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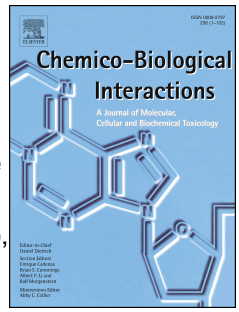

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Metformin administration induces hepatotoxic effects in paraoxonase-1-deficient mice

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ABSTRACT

Metformin is the first-line pharmacological treatment of diabetes. In these patients, metformin reduces body weight and decreases the risk of diabetes-related complications such as cardiovascular disease. However, whether metformin elicits beneficial effects on liver histology is a controversial issue and, as yet, there is no consensus. Paraoxonase-1 (PON1), an enzyme synthesized mainly by the liver, degrades lipid peroxides and reduces oxidative stress. PON1 activities are decreased in chronic liver diseases. We evaluated the effects of metformin in the liver of PON1-deficient mice which, untreated, present a mild degree of liver steatosis. Metformin administration aggravated inflammation in animals given a standard mouse chow and in those fed a high-fat diet. Also, it was associated with a higher degree of steatosis in animals fed a standard chow diet. This report is a cautionary note regarding the prescription of metformin for the treatment of diabetes in patients with concomitant liver impairment.

[Abstract word count = 148]

Key words: Antioxidants; hepatotoxicity; inflammation; metformin; NAFLD; paraoxonase; steatosis

Abbreviations: ALT: Alanine aminotransferase; AMPK: Adenosine monophosphate-activated protein kinase; AST: Aspartate aminotransferase; BAT: Brown adipose tissue; CD: Chow diet; eWAT: Epididimal white adipose tissue; FASn: Fatty acid synthase; FPLC: Fast protein liquid chromatography; GTT: Glucose tolerance test; HDL: High-density lipoproteins; HFD: High-fat and high-cholesterol diet; iWAT: Inguinal white adipose tissue; CCL2: Chemokine (C-C motif) ligand-2; pAMPK: Phosphorylated AMPK; PON1: Paraoxonase-1; vWAT: Visceral white adipose tissue

HIGHLIGHTS

- Studies have reported toxic effects of metformin in patients with liver disease
- PON1 is an antioxidant enzyme synthesized mainly by the liver
- We found that metformin administration increases steatosis and inflammation in PON1-deficient mice
- PON1 deficiency is associated with toxic effects of metformin in the liver

1. Introduction

Metformin (dimethylbyguanidine) is the first-line pharmacological treatment of diabetes. In these patients, metformin assists weight loss and reduces the risk of diabetes-related end-points such as microvascular disease, myocardial infarction (large vessel disease) and all-cause mortality. This drug has also been reported to elicit beneficial effects on liver histology, by reducing hepatic steatosis [1]. In normal mice fed with a high-fat diet, metformin has been reported to fully reverse hepatic steatosis and inflammation; effects that appear to be mediated by upregulation of hepatic adenosine monophosphate-activated protein kinase (AMPK) and, as well, to be associated with changes in lipogenic gene expression, such as fatty acid synthase (FASn) [2]. However, clinical studies investigating the effects of metformin on the liver have not reached a consensus [3-6]. Metformin possesses multiple pleiotropic effects [7-10], and one of the most important is to decrease oxidative stress by enhancing the hepatic levels of antioxidant enzymes such as paraoxonase-1 (PON1) [11,12]. PON1 is a lipolactonase synthesized, mainly, by the liver. It degrades oxidized phospholipids and, as such, plays a role in an organism's antioxidant system [13,14]. Preliminary observations from our laboratory suggest that PON1 is an important factor in explaining the beneficial effects of metformin in the liver [15].

Some reports have suggested that metformin may be useful in the treatment of hepatitis or hepatocellular carcinoma [16]. Conversely, however, several cases of metformin-induced aggravation of liver injury have been reported in patients with liver disease [17-20]; liver damage being documented as the elevation of serum liver enzymes, and improvement in liver function being documented following discontinuation of the drug for 1 week. Unfortunately, the mechanism by which metformin may induce liver injury is unknown. Severe liver impairment is associated with inhibited hepatic and circulating PON1 levels. Indeed, serum PON1 activity is strongly decreased in patients with chronic hepatitis or cirrhosis, and the magnitude of the decrease is related to the extent of liver damage [21,22]. Moreover, a study found a decreased hepatic PON1 activity related to enhanced lipid peroxidation and liver damage in rats with experimental fibrosis [23]. In addition, PON1 over-expression provided strong protection against the development of experimentally-induced liver disease [24].

With all these pointers in mind, the possibility that PON1 deficiency itself is associated with toxic effects of metformin in the liver warrants investigation. The objective of this study was to evaluate whether metformin elicits toxic effects in the livers of PON1-deficient mice fed a standard chow diet or a high-fat diet.

2. Methods

2.1. Experimental animals and dietary intervention

Male PON1-deficient mice of the C57BL/6J genetic background were the progeny of those provided to us by the Division of Cardiology of the University of California in Los Angeles [25]. These mice develop a mild degree of spontaneous liver steatosis even on a standard chow diet [26]. At 10 weeks of age, mice were fed a high-fat and high-cholesterol diet [HFD group; n=16; the diet contained w/w 20% fat and 1.00% cholesterol (Harlan, Barcelona, Spain)], or a chow diet [CD group; *n* = 16; the diet contained w/w 14% protein and 0.03 cholesterol (Harlan, Barcelona, Spain)]. The groups were further divided to receive metformin (*n* = 8) or placebo (regular drinking water; *n* = 8). Metformin (DIANBEN® 850 mg) was added to the water to achieve a dose of 166 $mg.Kg^{-}1.day^{-1}$. At 24 weeks of age, animals were sacrificed after an overnight fast. Liver, pancreas, visceral white adipose tissue (vWAT), epididimal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), and brown adipose tissue (BAT) were removed and weighed. Portions of tissue were stored at −80° C until needed for histological examination, at which stage the tissues were fixed for 24 h in 10% neutral-buffered formalin, embedded in wax, and microtome sectioned for microscopy. Glucose tolerance tests (GTT) were performed in all mice at one week before sacrifice. Glucose (2 $mg.g^{-1}$ of body weight) was administered as an intraperitoneal injection under anesthesia. Measurements of blood glucose concentrations were made at t = 0, 15, 30, 60 and 120 min. Glucose was measured with glucose strips adapted to the Accucheck sensor system (Roche Diagnostics).

Wild-type mice fed with chow diet or HFD and receiving metformin or placebo (n = 8, for each group) were used to investigate the effect of PON1-deficiency in liver histology. All procedures adhered to those described by the Helsinki accord on animal experimentation. The study protocol was accepted by the Ethics Committee on

Animal Experimentation of the Faculty of Medicine of the *Universitat Rovira i Virgili* (Reus).

2.2. Biochemical measurements

Following an overnight fast, blood samples were collected from anesthetized animals into blood collection tubes not containing anti-coagulant. Serum glucose, cholesterol and triglyceride concentrations together with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined by standard clinical laboratory procedures. Analysis of serum lipoprotein profiles was performed using fast protein liquid chromatography (FPLC), to evaluate differences in cholesterol and triglyceride distributions among the lipoprotein fractions in the different experimental groups. Briefly, a pooled serum (200 µL from each experimental group) was fractionated in a Superose 6/300 GL column (GE Healthcare Europe, Glattbrugg, Switzerland) equilibrated with phosphate buffer (NaPi) 50 mM, with NaCl 0.150 M, pH = 7.0 and eluted (500 µl fractions) with the same buffer. Cholesterol and triglycerides were measured in the eluted fractions using photometry, with reagents obtained from Beckman Coulter (Brea, CA, USA) and read with an automated microplate reader (BioTeK Instruments Inc., Winooski, VT, USA).

2.3. Histology analyses

Liver and eWAT sections of 2 µm thickness were stained with hematoxylin and eosin to evaluate histological alterations. Steatosis extent and eWAT adipocyte size were estimated by image analysis software (AnalySIS, Soft Imaging System, Munster, Germany). The degree of steatosis was further evaluated using a semi-quantitative score (percentage) of hepatocytes containing lipid droplets. The scores were arbitrarily dichotomized as 1: <33%; 2: 33−66%; 3: >66%, as previously reported [26]. Chemokine (C-C motif) ligand-2 (CCL2) expression was measured as a marker of inflammation using immunohistochemistry with specific antibodies from Santa Cruz Biotechnology (Heidelberg, Germany). F4/80 antigen was determined as a widely-accepted marker of macrophages, using specific antibodies from Serotec (Oxford, UK). For each sample, we included a negative control that was treated exactly as the test samples throughout, except with the primary antibody omitted from the incubations.

2.4. Western blot analysis

Using a Precellys 24 (Bertin Technologies, France) homogenizer, liver samples were homogenized in a lysis buffer containing an inhibitor of the proteases. FASn, AMPK, and its active form phosphorylated AMPK (pAMPK), were measured using specific antibodies from Cell Signaling Tech. (Danvers, MA, USA). Arginase and caspase-9 were measured using antibodies from Abcam Inc. (Cambridge, UK). Actin expression was used as control (antibodies from Sta. Cruz Biotech, CA, USA).

2.5. Statistical Analysis

Results are shown as means ± SD. Between group comparisons were with the Mann-Whitney *U* test. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Food intake and weight control

As expected, mice fed with HFD weighed more than animals fed with CD. Metformin administration did not produce any significant change in weight, nor in the cumulative food ingested in any of the animal groups (Fig. 1A). Metformin produced a significant increase of eWAT and vWAT weights, and a small reduction in liver weights in mice fed with CD, but not in animals fed with HFD. Metformin also produced a significant increase in pancreas weight in experimental groups of animals, relative to the group of control animals. We did not observe any significant differences in BAT and iWAT in relation to metformin administration (Fig. 1B).

3.2. Glucose tolerance test

Glucose tolerance was impaired in mice fed HFD compared to animals with CD, as shown by the areas under the curve of the GTT test results. Metformin administration significantly improved glucose tolerance in mice fed HFD, but did not produce any significant effect in mice fed CD (Fig. 1C).

3.3. Biochemical measurements

Metformin administration was associated with mild, but significant, reductions in baseline serum glucose concentration and AST activity in mice fed CD, and an important increase in serum triglycerides in animals fed HFD (Table 1). We did not

observe any significant change, associated with metformin administration, with respect to cholesterol or triglyceride distributions among lipoprotein fractions; neither in animals fed CD or those fed HFD (Supplementary Fig. 1).

3.4. Histological analyses

Hepatic steatosis scores were significantly increased in mice receiving metformin and CD compared to controls (CD and no metformin), while there was a trend, albeit statistically non-significant, towards a decrease in the scores in animals receiving HFD + metformin (Fig. 2A). With respect to eWAT, metformin administration was associated with a mild, but statistically significant, increase in adipocyte size in mice fed HFD but not in those fed CD (Fig. 2B). We did not observe any significant changes in iWAT, vWAT or BAT associated with metformin administration (data not shown). Metformin administration was associated with an increase in the staining of the pro-inflammatory marker CCL2 in CD as well as HFD-fed mice. However, the number of macrophages was increased only in animals fed HFD (Fig. 3).

3.5. Western blot analyses

Treatment with metformin produced a significant decrease in the pAMPK/AMPK ratio and in arginase expression in mice fed CD, and a significant decrease in FASn expression in mice fed HFD. With respect to caspase-9, we detected two bands of molecular weight 45 and 35 KDa. The 45 KDa band corresponded to the inactive form (procaspase-9). Mature procaspase-9 expression (35 KDa) was enhanced in mice fed HFD, compared to those fed CD. The administration of metformin in HFD mice produced an important reduction in procaspase-9 and a small reduction in caspase-9, while producing a significant increase in caspase-9 in CD animals. Arginase expression was significantly decreased in mice fed CD, and there was no significant change in HFD animals (Fig. 4).

3.6. Effect of metformin in liver histology in wild-type mice

To assess whether the deleterious effects of metformin were specific to PON1-deficient mice, we analyzed the influence of this product on hepatic steatosis and the number of macrophages in wild-type mice. Metformin administration did not produce any significant alteration in any of these parameters (Supplementary Fig. 2).

4. Discussion

Results of the present study show that metformin caused an aggravation of hepatic steatosis in the livers of PON1-deficient mice receiving CD, and a general increase in inflammation markers in animals fed either CD or HFD. Zhou et al. [27] reported that, in primary hepatocyte cultures, the activation of AMPK (measured as an increase of the ratio pAMPK/AMPK) was intimately associated with the pleiotropic actions of metformin. AMPK is activated by an enhancement in the intracellular AMP/ATP ratio resulting from an imbalance between ATP production and consumption. Further, metformin improved lipid metabolism by increasing fatty acid oxidation and inhibiting lipogenesis; an effect mediated, presumably, by AMPK activation [27-29]. Surprisingly, we did not observe an activation of AMPK in the liver of mice receiving metformin and fed either of the diets. We even found a decrease in pAMPK/AMPK ratio associated with metformin administration in mice fed CD. The explanation for these contradictory results might be due to our mice being PON1-deficient and having a certain degree of (mild) spontaneous steatosis. The effects of metformin in livers with steatosis remain unclear [30-32]. In our model, AMPK inactivation in mice receiving CD and metformin could explain the accumulation of fat, resulting in an increase in hepatic steatosis. Nevertheless, in mice receiving HFD, metformin administration produces the opposite effect i.e. a reduction in the accumulation of fat in the liver. This effect was associated with a reduction in FASn protein expression. Indeed, Kita et al. [32] had shown that hepatic FAS expression in metformin-treated mice was decreased. In our study, these observations were associated with an increase in eWAT adipocyte size. A possible explanation for this observation is that, in mice fed HFD, the channeling of fat towards an accumulation in eWAT is, perhaps, a defense mechanism to protect the liver.

Several studies have shown that metformin induces caspase-9 expression and apoptosis in several cell lines [33-35]. The caspase-9 findings are confirmed by the present investigation. For example, mice given CD and metformin had a significant increase in caspase-9 in its active form, while animals fed HFD had an important reduction in the expression of the inactive procaspase-9. However, the above-mentioned studies suggest that this effect is mediated through AMPK activation while

our results suggest that, on the contrary, AMPK is not necessary to explain the effects of metformin on caspase-9.

An unexpected result from the present investigation was that metformin administration caused pro-inflammatory changes in the livers of CD as well as HFD mice. All the animals had an increased presence of CCL2 in the liver. This chemokine is responsible for the recruitment of monocytes to sites of inflammation, followed by their differentiation to macrophages [36] and is considered pathognomonic of the onset of the inflammatory reaction. Previous studies from our group showed that it is a good marker of the severity of inflammation in patients with liver disease [37]. In addition, metformin was associated with an increase in the total number of macrophages in HFD-fed mice and, although the number of macrophages did not change in CD-fed animals, they had a significant decrease in arginase expression. Arginase is a marker of M2 macrophages (which play an anti-inflammatory role) and their decrease suggests an enhancement of the liver pro-inflammatory state [38].

We did not observe any significant deleterious effect of metformin administration with respect to the degree of steatosis or the number of macrophages in the livers of wild-type mice fed with either CD or HFD. This is not surprising since the beneficial effects of metformin in lean or obese mice have been documented extensively, already [39,40]. Indeed, the main goal of the present study was to show that these beneficial effects of metformin are completely reversed when PON1 is lacking (as in PON1-deficient mice).

In conclusion metformin administration in PON1-deficient mice produces significant undesirable effects in the liver. These effects vary depending on the diet administered. An increase in the severity of steatosis was observed in animals fed CD, together with an aggravation of inflammation irrespective of the diet administered. Since individuals with liver impairment have low hepatic and serum PON1 activities, this report is a cautionary note on the administration of metformin in these patients. In the case of therapeutic metformin in diabetes type 2, the advice would be regular monitoring of the patient to detect hepatic impairment and its progression.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1

Selected serum biochemical variables in PON1-deficient mice

Data presented as means (SD)

	Chow Diet			High Fat Diet		
	Control	Metformin	*P* value	Control	Metformin	*P* value
Glucose; mmol/L	14.9 (1.5)	12.9 (1.8)	0.0281	19.5 (3.2)	21.5 (3.8)	0.2766
Cholesterol; mmol/L	1.8 (0.2)	1.7 (0.2)	0.1949	3.8 (0.5)	3.7 (0.3)	0.7430
Triglycerides; mmol/L	0.7 (0.2)	0.5 (0.1)	0.0721	0.3 (0.1)	0.5 (0.2)	0.0148
ALT; μkat/L	2.8 (1.8)	1.6 (0.60)	0.2345	2.2 (1.0)	3.2 (1.5)	0.1996
AST; μkat/L	0.6 (0.2)	0.4 (0.1)	0.0426	0.6 (0.1)	1.0 (0.4)	0.1520
Bilirubin; μmol/L	3.4 (1.7)	3.3 (1.6)	0.1605	5.1 (1.4)	5.0 (1.3)	0.6730

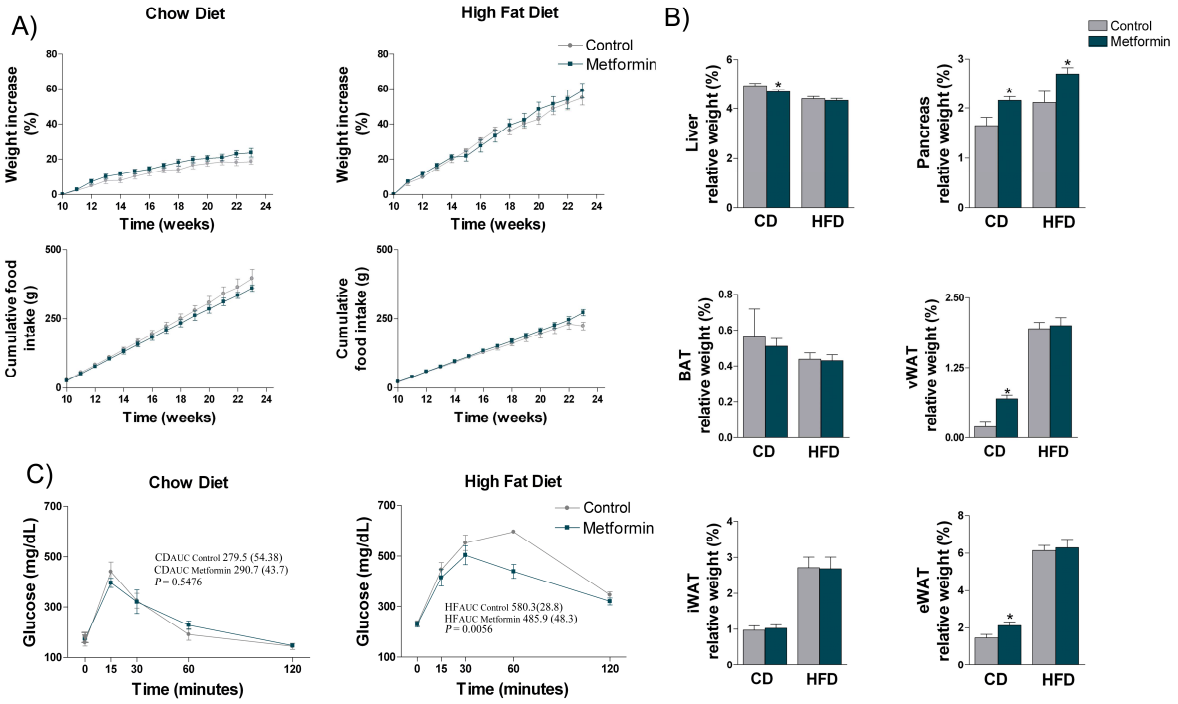
FIGURE LEGENDS

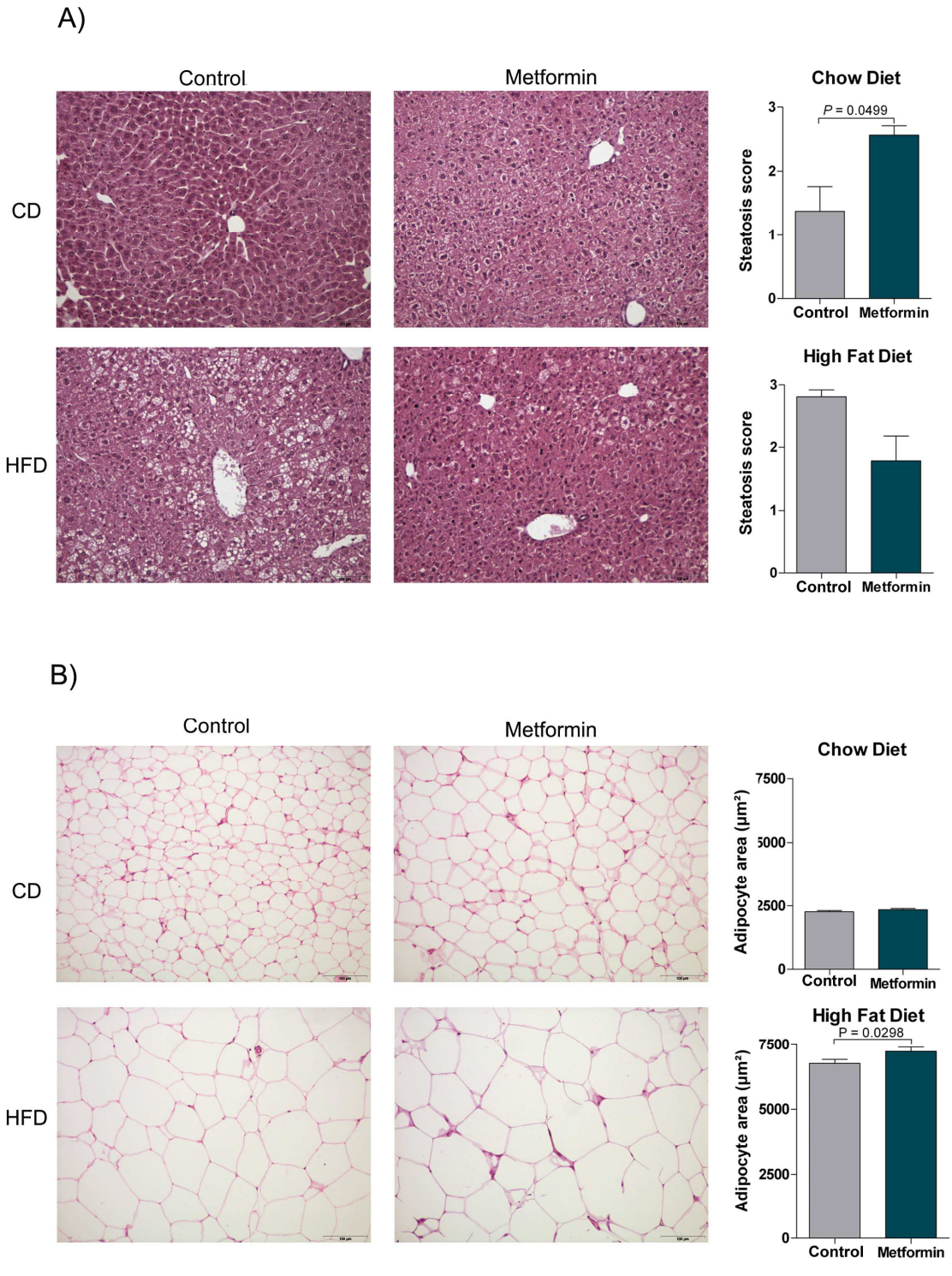
Fig. 1. Effects of metformin administration in PON1-deficient mice fed a chow diet (CD) and a high fat diet (HFD). A) Cumulative food intake and weight increase in mice having metformin administered and fed CD (left panel) and HFD (right panel) from 10 to 24 weeks of age. There were significant differences in weight increase between animals given CD or HFD at all time-points ($P < 0.01$). B) Relative weight of liver, pancreas, brown adipose tissue (BAT), visceral adipose tissue (vWAT), inguinal adipose tissue (iWAT) and epididimal white adipose tissue (eWAT) in mice fed CD or HFD. [a]$P < 0.05$, with respect to the control group; [b] $P < 0.01$, [c] $P < 0.001$, with respect to mice given CD diet. C) Metformin effect on blood glucose levels and area under the curve (AUC) in the glucose tolerance test in animals fed CD or HFD. AUC values are presented as means and SD

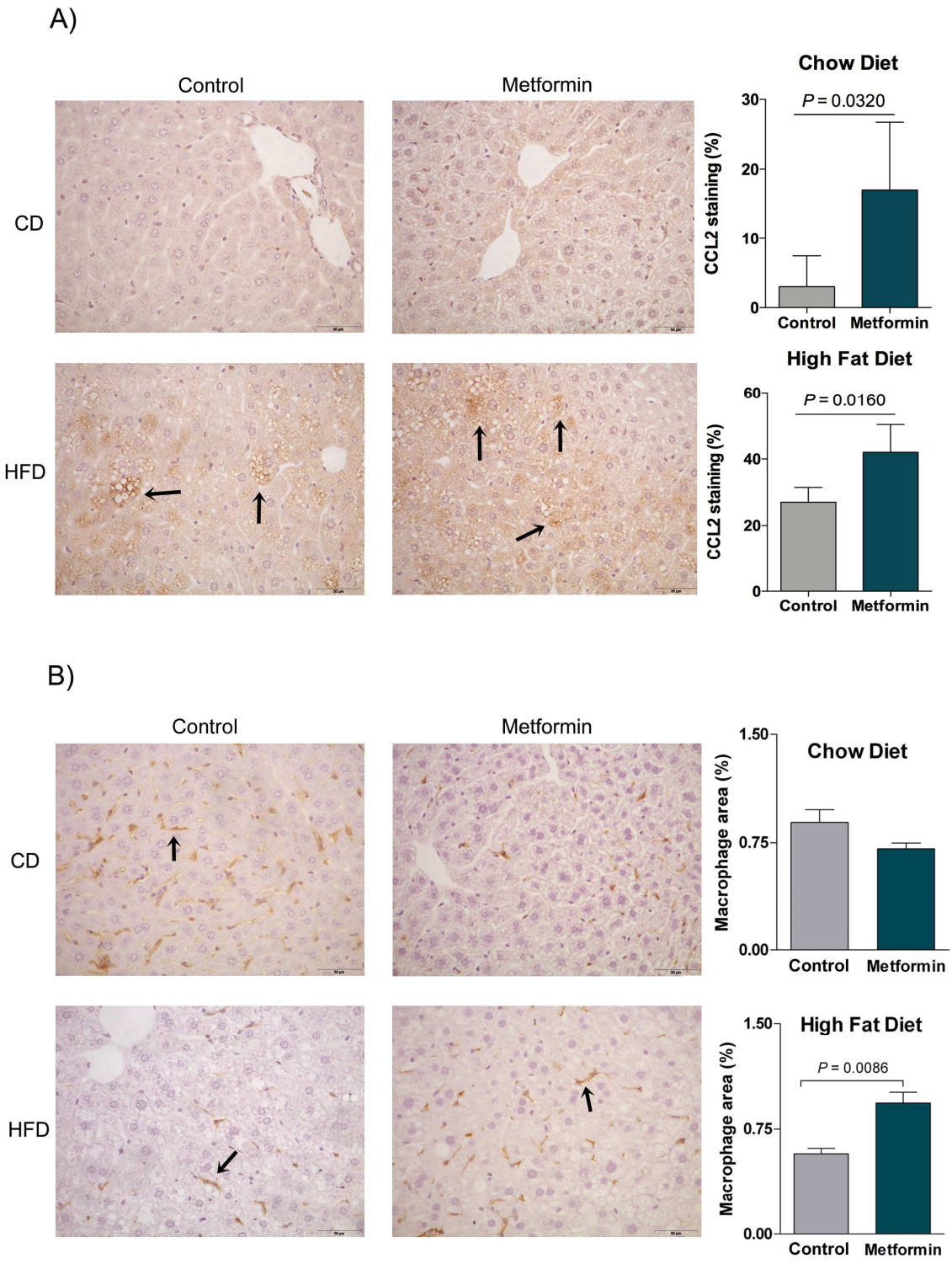
Fig. 2. Hematoxylin-eosin staining of the liver and eWAT of PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). The arrows show ballooning hepatocytes. Magnification x10

Fig. 3. Immunohistochemical analyses of liver tissues of PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). A) Immunochemical staining for CCL2. The arrows show positively-stained areas. B) Immunochemical staining for F4/80 and macrophage area quantification. The arrows show positive staining for F4/80. Magnification x20

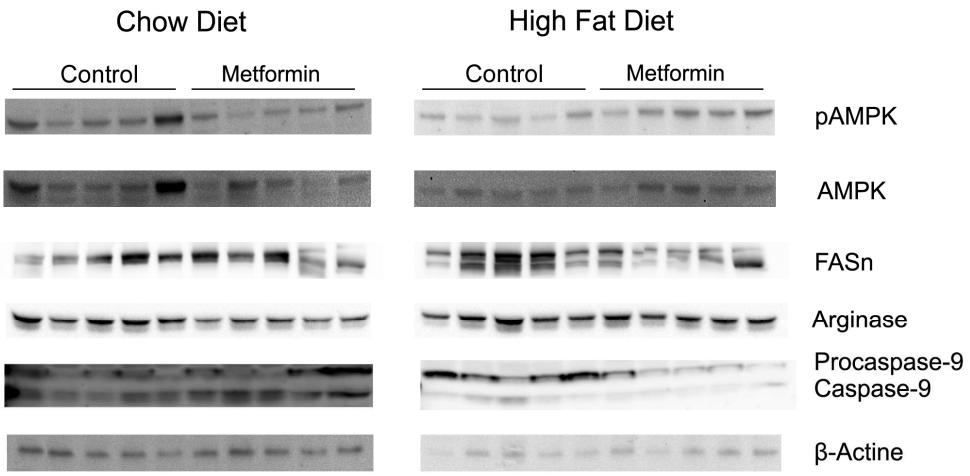
Fig. 4. Western blot analyses of liver in PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). A) Immunoblots for pAMPK, AMPK, FASn, arginase, procaspase-9 and caspase-9. B) Quantification of these immunoblots. Results are shown as arbitrary units (AU). [a] $P < 0.05$ with respect to the control group; [b] $P < 0.01$ with respect to mice given CD diet.



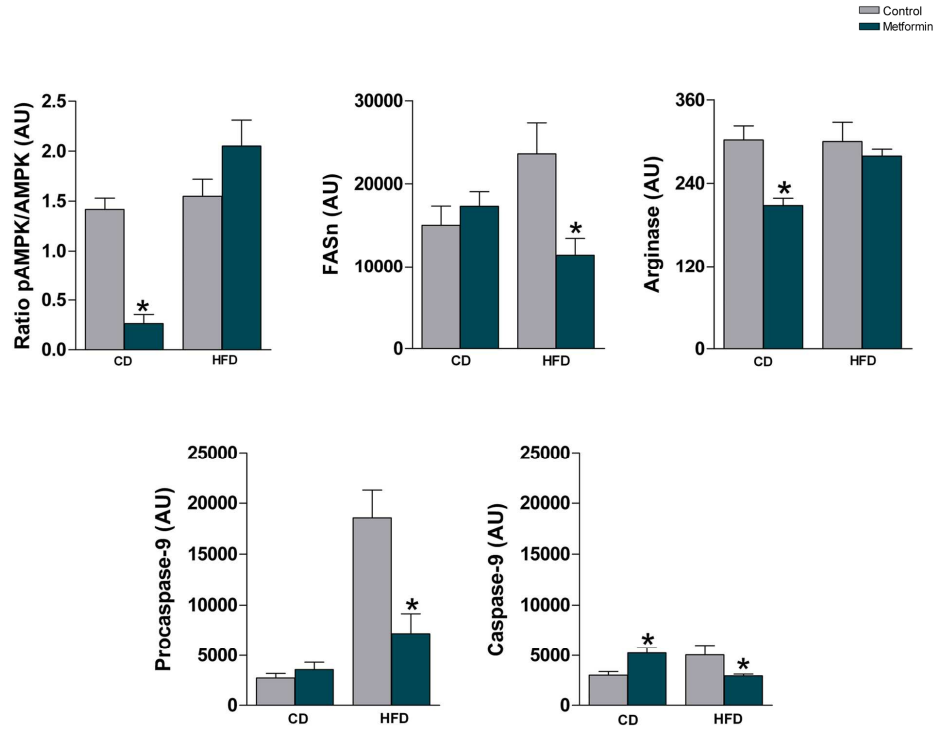

A)
Control
Metformin
CD
HFD
Chow Diet
P = 0.0499
Steatosis score
0
1
2
3
Control
Metformin
High Fat Diet
Steatosis score
0
1
2
3
Control
Metformin
B)
Control
Metformin
CD
HFD
Chow Diet
Adipocyte area (µm²)
0
2500
5000
7500
Control
Metformin
High Fat Diet
P = 0.0298
Adipocyte area (µm²)
0
2500
5000
7500
Control
Metformin


A)
Control
Metformin
CD
HFD
Chow Diet
P = 0.0320
CCL2 staining (%)
30
20
10
0
Control
Metformin
High Fat Diet
P = 0.0160
CCL2 staining (%)
60
40
20
0
Control
Metformin
B)
Control
Metformin
CD
HFD
Chow Diet
Macrophage area (%)
1.50
0.75
0.00
Control
Metformin
High Fat Diet
P = 0.0086
Macrophage area (%)
1.50
0.75
0.00
Control
Metformin

A)


Chow Diet
High Fat Diet
Control
Metformin
Control
Metformin
pAMPK
AMPK
FASn
Arginase
Procaspase-9
Caspase-9
β-Actine

B)


Control
Metformin
Ratio pAMPK/AMPK (AU)
FASn (AU)
Arginase (AU)
Procaspase-9 (AU)
Caspase-9 (AU)
CD
HFD