with previous studies,[17,18,35,36,56–61] we observed that the loss of Pgc-1α in the liver leads to significant hepatic pathology, including inflammation and fibrosis. In particular, by using a specific mouse model overexpressing Pgc-1a in the liver, Morris *et al.*[56] demonstrated an increased

mitochondrial function, increased FA oxidation and reduced TAG storage and secretion. Intriguingly, a short-term HFD administration can result in a decrease in hepatic respiratory capacity that is completely prevented by hepatic overexpression of Pgc-1α. Building upon these important findings, our research investigates whether the hepatic homozygous deletion of Pgc-1α can exacerbate the effects of long-term WD-inducing MASH. Specifically, by using a pure strain mouse model in which Pgc-1α is selectively ablated in the liver, we here explored the impact of prolonged WD exposure on liver pathology. Our results show that the hepatic absence of PGC-1α significantly worsens the hepatic response to long-term WD, promoting severe mitochondrial dysfunction, enhanced inflammation and fibrosis, which are hallmarks of MASH. This exacerbation is characterized by a marked impairment in mitochondrial function, reduced antioxidant capacity and increased oxidative stress, highlighting the critical role of PGC-1α in maintaining hepatic metabolic health under chronic dietary stress. Recently, Morris *et al.* demonstrated that liver-specific Pgc-1α heterozygosity increases susceptibility to short-term diet-induced weight gain in male mice. In line with our observation, they additionally demonstrated that the reduced liver-specific Pgc-1α results in reduced FA oxidation and respiratory capacity in isolated liver mitochondria of mice fed with a normal diet compared to wild-type mice.[57] However, as PGC-1α and PGC-1β share functional redundancy, it is possible that PGC-1β upregulation might partially counteract the mild deficiency of PGC-1α in the heterozygous mice. By contrast, to ensure that the observed effects are directly attributable to the absence of Pgc-1α in hepatic tissue, we measured the hepatic expression of Pgc-1β levels, observing no differences between the two genotypes, thus suggesting that in our model, PGC-1β may not be exerting a compensatory effect. Very recently, Shen *et al.* demonstrated that Pgc-1α mitigated hepatic steatosis induced by MCD diet.[58] However, the authors themselves depicted that the research they conducted showed a limitation related to the classical dietary model of MASH they used in the study, which deviated from human MASH systemic phenotypes. In the current study, we fed mice lacking Pgc-1α in the hepatocytes with a diet that recapitulates all the various endpoints of human MASH,[21] ensuring an ideal preclinical mice model of MASH. Moreover, Shen *et al.* demonstrated that hepatic-specific overexpression of Pgc-1α exerts a beneficial effect in the control of MASH by restoring NAD+ bioavailability and by expanding antioxidant capacity thus improving metabolic performance in the impaired liver.[58] However, protein function is the result of gene expression and post-translational modifications, therefore it is possible that the overexpression of a coactivator does not necessarily correlate with its transcriptional activity. By contrast, by using a pure strain mouse model in which Pgc-1α is selectively ablated in the liver, here we proved that the hepatic absence of Pgc-1α dramatically causes mitochondrial respiration impairment. Specifically, we demonstrate that these mice showed a considerable reduction of respiratory fluxes, promoting a bioenergetic hepatic harmful environment for MASH. Indeed, we detected a strong impairment of ROS scavenging capacity, thus fostering oxidative damage in the MASH scenario. In line with our results, it has been demonstrated that reduced hepatic Pgc-1α expression impairs ROS detoxification and results in pronounced oxidative damage, thus exacerbating liver steatosis and inflammation induced by an HFD in mice.[17]

5 | CONCLUSIONS

Our results demonstrate that the hepatic ablation of Pgc-1α decreases mitochondrial metabolism and respiratory capacity while increasing liver inflammation and fibrosis in the intricate scenario of MASH, independently of insulin resistance and obesity. Further mechanistic research is essential to establish the precise causative relationships between PGC-1α activity, mitochondrial dysfunction and liver disease.

AUTHOR CONTRIBUTIONS

Maria Arconzo, Gaetano Villani and Antonio Moschetta contributed to the conception and design of the study. Maria Arconzo, Elena Piccinin, Emanuela Pasculli, Marica Cariello, Nicolas Loiseau, Justine Bertrand-Michel, Hervé Guillou and Maria Laura Matrella were responsible for the acquisition of data. Maria Arconzo and Elena Piccinin contributed to the analysis and interpretation of data. Maria Arconzo and Antonio Moschetta were involved in drafting the article. Elena Piccinin and Gaetano Villani critically revised the article for important intellectual content. Antonio Moschetta gave final approval of the version to be published, managed the project administration and supervised the work.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

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SUPPORTING INFORMATION

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