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Paraoxonase 1 and dietary hyperhomocysteinemia modulate the expression of mouse proteins involved in liver homeostasis

Joanna Suszyńska-Zajczyk[1] and Hieronim Jakubowski[1,2,3]✉

[1]Institute of Bioorganic Chemistry, Poznań, Poland; [2]Department of Biochemistry and Biotechnology, University of Life Sciences, Poznań, Poland; and [3]Department of Microbiology, Biochemistry and Molecular Genetics, Rutgers-New Jersey Medical School, International Center for Public Health, Newark, NJ, USA

Homocysteine (Hcy), a product of methionine metabolism, is elevated by the consumption of a high-methionine diet that can cause fatty liver disease. Paraoxonase 1 (Pon1), a hydrolase expressed mainly in the liver and carried in the circulation on high-density lipoprotein, participates in Hcy metabolism. Low Pon1 activity is linked to fatty liver disease. We hypothesize that hyperhomocysteinemia and low Pon1 induce changes in gene expression that could impair liver homeostasis. To test this hypothesis, we analyzed the liver proteome of *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice fed a high methionine diet (1% methionine in the drinking water) for 8 weeks using 2D IEF/SDS-PAGE gel electrophoresis and MALDI-TOF mass spectrometry. We identified seven liver proteins whose expression was significantly altered in *Pon1*$^{-/-}$ mice. In animals fed with a control diet, the expression of three liver proteins involved in lipoprotein metabolism (ApoE), iron metabolism (Ftl), and regulation of nitric oxide generation (Ddah1) was up-regulated by the *Pon1*$^{-/-}$ genotype. In mice fed with a high-methionine diet, expression of four liver proteins was up-regulated and of three proteins was down-regulated by the *Pon1*$^{-/-}$ genotype. The up-regulated proteins are involved in lipoprotein metabolism (ApoE), energy metabolism (Atp5h), oxidative stress response (Prdx2), and nitric oxide regulation (Ddah1). The down-regulated proteins are involved in energy metabolism (Gamt), iron metabolism (Ftl), and catechol metabolism (Comt). Expression of one protein (Ftl) was up-regulated both by the *Pon1*$^{-/-}$ genotype and a high-methionine diet. Our findings suggest that Pon1 interacts with diverse cellular processes — from lipoprotein metabolism, nitric oxide regulation, and energy metabolism to iron transport and antioxidant defenses — that are essential for normal liver homeostasis and modulation of these interactions by a high-methionine diet may contribute to fatty liver disease.

Key words: Pon1, high-methionine diet, hyperhomocysteinemia, mouse liver proteome

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INTRODUCTION

Elevated homocysteine (Hcy) levels have long been known to be linked to liver disease. Indeed, fatty liver is a common finding in nutritionally induced hyperhomocysteinemia (HHcy) due to methionine (Met) overload, folate deficiency, or excessive alcohol intake (Werstuck *et al.,* 2001; Hirsch *et al.,* 2005; Kaplowitz *et al.,* 2007). Although there is evidence suggesting that Hcy-induced endoplasmic reticulum and oxidative stress mediates liver damage by promoting apoptotic cell death, inflammation, insulin resistance, and deregulated lipid metabolism (Kaplowitz *et al.,* 2007), the exact mechanism underlying Hcy-induced liver damage is unclear.

Paraoxonase 1 (PON1), named for its ability to hydrolyze the organophosphate paraoxon (Costa *et al.,* 2013), is expressed in the liver, kidney, brain, and colon (Mackness *et al.,* 2010), circulates in the blood attached to high-density lipoproteins (HDL), and is localized to all organs (Marsillach *et al.,* 2008). Clinical studies have linked PON1 activity to cardiovascular disease (Domagala *et al.,* 2006; Bayrak *et al.,* 2012) and it has been found that Pon1 protects against high-fat diet-induced atherosclerosis in mice (Shih *et al.,* 1998) and humans (Bhattacharyya *et al.,* 2008).

Several studies have also linked PON1 to liver disease. For example, serum PON1 activity decreases in patients with chronic hepatitis or cirrhosis and the magnitude of the decrease correlates to the extent of liver damage (Ferre *et al.,* 2002; Marsillach *et al.,* 2007). Serum PON1 activity is also lower in cows suffering from fatty liver compared with healthy animals (Farid *et al.,* 2013). In experimental rat cirrhosis model, the decrease in Pon1 activity is also correlated with the extent of liver damage (Ferre *et al.,* 2001). Furthermore, in mouse models, deletion of the *Pon1* gene increases the frequency of fatty liver in animals fed with a high-fat diet (Garcia-Heredia *et al.,* 2013) while overexpression of Pon1 protects against the development of liver disease induced by CCl_4 (Zhang *et al.,* 2008). Because *Pon1*-knockout animals exhibit elevated levels of oxidative stress markers, the hepatoprotective function of Pon1 has been suggested to be due to its ability to act as an antioxidant (Garcia-Heredia *et al.,* 2013). However, the mechanism underlying the anti-oxidative function of Pon1 is not clear (Perla-Kajan & Jakubowski, 2010; 2012).

Pon1 occupies a juncture between the metabolisms of HDL/PON1 and Hcy (Jakubowski, 2008b) that may ac-

✉e-mail: jakubows@njms.rutgers.edu

Abbreviations: Hcy, homocysteine; Met, methionine; Pon1, paraoxonase 1; Cbs, cystathionine β-synthase; ApoA-I, apolipoprotein A-I; ApoE, apolipoprotein E; Atp5h, ATPase subunit d; Comt, catechol-*O*-methyltransferase; Ddah1, dimethylarginine dimethylaminohydrolase 1; Ftl1, ferritin light subunit; Gamt, guanidinoacetate-*N*-methyltransferase; IEF/SDS-PAGE, isoelectric focusing/sodium dodecylsulphate polyacrylamide gel electrophoresis; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; Prdx2, peroxiredoxin 2.

count for their role in liver disease. For example, in humans Hcy is a negative determinant of HDL and PON1 activity (Lacinski *et al.,* 2004; Wehr *et al.,* 2009) and attenuates *Apoa1* and *Pon1* gene expression both in mice and humans (Robert *et al.,* 2003; Liao *et al.,* 2006; Jiang *et al.,* 2012). We found that HDL and purified PON1 protein have the ability to hydrolyze Hcy-thiolactone (Jakubowski 2000), thereby protecting against protein *N*-homocysteinylation in humans (Perla-Kajan & Jakubowski, 2010) and mice (Borowczyk *et al.,* 2012a). *N*-Homocysteinylation causes protein damage (Jakubowski, 1999; Glowacki & Jakubowski, 2004) and is linked to atherosclerosis (Perla-Kajan *et al.,* 2008), stroke (Undas *et al.,* 2004), and coronary artery disease (Undas *et al.,* 2005). *N*-Hcy-proteins, including *N*-Hcy-HDL (Jakubowski, 2002) and *N*-Hcy-ApoA-I (Ishimine *et al.,* 2010), are present at basal levels in normal human plasma and increase in hyperhomocysteinemic individuals (Jakubowski *et al.,* 2008). *N*-Hcy-proteins are also elevated in the liver and plasma of hyperhomocysteinemic *Cbs*$^{-/-}$ mice (Jakubowski *et al.,* 2009). *In vitro*, *N*-homocysteinylation of HDL and Pon1 causes a loss of their atheroprotective function *(Ferretti et al., 2003*). We have also found that the *Pon1*-null mice show impaired metabolic conversion of Hcy-thiolactone to Hcy, elevated brain Hcy-thiolactone levels, and increased susceptibility to the neurotoxic effects of Hcy-thiolactone (Borowczyk *et al.,* 2012a). Taken together, these findings indicate that Pon1 plays an important role in Hcy metabolism.

We hypothesize that interactions between Pon1 deficiency and HHcy impair liver homeostasis. To gain insight into the role of Pon1 in the liver and to identify metabolic pathways regulated by Pon1 and HHcy, we examined liver proteomes of *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice fed with a control diet or a high-Met diet (1% Met in drinking water) for 8 weeks using 2D IEF/SDS-PAGE gel electrophoresis and MALDI-TOF mass spectrometry.

MATERIALS AND METHODS

Mice and diets. Colonies of *Pon1*$^{-/-}$ mice on the C57BL/6J genetic background (Shih *et al.,* 1998) and wild type *Pon1*$^{+/+}$ littermates were bred and housed at the New Jersey Medical School Animal Facility. Female mice (n = 8 per group) were maintained on a standard rodent chow diet (LabDiet 5010, Purina Mills International, St. Louis, MO; contains 0.66% methionine). At 4 weeks of age, half of *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice were provided with 1% methionine in drinking water (high-Met diet) for 8 weeks (Velez-Carrasco *et al.,* 2008, Borowczyk *et al.,* 2012b). Four experimental groups of animals were studied: 1) *Pon1*$^{-/-}$ mice, control diet; 2) *Pon1*$^{+/+}$ mice, control diet; 3) *Pon1*$^{-/-}$ mice, high-Met diet; 4) *Pon1*$^{+/+}$ mice, high-Met diet. Supplementation of drinking water with 1% Met did not affect water intake by the mice. Consumption of 1% Met in drinking water did not affect body weight of the mice. Animal procedures were approved by the Institutional Animal Care and Use Committee at the Rutgers-New Jersey Medical School.

Genotyping. To establish the status of the *Pon1* locus, genomic DNA was isolated and genotyped by PCR using the *Pon1* forward primer p1 (5'-TGGGCTGCAGGTCTCAGGACTGA-3'), *Pon1* exon 1 reverse primer p2 (5'-ATAGGAAGACCGATGGTTCT-3'), and neomycin cassette reverse primer p3 (5'-TCCTCGTGCTTTACGGTATCG-3') (Shih *et al.,* 1998). Briefly, the 10 μL PCR mixture contained 100 ng purified mouse DNA, 5μl PCR MasterMix (Fermentas), 0.5 μL primer p1, p2, p3, 0.5 units of Taq polymerase (Fermentas) and water to 10 μL. The thermal cycling reaction was run for 34 cycles of 92°C for 30 s, 65°C for 40 s and 72°C for 90 s. The 144 bp amplicon from the *Pon1*$^{+/+}$ wild-type allele (obtained with p1, p2 primers) and the 240 bp amplicon from the *Pon1*$^{-/-}$ knockout allele (obtained with p1, p3 primers) were distinguished on a 1.5% agarose gel stained with SYBRSafe (Invitrogen) (Suszynska-Zajczyk *et al.,* 2014). Pon1 genotype was confirmed by enzymatic assays of serum paraoxonase (POase) and arylesterase (PhAcase) activities (Perla-Kajan & Jakubowski, 2010).

Enzymatic assys. POase and PhAcase activity assays were carried out at 25°C as previously described (Perla-Kajan & Jakubowski, 2010). Reactions were initiated by adding 5 μL serum to 500 μL mixtures containing 50 mM K-HEPES buffer (pH 7.4), 1 mM $CaCl_2$, and 2 mM paraoxon or 5 mM phenyl acetate. For POase activity assays, the generation of *p*-nitrophenol from paraoxon was monitored at 412 nm for 2-min time periods, and reaction rates (A_{412}/min) were calculated. For PhAcase activity assays, the generation of phenol from 5 mM phenyl acetate (PhAc) was monitored at 270 nm for 2 min, and rates (A270/min) were calculated from the first 0.5 min of the reaction. Controls in which Pon1 was inactivated with 1 mM EDTA were subtracted from the results (0.10–0.14 A_{270}/min and 0.0001A_{412}/min for reactions with PhAc and paraoxon, respectively).

Hcy assays. Total Hcy and *N*-Hcy-protein were assayed by HPLC-based methods with post-column derivatization and fluorescence detection as previously described (Jakubowski 2008a; Jakubowski *et al.,* 2008; Jakubowski *et al.,* 2009).

Protein extraction. Liver proteins were extracted using the phenol method (Faurobert *et al.,* 2007) as previously described (Suszynska-Zajczyk *et al.,* 2014a). Briefly, liver tissue was disintegrated by grinding with dry ice using a mortar and pestle. A 100 mg portion of the pulverized liver material was extracted with 0.9 mL of extraction buffer (0.5 M Tris/HCl pH 7.5, 50 mM EDTA, 0.1 M KCl, 0.7 M sucrose, 2% w/v DTT) containing protease inhibitors (Protease Inhibitor Mix, GE Healthcare) and 1 mL phenol containing 0.1 % hydroxyquinoline with vigorous shaking (10 min, 4°C). The mixture was centrifuged (12 000 ×*g*, 10 min, 4°C), the phenol layer collected, and extracted again with an equal volume of the extraction buffer. The phenol layer was separated by centrifugation, collected, and the proteins precipitated with 5 volumes of 0.1 M ammonium acetate in methanol (–80°C, 2 days). The protein pellet was collected by centrifugation (12 000 ×*g*, 10 min, 4°C), washed 3 times with 0.1 M ammonium acetate in methanol, followed by 5-min washes with 80% and 100% acetone, and allowed to air dry.

Liver protein samples were dissolved in IEF rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS). Insoluble material was removed by centrifugation (16 000 ×*g*, 20 min). Protein concentration was determined using a commercial 2-D Quant kit (GE Healthcare).

Two-dimensional IEF/SDS-PAGE. Liver protein separations and image analysis were carried out as previously described (Luczak *et al.,* 2011; Suszynska-Zajczyk *et al.,* 2014a). IPG strips (11 cm, pH 4–7, GE Healthcare) were rehydrated overnight in IEF buffer containing liver protein samples (0.3 mg/strip), 55 mM DTT, 0.5% (v/v) ampholite pH 4–10 (GE Healthcare). The strips were subjected to IEF on IPGphor III apparatus (GE Healthcare) using a ramping voltage (50–6000 V) to final 25 000 Vh. After IEF, IPG strips were incubated for 15 min in an equilibration buffer (6 M urea, 2% w/v SDS, 30% v/v

glycerol, 50 mM Tris/HCl, pH 8.8) containing 1% w/v DTT during the first equilibration step and 2.5% iodoacetamide w/v during the second equilibration step. The second dimension was carried out using 11% polyacrylamide gels (24 × 24 cm) on an Ettan DALT six system (GE Healthcare) according to the manufacturer's instructions. For each sample, a 2D analysis was repeated three times. After electrophoresis, gels were stained with Blue Silver overnight (Candiano *et al.,* 2004) and scanned using an Umax scanner and LabScan software (GE Healthcare).

The images were analyzed using the Image Master Platinum software version 7.0 (GE Healthcare). Spots were detected automatically without filtering. Gel patterns were automatically matched between groups. In addition, all individual matched spots were validated manually to ensure the correctness of spot matching. For each identified protein, the relative abundance (% Volume) was calculated from its area and intensity divided by the total volume of all protein spots on a gel. This procedure corrects for small variations between individual gels due to protein loading and staining (Luczak *et al.,* 2011).

Mass spectrometry. Mass spectrometry analyses have been carried out as previously described (Luczak *et al.,* 2011; Suszynska-Zajczyk *et al.,* 2014b). Briefly, protein spots were manually excised from gels using Pasteur pipets, transferred to Eppendorf tubes, de-stained by series of washes with 50 mM ammonium bicarbonate, 25 mM ammonium bicarbonate/50% acetonitrile, and dehydrated with neat acetonitrile according to a procedure described in (Shevchenko & Shevchenko, 2001). The dried gel pieces were digested with 10 μL 20 ng/μL trypsin (Promega), 25 mM ammonium bicarbonate (37°C, 16 h). Tryptic peptides were recovered from gel pieces by adding acetonitrile (to 10%), sonication in an ultrasound bath for 5 min, followed by 0.5 h incubation at 4°C.

The proteins were identified using UltrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Germany) mass spectrometer operating in reflector mode. Positively charged ions in the m/z range 850–3500 were analyzed. 0.5 μL of the sample was co-crystallized with a CHCA matrix and spotted directly on MALDI AnchorChip 800 nm target (Bruker Daltonics). For data validation, external calibration was performed with a standard mixture of peptides with masses ranging from 700 to 3500 Da (Peptide Calibration Standards 1 – Bruker). Standards were spotted on calibration spots and calibration was performed after each four samples (samples surrounding calibration spot). Flex control v 3.3 was used for the acquisition of spectra and all further data processing was carried out using Flex analysis v 3.3. Monoisotopic peptide masses were assigned and used for databases search. Additionally five most intensive peaks for each sample were chosen to be fragmented in LIFT mode. MS and MS/MS spectra acquired for each sample were combined and used for Mascot MS/MS Ion Search. For data processing and Mascot (Matrix Science, London, UK) analysis Bruker BioTools 3.2 package was employed. The proteins were identified against UniProtKB/Swiss-Prot protein database. The protein search was done using the following search parameters: MS mass tolerance ±0.2 Da, MS/MS mass tolerance 0.5 Da, one allowed missed cleavage, cysteine treated with iodoacetamide to form carbamidomethyl-cysteine and methionine in the oxidized form.

Data treatment and statistical analysis. For each animal in the four experimental groups (4 animals/group), the analyses were repeated 2–3 times. The relative abundance of each protein spot (% Volume) was calculated as its volume divided by the total volume of all spots (Luczak *et al.,* 2011). Data are expressed as mean ±S.D. Data for each protein spot had a normal distribution. The differences between the groups were analyzed by ANOVA. Unpaired Student's *t*-test was used to test differences between two groups. Statistical analyses were carried out using Statistica 8.0 software.

RESULTS

Dietary hyperhomocysteinemia in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice

To identify genes regulated by *Pon1* genotype and to examine the interaction between the *Pon1* genotype and HHcy, we analyzed liver proteomes of *Pon1*$^{-/-}$ mice and their *Pon1*$^{+/+}$ littermates in the absence and presence of HHcy. We used a mouse model of dietary HHcy in which feeding a high-Met diet elevates plasma Hcy (Zhou *et al.,* 2001), and leads to hepatic steatosis after prolonged (16–20-week) exposure (Werstuck *et al.,* 2001). The extent of HHcy was assessed by measurements of plasma tHcy and *N*-Hcy-protein levels. Plasma tHcy levels in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice fed a standard chow diet were 8.5±1.9 μM and 7.4±2.2 μM, and increased to 48±16 μM and 77±45 μM, respectively, in animals fed with a high-Met diet for 8 weeks. These levels of HHcy are known to induce the accumulation of cholesterol and triglycerides in mouse livers with no apparent fibrosis or necrosis after 16–20 weeks (Werstuck *et al.,* 2001). Plasma *N*-Hcy-protein levels increased from basal levels of 1.4±0.5 μM (*Pon1*$^{-/-}$) and 1.2±0.4 μM (*Pon1*$^{+/+}$) to 3.8±1.8 μM and 5.4±2.9 μM in hyperhomocysteinemic *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice, respectively.

Identification of differentially expressed proteins in *Pon1*$^{-/-}$ mouse liver

Mouse liver protein separation by IEF/SDS-PAGE yielded several hundred distinct protein spots (Fig. 1),

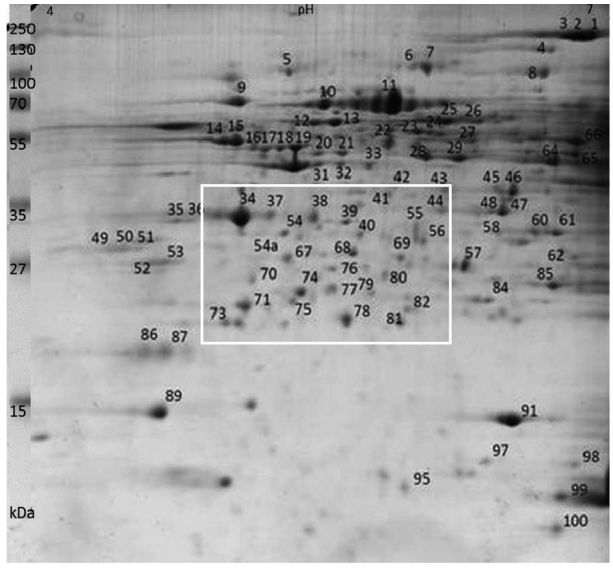

Figure 1. Representative IEF/SDS-PAGE gel showing the liver proteome of a wild type C57BL/6J mouse.
Left to right: IEF pH gradient from pH 4 to pH 7. Top to bottom: SDS-PAGE; molecular weight markers, 10 to 100 kDa, shown on the left. Numbers indicate spots of proteins whose identity has been established by mass spectrometry. White rectangle outlines an area containing proteins whose expression was affected by the *Pon1* genotype and/or high-Met diet. Identities of the differentially expressed proteins are described in Table 1. Identities of other proteins are described in ref. (Suszynska-Zajczyk *et al.,* 2014a).

Table 1. Characteristics of C57BL/6J mouse liver proteins regulated by *Pon1*$^{-/-}$ genotype identified by proteomic analyses[1]

Spot No.	Protein name	Theoretical Mass (kDa)	pI	Sequence coverage (%)	Matched peptides (n)	Gene name	Accession No.	Score	% Volume
39	Apolipoprotein E	35.9	5.46	35	13	Apoe	P08226	119	0.30
41	Dimethylarginine dimethylaminohydrolase 1	31.6	5.63	39	10	Ddah1	Q9CWS0	103	0.15
73	Peroxiredoxin-2	21.9	5.20	35	5	Prdx2	Q61171	93	0.37
75	Catechol-O-metyltransferase	29.7	5.52	37	8	Comt	O88587	89	0.21
76	Guanidinoacetate *N*-methyltransferase	26.6	5.43	31	5	Gamt	O35969	69	0.20
77	Apolipoprotein A-I	30.4	5.52	30	8	Apoa1	Q00623	81	0.56
78	ATP synthase subunit d, mitochondrial	18.7	5.52	50	9	Atp5h	Q9DCX2	142	0.71
81	Ferritin light chain 1	20.8	5.66	49	8	Ftl1	P29391	128	0.34

[1]Spot numbers are identical to those shown in Fig. 1. Values of the % Volume are averages of 6 measurements for indicated protein in wild type *Pon1*$^{+/+}$ mice.

100 of which have been identified by MALDI-TOF mass spectroscopy (Suszynska-Zajczyk *et al.,* 2014). Eight of these proteins were found to have significantly changed expression in response to *Pon1*$^{-/-}$ genotype and/or a high-Met diet. The expression levels of the other identified proteins were not altered by the *Pon1* genotype or high-Met diet. Characteristics of the differentially expressed proteins are shown in Table 1. Close-up views of representative IEF/SDS-PAGE separations of differentially expressed proteins are shown in Fig. 2. Quantification of the levels (% Volume) for each of the differentially expressed protein is shown in Fig. 3.

Liver proteins regulated by *Pon1* genotype

In mice fed with a standard chow diet the differential expression (*Pon1*$^{-/-}$ *vs.* *Pon1*$^{+/+}$ mice) of three liver proteins was higher (1.20 to 1.27-fold, $P<0.01$) (Table 2). The proteins up-regulated by the *Pon1*$^{-/-}$ genotype are involved in lipoprotein metabolism (apolipoprotein E, ApoE), regulation of nitric oxide generation (dimethylarginine dimethylaminohydrolase 1, Ddah1), and iron metabolism (ferritin light chain, Ftl) (Table 2).

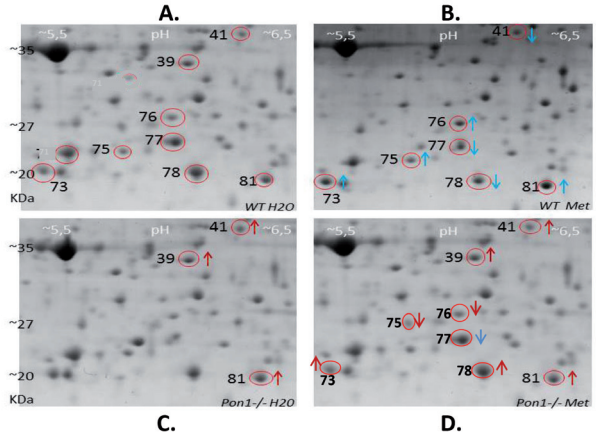

Figure 2. Close-up views of representative IEF/SDS-PAGE gels showing mouse liver proteins whose expression was affected by *Pon1* genotype and/or high-Met diet.
Analyses were carried out for the following groups of mice: panel A – *Pon1*$^{+/+}$, control diet; panel B – *Pon1*$^{+/+}$, high-Met diet; panel C – *Pon1*$^{-/-}$, control diet; panel D – *Pon1*$^{-/-}$, high-Met diet. Up and down arrows indicate the direction of the change dependent of *Pon1* genotype (panels C and D) and high-Met diet (panel B).

Liver proteins regulated by high-Met diet

In wild type *Pon1*$^{+/+}$ mice, 1%-Met diet significantly down-regulated three liver proteins (apolipoprotein A-I – ApoA-I, Atp5h, and Ddah1; –1.14 to –1.47-fold, $P<0.001$) and up-regulated four proteins (Gamt, Ftl, Prdx2, catechol-*O*-methyltransferase — Comt (1.22 to 1.68-fold, $P<0.01$; Table 2). With the exception of Ftl and Ddah1, the expression of these proteins was not significantly altered by the *Pon1*$^{-/-}$ genotype in mice fed a control diet (Table 2).

Liver proteins regulated by *Pon1* genotype and high-Met diet

The expression of four proteins: Gamt, Atp5h, Prdx2, and Comt, became dependent on the *Pon1*$^{-/-}$ genotype only in mice fed with high-Met diet and was decreased –1.45 to –2.04-fold, $P<0.001$ (Gamt, Comt) or increased 1.15 to 1.32-fold, $P<0.01$ (Atp5h, Prdx2) in *Pon1*$^{-/-}$ animals (Table 2). The expression of one protein, ApoE, was dependent on the *Pon1*$^{-/-}$ genotype regardless of the presence or absence of the dietary HHcy (Table 2). For one protein, Ftl, direction of the regulation by the *Pon1*$^{-/-}$ genotype was dependent on the diet: the up-regulation of Ftl by the *Pon1*$^{-/-}$ genotype, observed in mice fed with the standard chow diet, was changed to down-regulation in animals fed with high-Met diet (Table 2).

Western blot analysis was performed for ApoA-I and Ftl to validate the IEF/SDS-PAGE results. As shown in Fig. 4, ApoAI was lowered by high-Met diet both in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice (Fig. 4A), similar to the results obtained by the IEF/SDS-PAGE analysis (Fig. 4D). Western blot analysis also showed that Ftl was elevated by high-Met diet in *Pon1*$^{+/+}$ mice, but not in *Pon1*$^{-/-}$ animals (Fig. 4B), consistent with the results of the IEF/SDS-PAGE analyses (Fig. 4E).

High-Met diet reduces serum Pon1 levels

Hepatic Pon1 expression and activity (Robert *et al.,* 2003) as well as serum Pon1 activity (Jiang *et al.,* 2012)

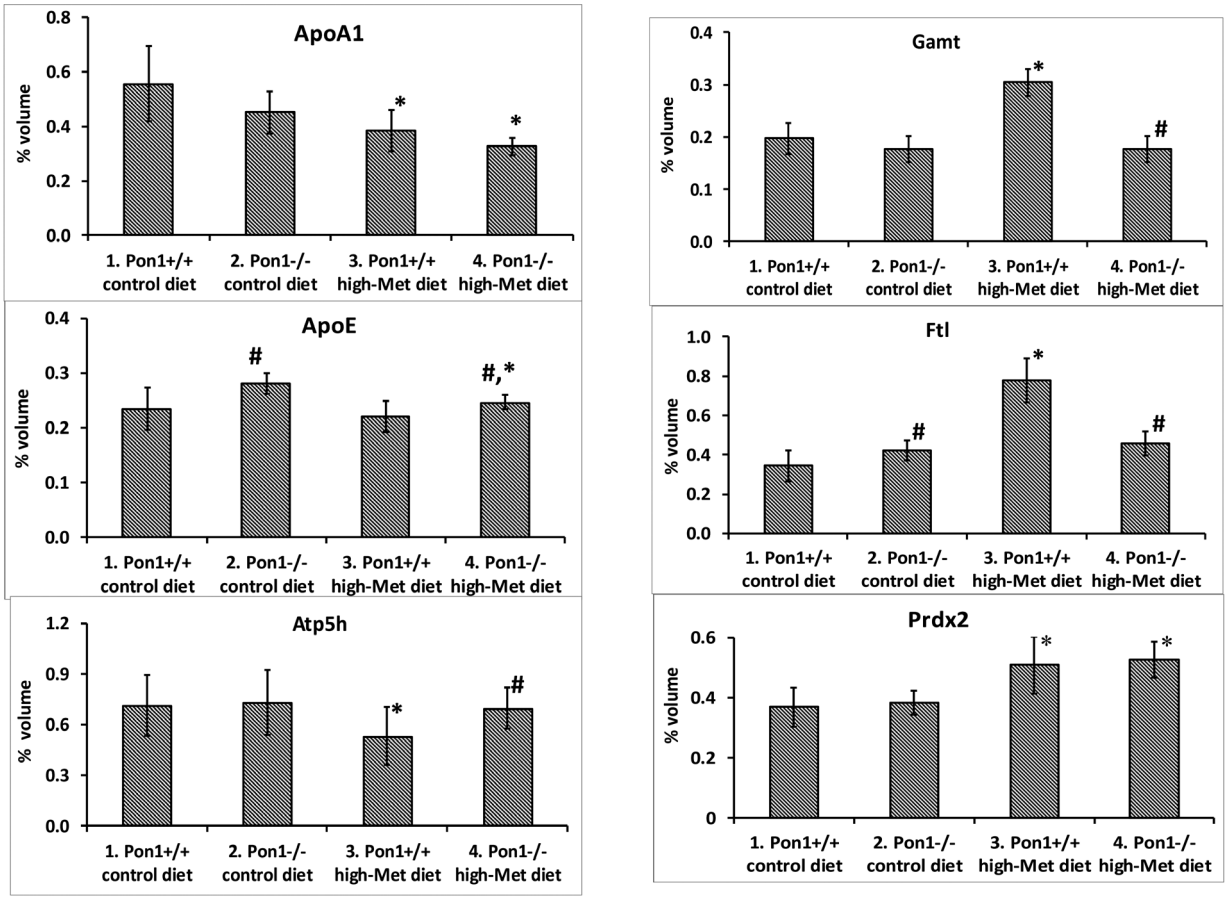

Figure 3. Mouse liver protein expression levels as a function of *Pon1* genotype and/or high-Met diet.
Each panel shows expression level (% volume) for the indicated liver protein determined for the following groups of mice: 1 – *Pon1*$^{+/+}$, control diet; 2 – *Pon1*$^{-/-}$, control diet; 3 – *Pon1*$^{+/+}$, high-Met diet; 4 – *Pon1*$^{-/-}$, high-Met diet. Symbols # and * indicate significant ($P<0.05$) effects of the *Pon1*$^{-/-}$ genotype and high-Met diet, respectively.

were reported to be significantly reduced in severely hyperhomocysteinemic *Cbs*$^{-/-}$ mice. Hepatic Pon1 activity is also reduced in *Cbs*$^{+/-}$ mice fed with 0.5% Met in drinking water for 8 weeks; these mice have mild HHcy with plasma tHcy = 15 μM (Robert *et al.,* 2003) . Thus, it is likely that dietary hyperhomocysteinemia with plasma tHcy = 77 μM in our wild type *Pon1*$^{+/+}$ mice would also lead to reduced Pon1 expression. To examine this possibility, we measured Pon1 activity in serum from wild type *Pon1*$^{+/+}$ mice fed with a hyperhomocysteinemic high-Met diet. We found that POase and PhAcase activities were significantly reduced in wild type mice that were fed with a high-Met-diet (by 18–22% in females and 40% in males; Table 3). Consistent with previous work (Shih *et al.,* 1998), *Pon1*$^{-/-}$ mice did not exhibit any Pon1 activity (Table 3). The reduction of Pon1 activity by HHcy may contribute to altered protein expression induced by a high-Met diet.

DISCUSSION

In the present work we used *Pon1*$^{-/-}$ mice in a proteomic study to discover metabolic pathways regulated by the *Pon1* genotype and high-Met diet in the liver. We found that: 1) liver proteins involved in lipid homeostasis (ApoE), energy metabolism (Atp5h, Gamt), iron transport (Ftl), oxidative stress response (Prdx2), catechol metabolism (Comt), and nitric oxide regulation (Ddah1) were regulated by the *Pon1*$^{-/-}$ genotype; 2) high-Met diet interacts with the *Pon1*$^{-/-}$ genotype to modify its effects on protein expression; 3) proteins involved in

Table 2. Differentially expressed liver proteins regulated by *Pon1*$^{-/-}$ genotype and/or high-Met diet.

Protein description (Spot #)[1]	Gene name	Fold change *Pon1*$^{-/-}$ *vs. Pon1*$^{+/+}$		Fold change 1% Met *vs.* std diet	
		Control diet	1%-Met diet	Pon1$^{+/+}$	Pon1$^{-/-}$
Lipoprotein metabolism Apolipoprotein A1 (#77)	Apoa1	–1.11	–1.05	–1.47[a]	–1.39[a]
Apolipoprotein E (#39)	ApoE	1.20[b]	1.11[c]	–1.06	–1.14[a]
Energy metabolism ATP synthase subunit d (#78)	Atp5h	1.03	1.32[b]	–1.35[b]	–1.05
Guanidinoacetate *N*-methyltransferase (#76)	Gamt	1.05	–2.04[a]	1.62[a]	–1.32[b]
Iron metabolism and homeostasis Ferritin light chain (#81)	Ftl	1.27[b]	–1.70[c]	2.29[a]	1.09
Oxidative stress response Peroxiredoxin 2 (#73)	Prdx2	1.07	1.15[b]	1.28[a]	1.37[a]
Catechol metabolism Catechol-*O*-methyl transferase (#75)	Comt	1.03	–1.45[a]	1.36[a]	–1.06
Nitric oxide generation Dimethylarginine dimethylaminohydrolase 1 (#41)	Ddah1	1.23[a]	1.44[a]	–1.14[a]	1.03

[1](Spot #) refers to the numbering on the IEF/SDS-PAGE gels in Figs. 1 and 2. Significantly different: [a]$P<0.001$, [b]$P<0.01$, [c]$P<0.05$.

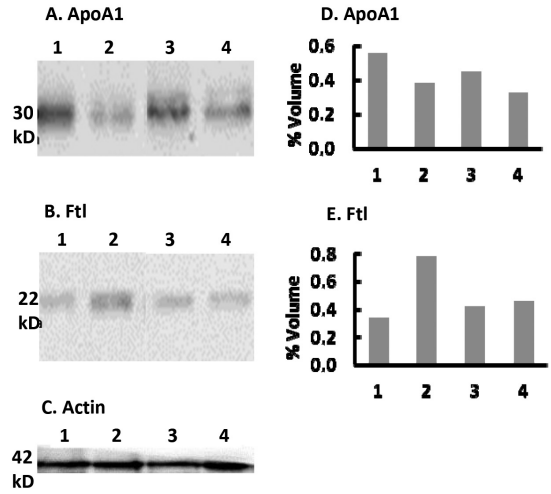

Figure 4. Western blot validation of IEF/SDS-PAGE results.
Panels A and B show Western blots for ApoA-I and Ftl, respectively. Panel C shows Western blot for actin that indicates equal loading in the four lanes. Panels D and E show '% Volume' for ApoA-I and Ftl, respectively from IEF/SDS-PAGE analyses of the same samples. The following groups of mice were studied: lane 1 – *Pon1*$^{+/+}$, control diet; lane 2 – *Pon1*$^{+/+}$, high-Met diet; lane 3 – *Pon1*$^{-/-}$, control diet; lane 4 – *Pon1*$^{-/-}$, high-Met diet.

energy metabolism (Atp5h, Gamt), oxidative stress response (Prdx2), and catechol metabolism (Comt) show the *Pon1*$^{-/-}$ genotype-dependent expression only in mice fed with a high-Met diet. Alterations in the liver proteome, observed in the present work after 8 week exposure to HHcy, may precede the development of fatty liver disease that is observed after 16–20 week-long exposure (Werstuck *et al.,* 2001).

The function of Pon1 in the liver has not been fully explored. In the present work we identified three liver proteins: ApoE, Ftl, and Ddah1, whose expression was increased in *Pon1*$^{-/-}$ mice fed with a control diet, compared with *Pon1*$^{+/+}$ animals (Table 2). This finding suggests that in the absence of HHcy Pon1 interacts with proteins involved in lipid metabolism (ApoE), iron metabolism (Ftl), and nitric oxide generation (Ddah1).

In *Pon1*$^{-/-}$ mice fed with a hyperhomocysteinemic high-Met diet, three liver proteins with decreased expression (Gamt, Ftl, and Comt) and four with increased expression (ApoE, Atp5h, Prdx2, Ddah1) were identified (Table 2). Effects of *Pon1*$^{-/-}$ genotype on protein expression were more pronounced and had a greater magnitude (up to 204%) in mice fed with hyperhomocysteinemic diet, compared with a control diet (up to 27%). HHcy alone changed the expression of one protein (Ftl) that was also affected by the *Pon1*$^{-/-}$ genotype, as well as of four proteins (ApoA-I, Gamt, Prdx2, and Comt) that were not affected by the *Pon1*$^{-/-}$ genotype alone. Taken together, these findings indicate that a high-Met diet and *Pon1* genotype have distinct effects on protein expression and that there is an interaction between the hyperhomocysteinemic diet and *Pon1*$^{-/-}$ genotype that modulates protein expression.

Our previous work has shown that the inactivation of the *Pon1* gene lowers the expression of the antioxidant defense proteins Sod1, Prdx2 and DJ-1 in the brain (Suszyńska-Zajczyk *et al.,* 2014b). In contrast, in the present work we found that in the liver the expression of Sod1, Prdx2 and DJ-1 was not affected by the *Pon1*$^{-/-}$ genotype. Taken together, these findings suggest that the antioxidant function of Pon1 is organ-specific, *i.e.* different in the liver and in the brain. While interactions of Pon1 with antioxidant defense proteins can contribute to the antioxidant function in the brain (Suszyńska-Zajczyk *et al.,* 2014), such interactions are not perceptible in the livers of mice fed with a normal chow diet. However, it remains to be determined whether the expression of antioxidant defense proteins is altered in livers of *Pon1*$^{-/-}$ mice fed with a high-fat diet that exhibit liver steatosis and elevated levels of oxidative stress markers (Garcia-Heredia *et al.,* 2013).

Atp5h is a mitochondrial ATP synthase that catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. The ATP synthase system is disturbed under pathophysiological conditions (Das, 2003), including methylmalonic aciduria due to the dysfunction of methylmalonyl CoA mutase, a vitamin B_{12}-dependent enzyme. Toxic metabolites, such as Hcy and methylmalon-

Table 3. Serum Pon1 activities in *Pon1*$^{+/+}$ mice are significantly reduced by dietary hyperhomocysteinemia.

Genotype	Diet	POase, A_{412}/min		PhAcase, A_{270}/min	
		Female (n=5)	Male (n=4)	Female (n=5)	Male (n=3–7)
Pon1$^{+/+}$	Std chow	0.040±0.008	0.020±0.004	0.50±0.05	0.22±0.02
Pon1$^{+/+}$	Std chow + 1% Met in drinking water, 8 weeks	0.031±0.003[a]	0.012±0.003[b]	0.41±0.02[c]	0.13±0.04[d]
Pon1$^{-/-}$	Std chow		0.0005±0.0001	0.04±0.02	–0.02±0.02

Significantly different from controls: [a]P<0.001, [b]P=0.007, [c]P=0.006, [d]P=0.0005

ic acid, accumulate in tissues and body fluids in aciduria. These metabolites compromise ATP synthase activity, which limits ATP generation and may lead to 'slow onset' excitotoxicity and finally cell death (Das, 2003). Our finding that Atp5h is up-regulated by the *Pon1*$^{-/-}$ genotype and a high-Met diet suggests that Pon1 interacts with ATP synthase to maintain adequate energy generation in the hyperhomocysteinemic liver.

We found that two methyltransferases, Gamt and Comt, are down-regulated by the *Pon1*$^{-/-}$ genotype in mice fed with a high-Met diet and up-regulated by the diet in *Pon1*$^{+/+}$ animals. Gamt methylates guanidinoacetate using S-adenosylmethionine (AdoMet) in creatine synthesis in the liver and kidney. Creatine is transported through the blood, and taken up by tissues with high energy demands, such as the brain and skeletal muscle. Creatine synthesis is an important determinant of plasma Hcy (Brosnan *et al.,* 2004) and, as the major user of AdoMet methyl groups, accounts for ~75% of Hcy formation (Mudd *et al.,* 1980). This is reflected by a positive correlation between plasma Hcy and creatine in humans (Rauh *et al.,* 2001). Comt regulates one of the major degradation pathways of catecholamine neurotransmitters by catalyzing the transfer of a methyl group from AdoMet to catecholamines. Comt is also important in the metabolism of catechol drugs used in the treatment of hypertension, asthma, and Parkinson's disease (PD). L-Dopa treatment is associated with the increase in plasma Hcy level in patients with PD (De Bonis *et al.,* 2010). Concomitant treatment with a Comt inhibitor attenuates L-dopa-induced elevation of Hcy level (Hu *et al.,* 2013). Our present findings that Gamt and Comt are up-regulated by a high-Met diet and down-regulated by the *Pon1*$^{-/-}$ genotype only in animals fed with a high-Met diet, suggest that Pon1 is required for creatine biosynthesis and catechol degradation, respectively, in hyperhomocysteinemic animals.

Of the liver proteins that were not affected by the *Pon1*$^{-/-}$ genotype alone, three were up-regulated (Gamt, Prdx2, and Comt) and two (ApoA-I and Atp5h) were down-regulated in mice fed with a hyperhomocysteinemic high-Met diet. Altered expression of one of these proteins has been observed previously in other models of HHcy. For example, Prdx2 is up-regulated in livers of wild type mice fed with 0.5% Met in drinking water for 2 weeks (DiBello *et al.,* 2010). Hepatic and plasma ApoA-I are significantly reduced in *Cbs*$^{-/-}$ mice (Jiang *et al.,* 2012), while ApoA-I synthesis is inhibited in severely hyperhomocysteinemic *Cbs*$^{-/-}$*ApoE*$^{-/-}$ mice (Liao *et al.,* 2006). High-density lipoprotein cholesterol is significantly reduced in wild type C57BL/6 mice fed with a 2%-Met in a chow diet for 5 weeks (Velez-Carrasco *et al.,* 2008). Plasma ApoA-I is also significantly reduced in human CBS deficiency (Jiang *et al.,* 2012) and is negatively correlated with plasma Hcy in coronary artery disease patients (Liao *et al.,* 2006).

We identified three proteins, Ddah1, ApoE, and Ftl that were up-regulated by the *Pon1*$^{-/-}$ genotype in mice fed with a control diet. Ddah1 regulates nitric oxide synthesis by removing an inhibitor, asymmetrical dimethylarginine (ADMA), generated by protein degradation (Ogawa *et al.,* 1987). Hcy inhibits Ddah1 activity, causes ADMA accumulation, which in turn inhibits nitric oxide synthase activity, thereby contributing to endothelial dysfunction (Stuhlinger & Stanger, 2005). Our findings that Ddah1, ApoE, and Ftl were up-regulated by the *Pon1*$^{-/-}$ genotype suggest that the absence of Pon1 induces protective responses that enhance nitric oxide generation, iron transport, and ApoE synthesis.

Our present finding that a high-Met diet and the *Pon1*$^{-/-}$ genotype up-regulate the expression of Ftl reveals an additional level of complexity in the ferritin's function. Ferritin is known to participate in one carbon metabolism, which is intimately linked with Hcy metabolism. Specifically, rat ferritin catalyzes folate turnover *in vitro* and *in vivo* and may be an important factor in regulating intracellular folate concentrations (Suh *et al.,* 2000) while heavy chain ferritin regulates expression of serine hydroxymethyltransferase by a posttranscriptional mechanism (Oppenheim *et al.,* 2001; Woeller *et al.,* 2007). These processes regulate Hcy levels, and are expected to affect Hcy-thiolactone levels, which in turn would be reflected in the extent of protein *N*-homocysteinylation (Jakubowski *et al.,* 2000; Jakubowski *et al.,* 2008). Furthermore, human and equine ferritins contain substoichiometric amounts of *N*-linked Hcy (Jakubowski, 2008a). Because HHcy is often caused by folate deficiency, up-regulation of Ftl by high-Met diet might represent a regulatory mechanism that restores folate homeostasis. In this context, our observation that Ftl is also up-regulated by the *Pon1*$^{-/-}$ genotype in mice fed with a control diet (Table 2) suggests that Hcy-thiolactone is involved in Ftl up-regulation.

Although we have not determined how the absence of Pon1 affects the liver protein expression, our findings suggest that Hcy-thiolactone could contribute to specific protein down-regulation in *Pon1*$^{-/-}$ mice fed with a high-Met diet. This suggestion is based on our previous findings showing that while HHcy elevates Hcy-thiolactone and other Hcy metabolites, *Pon1*$^{-/-}$ genotype elevates only Hcy-thiolactone (Borowczyk *et al.,* 2012a). A possible mechanism of reduced expression of specific proteins in the livers of *Pon1*$^{-/-}$ mice could involve modification by Hcy-thiolactone protein *N*-homocysteinylation followed by increased proteolytic turnover of *N*-Hcy-proteins (Glowacki *et al.,* 2010; Zaabczyk *et al.,* 2011). In this scenario, Gamt, Comt, and Ftl would be targeted for *N*-homocysteinylation in *Pon1*$^{-/-}$ mice while ApoA-I, Atp5h, and Ddah1 would be targeted in wild type mice fed with high-Met diet. Indeed, in humans Ftl is known to contain *N*-linked (Jakubowski, 2008a) Hcy while ApoA-I is known to undergo *N*-homocysteinylation (Jakubowski,

2002) and plasma *N*-Hcy-ApoA-I is positively correlated with Hcy levels (Ishimine *et al.,* 2010). Thus, it would be interesting to determine whether *N*-homocysteinylation status of specific liver proteins is altered in *Pon1*$^{-/-}$ mice.

In conclusion, our findings suggest that Pon1 interacts with diverse cellular pathways that are essential for normal liver homeostasis — from lipoprotein and energy metabolisms, nitric oxide regulation to iron metabolism and antioxidant defenses — and that modulation of these interactions by HHcy underlies the involvement of Hcy in the liver disease. Our findings also suggest that Pon1 has a protective role in the liver, particularly in HHcy.

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