INFLUENCE OF PARAOXONASE-1 DEFICIENCY ON METABOLIC ALTERATIONS AND INFLAMMATION

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PhD Thesis Dissertation

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I STATE that the present study, entitled **"influence of paraoxonase-1 deficiency on metabolic alterations and inflammation"**, presented by **Anabel García Heredia** for the award of the degree of Doctor, has been carried out under my supervisión at the **Department of Medicine and Surgery** of this University.

Reus, 1st September 2016

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"Our passion for learning is our tool for survival"

"Somewhere, something incredible is waiting to be known"

Carl Sagan

A mi familia

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Abbreviations

8-oxo-dG: 8-oxo-2´deoxyguanosine

A: Alanine

ABCA1: ATP-binding cassette transporter A1

AMPK: AMP-activated protein kinase

Apo A-I: Apolipoprotein A-I

C: Cysteine

CAM: Cell adhesion molecules

CCL2: C-C chemokine ligand 2

CD99: Cluster of differentiation 99

CV: Central vein

CVDs: Cardiovascular diseases

ER: Endoplasmic reticulum

FAS: Fatty acid synthase

G: Glycine

GPCR: G protein-coupled receptors

GSH: Glutathione

GSSG: Glutathion disulphide

H_2O_2: Hydrogen peroxide

HDL: High density lipoprotein

HNE: 4-hydroxy-2-nonenal

ICAM: Intercellular adhesion molecule

ICAM-1: Intercellular adhesion molecule 1

IL-1β: Interleukin-1β

JAMs: Juntional adhesion molecules

L: Leucine

LDL: Low density lipoprotein

M: Methionine

MCP-1: Monocyte chemoattractant protein-1

MDA: Malondialdehyde

MS: Metabolic síndrome

MUFA: monounsaturated fatty acid

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NCD: Non-communicable diseases

NLRs: NOD-like receptors

NOD: Nucleotide-binding oligomerization-domain protein

$O_2^{\bullet-}$: Superoxide

oxLDL: Oxidized low density lipoprotein

PCAM: Platelet endothelial cell adhesion molecule

PON: Paraoxonase

PT: Portal trial

PUFA: polyunsaturated fatty acid

Q: Glutamine

R: Arginine

ROS: Reactive oxygen species

TCA: TricarboxYlic acid cycle

S: Serine

SMCs: Smooth muscle cells

SOD: Superoxide dismutase

TBBL: 5-thiobutylbutyrolactone

TGF-β: Transforming growth factor-β

TLRs: Toll-like receptors

TNF-α: Tumor necrosis alpha

UPR: Unfolded protein response

VCAM-1: Vascular cell adhesion molecules

VLDL: Very low density lipoproteins

CONTENTS

ABSTRACT

Abstract

Non-communicable diseases (NCD) are, by definition, those chronic diseases that are non-infectious and non-transmissible. The most common NCD are diabetes, cancer, cardiovascular disease, chronic respiratory disease and neurological disease. All of them together are the commonest cause of death and disability in the modern world. They share common molecular mechanisms including oxidative stress, inflammation and metabolic alterations. Probably, NCD complications are interrelated, leading to an increased risk to develop other comorbidities such as non-alcoholic fatty liver disease (NAFLD) and atherosclerosis.

NAFLD comprehends a broad spectrum of liver damage from hepatic steatosis to non-alcoholic steatohepatitis (NASH), which could progress to cirrhosis, hepatocellular carcinoma and liver failure. In addition, NAFLD is associated with insulin resistance and other metabolic risk factors such as diabetes mellitus, dyslipidemia, central abdominal obesity and cardiovascular disease.

The paraoxonase (PON) family of enzymes is an important endogenous antioxidant system implicated in several biochemical pathways: protection against oxidative damage and lipid peroxidation, contribution to innate immunity, bioactivation of drugs, detoxification of reactive molecules, modulation of endoplasmic reticulum stress, and regulation of cell proliferation/apoptosis.

Taking all these data into account, we hypothesize that PON1 deficiency is associated to severe metabolic disturbances that may be related to inflammation and the comorbidities of some NCD, such as NAFLD and atherosclerosis.

Consequently, **Study 1**, published in *Journal Proteomic Research*, investigated the effect of PON1 deficiency on histological alterations and hepatic metabolism in mice fed a high-fat high-cholesterol diet. The deficiency of PON1 was associated with hepatic steatosis. Moreover, these alterations were accompanied by important metabolic alterations, and with increased oxidative stress and inflammation. Thereby, PON1 seems to have an important protective role in the liver which may have clinical relevance since reduced serum and liver PON1 activity is an early alteration in patients with liver impairment.

Abstract

NAFLD has been demonstrated to be related to subclinical manifestations of atherosclerosis. This pathology involves a complex interaction among endothelial cells of the arterial wall, blood cells, and circulating lipoproteins. In addition, PON1 accumulates in the artery wall during the atherosclerotic process, and PON1 deficient mice fed with a proatherogenic diet have greater levels of oxidized low density lipoprotein (oxLDL) and larger atheromatous plaques. Accordingly, **Study 2**, published in *Mediators of Inflammation*, studied the influence of PON1 on metabolic alterations when oxLDL is incubated with endothelial cells. The results obtained showed that HDL from PON1 deficient mice has an impaired capacity to protect endothelial cells from oxLDL. We detected important metabolic disturbances (impaired glycolysis, tricarboxylic acid cycle, phospholipid metabolism, and activation of apoptotic pathways).

Oxidative stress and insulin resistance have been assumed to be driving factors in the progression of NAFLD to NASH, and both are recognized contributors to type 2 diabetes. In addition, the accumulation of fatty acid in the livers triggers to hepatic insulin resistance that led to accelerated atherosclerosis. Beneficial lifestyle changes led to ameliorate insulin resistance and hepatic steatosis. Metformin, an activator of the 5´adenosine monophosphate-activated protein kinase, regulates hepatic lipid metabolism by inducing adipose triglyceride lipases in patients with diabetes. However, there are controversial results about the effects of metformin in patients with liver impairment. Therefore, in **Study 3**, published in *Chemico-Biological Interactions*, we wanted to evaluate how the treatment with metformin affects the liver of PON1 deficient mice fed a standard chow diet or high-fat diet. The results showed that metformin administration produces undesirable effects in the liver of PON1 deficient mice. Metfomin administration aggravated inflammation in animals given both diets. Also, it was associated with a higher degree of steatosis in animals fed a standard chow diet. This report was a cautionary note about the prescription of metformin for the treatment of diabetes in patients with concomitant liver impairment.

INTRODUCTION

1. Oxidative stress and inflammation

Non-communicable diseases (NCD) are, by definition, those chronic diseases that are non-infectious and non-transmissible. They are of long duration and generally slow progression. The most common NCD are diabetes, cancer, cardiovascular disease, chronic respiratory disease and neurological diseases and all of them together are the commonest cause of death and disability in the modern world. In 2012 they caused 68% of all of them (1, 2). NCD share common molecular mechanisms that play an important role in their onset and development. These mechanisms include oxidative stress, inflammation and mitochondrial alterations (3-8).

The term “oxidative stress” begun to be used frequently in the 1970s, but its conceptual origins can be traced back to the 1950s used by researchers pondering the toxic effects of ionizing radiation, free radicals, and the similar toxic effects of molecular oxygen and the potential contribution of such processes to the phenomenon of aging (9). In general, oxidative stress is considered as the consequence of an imbalance between pro- and antioxidant species, which often result into indiscriminate and global damage in the organism (10, 11). Elderly people are more susceptible to oxidative stress and this phenomenon depends, almost in part, from an impaired performance of their endogenous antioxidant systems (12, 13).

Reactive oxygen species (ROS) comprise both, non-radical species such as hydrogen peroxide (H_2O_2) and free radicals species such as superoxide ($O_2^{\bullet -}$) (14). These molecules, persistently produced in the cell, are involved in physiological events such as primary immune defense, cell differentiation and signaling (15-17). Indeed, some ROS such as H_2O_2 are versatile players of the molecular signaling machinery because they are small, highly diffusible, and can be rapidly generated and degraded (18). On the other hand, ROS are able to oxidize different biomolecules in the cell, leading to a sequence of chain reactions that may end up in molecular and cellular damage (17, 19).

The most extensively studied DNA lesion produced by oxidative stress is the formation of 8-oxo-2’-deoxyguanosine (8-oxo-dG). The permanent modification of genetic material resulting from this oxidative damage represents the first step in mutagenesis, carcinogenesis and ageing. ROS

results in an attack not only on DNA, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. The major products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats, and HNE is weakly mutagenic but appears to be the major toxic product of lipid peroxidation (20, 21). Lipid peroxidation is commonly used as an indicator of oxidative stress in cells and tissues. On the other hand, proteins are also targets of oxidative modifications in cells. Oxidized proteins are accumulated under specific conditions (i.e. aging) and their accumulation depends on the balance between their generation and their elimination by protein degradation or repair pathways. Oxidized proteins often lose their biochemical function with downstream effects on various cellular processes. Certain oxidation processes are reversible and therefore the oxidized amino acid products can be reduced to their initial amino acid. Such modifications include oxidative cysteine and methionine products; specific enzymatic systems can recycle them into their reduced form. Irreversible oxidation products refer mostly to hydroxylated and carbonylated amino acid derivates (22).

The balance between beneficial and detrimental effects of ROS is preserved in the cell by the activity of a complex array of non-enzymatic and enzymatic detoxification mechanisms collectively known as antioxidants (23, 24).

1.1. Enzymatic Antioxidant Systems

- *Superoxide dismutase (SOD)*

It is an enzyme that catalyzes the dismutation of the superoxide anion to hydrogen peroxide, which is then decomposed by catalases primarily located in the peroxisomes (25). Two forms of SOD are known. SOD-1 contains copper and zinc and is also known as Cu-ZnSOD. This enzyme is primary located in the cytosol but also in the nucleus, and is a homodimeric protein. Copper is essential for the catalytic reaction, while zinc is important for maintaining the structure of the protein. SOD-2, also known as manganese-dependent superoxide dismutase MnSOD, is found in the mitochondrial matrix (26).

- *Catalase*

It is located in the liver, erythrocytes, kidneys and central nervous system. The principal function of this enzyme is convert H_2O_2 to water and molecular oxygen.

- *Glutathion peroxidase*

It is an important enzyme in cellular antioxidant defense systems, detoxifying peroxides and hydroperoxides. Its function is to reduce H_2O_2 to water, oxidizing two molecules of glutathione (GSH) to glutathione disulphide (GSSG), which is converted back to GSH by the enzyme, glutathione reductase using NADPH (12, 26).

- *Paraoxonases (PON)*

It is a family of three enzymes termed PON1, PON2 and PON3. They have multifunctional roles in various biochemical pathways such as protection against oxidative damage and lipid peroxidation, contribution to innate immunity, detoxification of reactive molecules, bio-activation of drugs, modulation of endoplasmic reticulum (ER) stress and regulation of cell proliferation/apoptosis (27).

1.2. Non-enzymatic Antioxidant Systems

Antioxidant compounds such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), GSH, carotenoids, flavonoids, polyphenols and others, play an important role in the antioxidant defense systems.

1.3. Oxidative stress leads to inflammation

When the equilibrium between oxidative stress and antioxidants is altered in the cells of tissues and organs, it promotes an inflammatory response that plays a vital role in host defense.

Inflammation is an adaptive response of the innate immune system that is triggered by noisome stimuli and injury, such as infection and tissue injury (28, 29). Generally, it is a controlled process with the objective of

restoring homeostasis, but in occasions leads to tissue damage, fibrosis and losses of cellular function.

Briefly, the inflammatory response to infection or tissue injury produces a coordinated release of leukocytes to the site of infection or injury (28, 29). This phenomenon is well characterized in microbial infections, where the receptors of the tissue-resident macrophages and mast cells, such as NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs) and toll-like receptors (TLRs) trigger to the production and liberation of a variety of inflammatory mediators, such as cytokines, chemokines, eicosanoids, vasoactive amines and products of proteolytic cascades. The principal effect of these mediators is an open access to the blood vessels, through the postcapillary venules. Having done that, the endothelium of the blood vessels is activated and leads to the selective extravasation of neutrophils through the junction of endothelial-cell selectins with integrins and chemokine receptors on leukocytes, which happens at the endothelial surface, as much as in the extravascular spaces (30).

Once the neutrophils go to the affected place, they become activated, either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells. The neutrophils try to eliminate the invading agents by liberation the toxic contents of their granules, which include ROS and reactive nitrogen species, cathepsin G, proteinase 3 and elastase (Figure 1) (31). This action does not discriminate between microbial and host targets, so collateral damage to host tissues is inevitable.

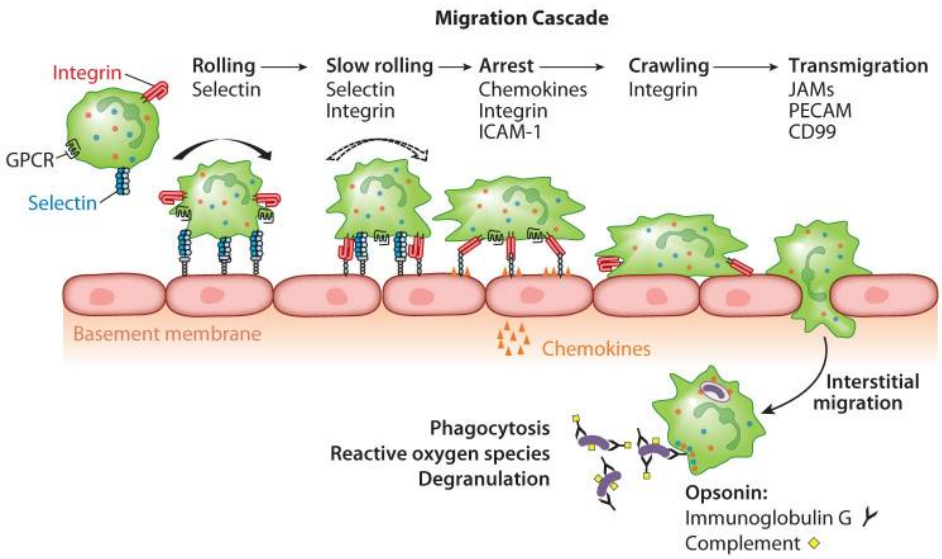


Figure 1. Schematic illustration of neutrophil recruitment. G protein–coupled receptors (GPCR); Intercellular Adhesion Molecular 1 (ICAM-1); Junctional Adhesion Molecules (JAMs); Platelet endothelial cell adhesion molecule (PCAM); cluster of differentiation 99 (CD99). Adapted from Mayadas et al. (32)

A victorious acute inflammatory response results in the elimination of the pathogens followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages. In this situation, the pro-inflammatory prostaglandins change to lipid mediators (lipoxins, protectins and resolvins), which are anti-inflammatory; this transition is crucial for the resolution of inflammation. The recruitment of neutrophils is inhibited by the lipoxins, and in addition, promotes the recruitment of monocytes, which remove dead cells and initiate tissue remodeling. Protectins, resolvins, growth factors, transforming growth factor-β (TGF-β) are produced by macrophages and also have an important role in the resolution of inflammation, including the initiation of tissue repair (33).

If the acute inflammatory response is not resolved, the inflammatory process persists and acquires new characteristics. The macrophages and T cells try to restore the homeostasis and if the result of this combination is still not sufficient, a chronic inflammatory state results, leading to the formation of granulomas and tertiary lymphoid tissues (34).

2. Paraoxonases

There are evidences relating chronic inflammation and deregulation of oxidative stress as main pathophysiological hallmarks in diabetes, cancer, cardiovascular disease, chronic respiratory disease and neurological diseases (3-8).

The paraoxonase family consists of three members termed PON1, PON2 and PON3. They are implicated in various biochemical pathways such as protection against oxidative damage and lipid peroxidation, contribution to innate immunity, bioactivation of drugs, detoxification of reactive molecules, modulation of ER stress, and regulation of cell proliferation/apoptosis (27). PON genes are located adjacent to each other on the long arm of chromosome 7 (7q21.3-q22.1) in humans and chromosome 6 in mice (35, 36). PON1 was the first identified enzyme of the family. In 1953, Aldridge was evaluating the rates of hydrolysis of organophosphate insecticides in different tissues of rabbits and rats and observed that one of the organophosphate insecticides, parathion, had a high rate of degradation in serum of rabbits, and that this compound was cleaved by an esterase. The esterases were classified depending of its hydrolysis capacity into A-esterase (hydrolyze substrates) and B-esterases (inhibited by interaction with substrates) (37, 38). The name of the paraoxonases comes from the PON1 capacity of hydrolyze paraoxon, the toxic metabolite of parathion. Years later, the other two members of the family were identified and consequently termed PON2 and PON3 (35). *PON1* and *PON3* genes are expressed in the majority of tissues, and their protein products are found in circulation bound to high density lipoproteins (HDL) (39-41). Contrarily, PON2 protein is an intracellular enzyme not found in plasma (42).

2.1. Paraoxonase 1

Initially, PON1 was the studied for its capacity to hydrolyze organophosphate compounds and, consequently, by its protective role against poisoning by organophosphate derivatives (37, 38). The physiological role of PON1 is related to its ability to hydrolyze lipid peroxides and alterations in serum PON1 activity are observed in diseases involving oxidative stress (protects against oxidation) (43), inflammation (PON1 as a negative acute phase protein) (44) and liver diseases (synthesized in this organ) (45). The enzyme is a calcium-dependent esterase, its molecular weight is 43-45 kDa and it is composed of 354 amino acids.

PON1 is considered as a "promiscuous" enzyme on account of the ability to hydrolyze many other substrates such as other organophosphorous compounds, non-phosphorous arylesters and also lactones; the latter being considered as its primary substrates. Therefore, PON1 can be evaluated by its different activities:

- *Paraoxonase activity:*
  The enzyme hydrolyzes organophosphorous compounds such as paraoxon, soman, sarin and others.
- *Lactonase activity:*
  PON1 hydrolyzes aromatic and aliphatic lactones such as 5-thiobutyl butyrolactone (TBBL) or other lactones such as dihydrocoumarin.
- *Arylesterase activity:*
  When the substrate hydrolyzed is an aromatic ester such as phenyl acetate or 4 (p)-nitrophenyl acetate (46).

For a long time, it has existed confusion with respect to the structure and mechanism-of-action of this enzyme, because its purification is unstable and often contaminated. Harel et al. (47) described the structure of PON1 through directed evolution methodology, and proposed that PON1 is a six-bladed β propeller with a unique active site that is also important in HDL binding (Figure 2). Briefly, the directed evolution methodology to replicate the evolutionary process in the laboratory by artificially inducing mutations in the gene-of-interest, followed by selection and amplification of the variants which show an enhancement of the desired characteristics.

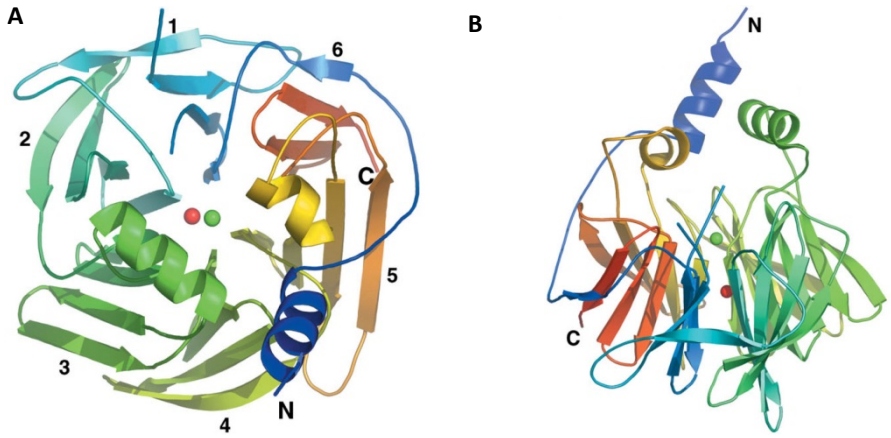


Figure 2. Overview of PON1 structure. A) View of the six-bladed β-propeller from above. The top of the propeller is, by convention, the face carrying the loops connecting the outer β-strand of each blade (strand D) with the inner strand of the next blade. Shown are the N and C termini and the two calcium atoms in the central tunnel of the propeller. B) A side view of the propeller with the three helices at the top (H1-H3). Adapted from Harel et al. (47)

Due to its capacity to hydrolyze a wide range of substrates research was conducted to distinguish the native or "ancestral" function of this enzyme. Once more, directed evolution studies, together with structure-function studies, identified the primordial function of PON1 as that of a lipolactonase (48-51) which subsequently evolved to new substrate specificities. Lactones are ubiquitous in nature and are produced in all five kingdoms (Monera, Protista, Plantae, Fungi and Animalia) (52). They show a wide phylogenetic diversity and have the ability to affect cellular signaling, growth and differentiation. The endogenous and exogenous lactones are metabolized by lactonases, then altering their biological activity and/or distribution (53). Consequently, it is probable that lactonase is the primary activity of PON1, while the paraoxonase activity arose as a promiscuous activity during their evolution.

The three activities, paraoxonase, lactonase and arylesterase, share the same active site; however, different residues in the active site are involved in their activities (47, 54, 55). For example, the residues His^{115}-His^{134} are involved in the lactonase and arylesterase catalytic activity and mutations in one of both residues dramatically decreases or abolishes both activities (54). Rosenblat et al. (56) reported that, in addition to its ability to degrade lipid peroxides,

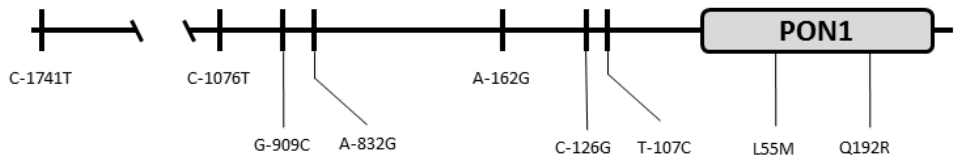
PON1 also increases cholesterol efflux from macrophages, which may further contribute to the PON1 antiatherogenic effects.

Several genetic polymorphisms can influence the expression, concentration and specific activity of the PON family. Genetic factors, including polymorphisms, affected more than 60% of phenotypic variability in PON1 activity, while demographic environmental factors (such as age and sex) reported for only 1-6% of variety and metabolic covariates for 4-19% (57). The most studied polymorphisms of PON1 are $PON1_{192}$ and $PON1_{55}$, both affecting PON1 coding region. In the case of $PON1_{192}$, there is a substitution to a glutamine (Q) for arginine (R) at position 192, leading to three phenotypic groups: QQ representing low, QR intermediate and RR high PON1 activities (58-60). Then, the polymorphism $PON1_{192}$ does not affect PON1 protein concentrations, but the PON1 activities(61). When analyzing serum PON1 activity it is important to consider the type of substrate used, due to the fact that a given genotype can produce opposite effects on PON1 activity, depending on the substrate employed. For example, the highest arylesterase and paraoxonase activity was found in RR individuals, but highest lactonase activity was observed in QQ individuals (57, 62).

The $PON1_{55}$ polymorphism is due to amino acid substitutions at position 55, a leucine (L) to methionine (M). When the allele that contains leucine is present, the PON1 concentration is significantly higher (63). Various reports highlighted the importance of the PON1 genotypes reporting an association between R and L alleles and a higher risk for cardiovascular disease (64-66). The allele frequencies varied greatly among populations of different geographical/ethnic groups (67, 68).

Moreover, several polymorphisms have been described in the promoter region of the *PON1* gene: $PON1_{-162}$, $PON1_{-832}$, $PON1_{-909}$, $PON1_{-1076}$, and $PON1_{-1741}$, which seem to be significantly associated with changes in PON1 serum enzyme activity (Figure 3) (69).

A)



B)

Polymorphism	Genotype frequency				Allele frequency		
$PON1_{192}$	QQ/QR/RR[a]	0.51	0.41	0.08	Q/R	0.71	0.29
$PON1_{55}$	LL/LM/MM[a]	0.36	0.47	0.17	L/M	0.59	0.41
$PON1_{-162}$	GG/GA/AA	0.59	0.36	0.05	G/A	0.77	0.23
$PON1_{-832}$	CC/CT/TT	0.52	0.42	0.06	C/T	0.73	0.27
$PON1_{-909}$	CC/CG/GG	0.35	0.48	0.17	C/G	0.59	0.41
$PON1_{-1076}$	AA/AG/GG	0.59	0.36	0.05	A/G	0.77	0.23
$PON1_{-1741}$	CC/CT/TT	0.39	0.47	0.14	C/T	0.62	0.38

Figure 3. A) Schematic representation of the most prevalent polymorphisms of *PON1*; **B**) Distribution of *PON1* genotypes in the healthy population. [a] Abbreviations of the coding region polymorphisms represent changes in the protein amino acid sequence (A, Ala; C, Cys; G, Gly; L, Leu; M, Met; Q, Gln; R, Arg), while those of the promoter region represent changes in the nucleotide sequence. Adapted from Marsillach et al. (69).

2.2. Paraoxonase 2

Although PON1 has been the most extensively studied enzyme, PON2 is thought to be the oldest member of the PON family, regarding structural homology and predicted evolutionary distance between them. (70).

PON2 is an intracellular protein, with a molecular weight of 44 kDa. This enzyme is not active against organophosphate substrates but has lactonase activity (71).

Research into the physiological role of PON2 and its possible implication in human pathophysiology is a young, yet promising, field. Nevertheless, like PON1, it has been involved in oxidative stress, inflammation and quorum-sensing regulation. Ng et al. (42) reported that PON2 was not present in circulation, at least not at levels measurable by the current methods, while its gene expression was detected in several human tissues with a primary

localization in the plasma membrane, suggesting functions that are different from those observed for PON1 and PON3. The authors, by using cells transfected with the human *PON2* gene, demonstrated that these cells have a higher antioxidant capacity and are less effective in oxidizing low density lipoprotein (LDL) that those cells that were not transfected. Furthermore, several studies in genetically modified mice demonstrated again the antiatherogenic properties of PON2. PON2 deficient mice present higher tissue levels of lipid hydroperoxides, leading to an increase in macrophages migration into the arterial wall, and consequently, more severe atherosclerosis lesions than control mice. Moreover, LDL isolated from theses mice was more susceptible to oxidation and triggered more monocyte chemotaxis. Macrophages isolated from PON2 deficient mice are more susceptible to oxidative stress and they also show an increase in tumor necrosis alpha (TNF-α) and interleukin-1β (IL-1β) gene expression after LPS-induced inflammation (72). PON2 also protects against macrophage triglyceride accumulation, inhibiting their conversion to foam cells (73, 74).

In addition, PON2 has the capacity to hydrolyze various bacterial products, such as acylhomoserine lactones, that permit bacteria to communicate and coordinate their infection. This process is known as *quorum sensing* and it is intensified in PON2 deficient mice (75, 76).

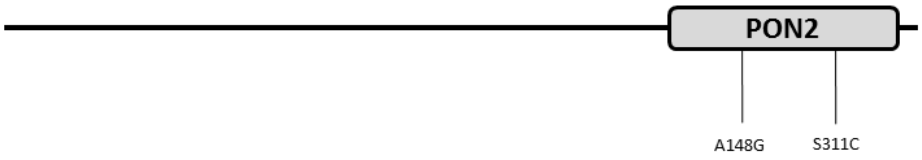
On the other side, PON2 plays an important role in mitochondrial oxidative stress and ER stress, which are involved in cell apoptosis, premature aging and cancer (77). Several studies showed an increase in *PON2* gene expression in prostate cancer, hepatocellular carcinoma and acute lymphoblastic leukemia (78-80). Altenhofer et al. (81) showed that PON2 reduced superoxide liberation from the inner mitochondrial membrane, irrespective whether resulting from complex I or complex III of the electron transport chain by modulating quinones. Rosenblat et al. (82) observed that PON2 was located inside the membranes of ER and mitochondria in murine macrophages.

Horke et al. (83) were the first to determine that PON2 reduces ER stress-induced caspase activation. They observed that PON2 was associated with the nuclear membrane and ER and was induced at both the promoter and protein levels by ER stress pathway unfolded protein response. One year later, the same authors demonstrated that PON2 protects against ER stress mediated cell death by regulating calcium homeostasis (84). It has been

observed by Devarajan et al. (85) that macrophage PON2 modulates calcium homeostasis and cell survival under ER stress conditions and is enough to prevent the development of atherosclerosis in PON2 and apoE double-deficient mice fed with a Western diet. Their observations suggest that PON2 regulates the mechanisms that link mitochondrial dysfunction, ER stress and the development of atherosclerosis.

Two *PON2* gene polymorphisms, have been described, $PON2_{148}$ (A/G) and $PON2_{311}$ (S/C). $PON2_{148}$ polymorphism involves an amino acid substitution of glycine for alanine at position 148 in the coding region and $PON2_{311}$ involves a substitution of serine for cysteine at position 311 in the coding region (Figure 4).

A)



B)

Polymorphism	Genotype frequency				Allele frequency		
$PON2_{311}$	SS/SC/CC[a]	0.62	0.34	0.04	S/C	0.79	0.21
$PON2_{148}$	AA/AG/GG[a]	0.63	0.34	0.03	A/G	0.80	0.20

Figure 4. A) Schematic representation of the most prevalent polymorphism of *PON2*; **B**) Distribution of *PON2* genotypes in the healthy population. [a] Abbreviations of the coding region polymorphisms represent changes in the protein amino acid sequence (A, Ala; C, Cys; G, Gly; S, Ser), while those of the promoter region represent changes in the nucleotide sequence. Adapted from Marsillach et al. (69)

Hegele et al. (86) reported that $PON2_{148}$ polymorphism was associated with glucose handling in patients with noninsulin-dependent diabetes mellitus in a genetically isolated Canadian population; GG individuals show a worst fasting hyperglycemia. In addition, both polymorphisms were associated with variations in serum cholesterol and apolipoprotein A-I concentration in another genetically isolated Canadian population (87), and $PON2_{311}$ was found to affect cardiovascular disease risk in Asian Indians (88). Recently

studies showed that $PON2_{148}$ and $PON2_{311}$ polymorphisms affect their capacity to hydrolyze several lactones (89).

Despite these results, a recent meta-analysis including seven studies with a great number of cases and controls did not observe any significant correlation between $PON2_{311}$ polymorphism and the risk for ischemic stroke (90). Furthermore, another study did not show any significant association between *PON2* gene polymorphisms, diabetes mellitus, cardiovascular disease, or renal disease in Mexicans participants (91).

2.3. Paraoxonase 3

PON3 is the latest identified enzyme of the PON family and the worst characterized. Its molecular weight is approximately 44 kDa and, as PON1, it is bound to HDL but in a lower concentration (92). In addition, as PON2, it is not able to hydrolyze paraoxon or other xenobiotic compounds, but has lactonase activity (71, 92).

Several studies report that PON2 and PON3 have a similar function and a similar cell-type association (93-95). Studies *in vitro* demonstrated that PON3 is able to prevent the oxidation of LDL (94). In addition, Shih et al. (96) reported that high PON3 expression significantly decreases atherosclerotic formation and adiposity in modified mice.

Interestingly, Schweikert et al. (97) showed that isolated mitochondria from PON3 deficient livers have an impaired function compared to the control mice. Moreover, the authors demonstrated that there is an increase PON3 expression in human tumors and that PON3 reduces mitochondrial superoxide formation by sequestering ubisemiquinone in cancer cells, triggering to improved cell death resistance.

PON3 is also a polymorphic enzyme, and its polymorphisms have been described recently. They are located in the promoter region, and its physiological role is still not clear (Figure5).

A)

B)

Polymorphism	Genotype frequency				Allele frequency		
$PON3_{-567}$	CC/CT/TT	0.58	0.37	0.05	C/T	0.76	0.24
$PON3_{-665}$	AA/AG/GG	0.59	0.37	0.04	A/G	0.77	0.23
$PON3_{-746}$	CC/CT/TT	0.58	0.37	0.05	C/T	0.77	0.23
$PON3_{-4105}$	GG/GA/AA	0.62	0.34	0.04	G/A	0.79	0.21
$PON3_{-4970}$	TT/TG/GG	0.62	0.35	0.03	T/G	0.79	0.21
$PON3_{-4984}$	AA/AG/GG	0.62	0.34	0.04	A/G	0.79	0.21

Figure 5. A) Schematic representation of the most prevalent polymorphism of *PON3*; **B**) Distribution of *PON3* genotypes in the healthy population. [a] Abbreviations of the coding region polymorphisms represent changes in the protein amino acid sequence (A, Ala; C, Cys; G, Gly; T, Thr), while those of the promoter region represent changes in the nucleotide sequence. Adapted from Marsillach et al. (69)

3. Paraoxonase 1 and non-communicable diseases.

NCD are underpinned by oxidative stress and inflammation; these phenomena are inextricably associated. Chronic inflammation is linked with oxidation, anti-inflammatory cascades are associated to reduce oxidation, increased oxidative stress produces inflammation, and redox balance inhibits the inflammatory cellular response. Whether or not oxidative stress and inflammation represent the cause or consequence of cellular pathology, they

participate significantly to the pathogenesis and development of NCD. Probably, NCD complications are related, increasing their risk to developing other comorbidities. For example, patients with obesity have an increased risk of developing atherosclerosis, type 2 diabetes mellitus, cancer, and other age-related diseases.

3.1. Cardiovascular disease and PON1

Cardiovascular diseases (CVDs) include a class of disorders that comprise heart and systemic blood vessels (98). Lifestyle, genetic, epigenetic and environmental factors may influence the risk of developing CVDs.

The initial pathological events of CVDs are difficult to ascertain due to their multifactorial background, and that they are often subclinical. Inflammation is considered to play an important role in the disease initiation and progression (99).

The most common pathological process that triggers to CVDs (myocardial infarction, heart failure, stroke and claudication) is atherosclerosis (98). Atherosclerosis is defined as a chronic and progressive disease characterized by an inflammatory response of arterial wall to injuries promoted by risk factors such as diabetes, dyslipidemia, hypertension and others (100). The major risk factor of atherosclerosis is hypercholesterolemia. Actually, it is well-known that atherosclerosis is the accumulation of fat in arterial walls and, in addition it is a complex process involving both innate and adaptive immune processes (100, 101).

Briefly, atherogenic process starts in places where endothelium is submitted to shear stress such as aortic arch, aortic root, renal arteries and superior mesenteric artery. Consequently, an endothelial dysfunction and alteration of the intimal layer permeability are observed, triggering to LDL migration to sub endothelial space (102). The endothelium activated by risk factors leads to expression of molecules such as E-selectin, intercellular adhesion molecule (I-CAM) and vascular cell adhesion molecules (VCAM), which attract monocytes. Once the monocytes have migrated to the media layer of the artery, differentiate into tissue macrophages. They express scavenger receptors and phagocyte oxidized LDL (oxLDL) yielding foam cells (103). The inflammatory cells release growth factors and cytokines that contribute to

the formation of a fibrous cap of smooth muscle and extracellular matrix around the lipid core, which compromises lumen of vessels (100, 101, 104). Therefore, the rupture of the plaque represents the major problem event associated with atherosclerosis (Figure 6).

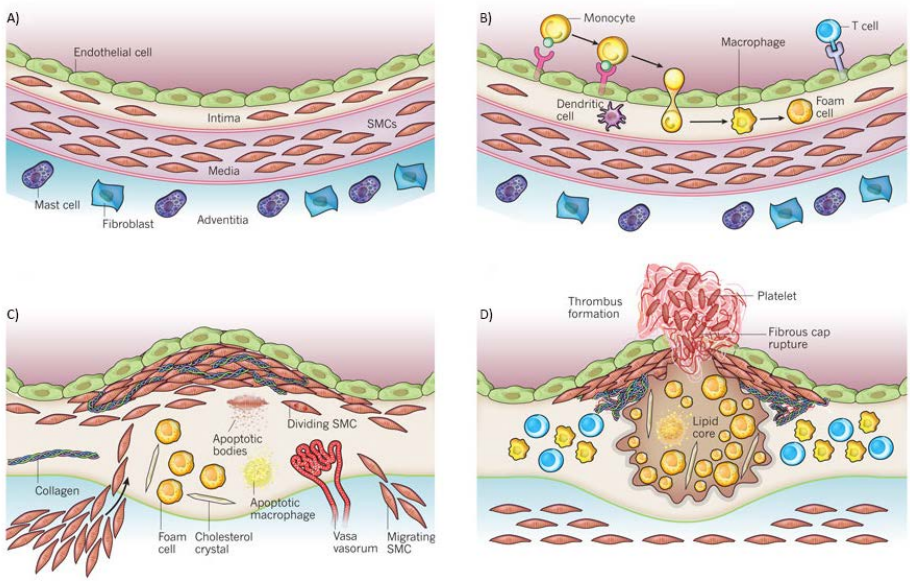


Figure 6. Development of atherosclerotic lesions. A) Representation of the normal artery, containing three layers (inner layer, tunica intima and adventitia). The human intima has resident smooth muscle cells (SMCs). The tunica media has SMCs embedded in a complex extracellular matrix; B) Recruitment of monocytes by the activated endothelium, their differentiation to macrophages, and their uptake of lipid turning into foam cells; C) Migration of SMCs from the media to the intima and synthesis of extracellular matrix macromolecules (collagen, elastin and proteoglycans). Occasionally, macrophages and SMCs can die leading to liberation of lipids and their accumulation in the central region of a plaque, called it as necrotic core. Advancing plaques also contain cholesterol crystals and microvessels; D) Rupture of the atherosclerotic plaque triggering to the thrombus that it can obstruct blood flow. Adapted from Libby et al. (104).

The atherosclerotic plaques are characterized by having necrotic areas, calcification, accumulation of modified lipids and foam cells and also other types of cells (T cells, endothelial cells, smooth muscle cells and vascular dendritic cells) (105). The accumulation of monocytes/macrophages at the lesion site is a key factor in the atherogenic process and involves various steps, such as the expression of adhesion molecules and chemotactic factors triggering the monocyte recruitment, and consequently the activation and

differentiation processes together with proliferation and immobilization of macrophages in the inflamed plaque (106).

In particular, the chemokine (C-C motif) ligand 2 (CCL2), formerly termed monocyte chemoattractant protein-1 (MCP-1), is intimately implicated in the inflammatory reaction. This chemokine regulates the migration of monocytes into tissues and their subsequent differentiation into macrophages (107). CCL2 can be secreted by several cells such as endothelial cells, T cells, smooth muscle cells, monocytes, macrophages and foam cells, perpetuating inflammation and lipid accumulation in atheroma (108).

Early studies reported that CCL2 is present in macrophage-rich atherosclerotic plaques in humans (109) and primates (110). Oxidized lipids have long been implicated as mediators of atherosclerosis and foam cell formation (111). Studies by Cushing et al. (112) demonstrated that minimally ox-LDLs, but not native LDLs, induced CCL2 production in vascular wall cells such as endothelial cells and smooth muscle cells. CCL2 thus emerged as a possible molecular link between oxidized lipoproteins and foam cell recruitment to the vessel wall. In addition, it is reported by Stephen et al. (103) that CCL2 induces a diminution in the expression of LDL receptors and an increase in the scavenger receptor (responsible to phagocytosis of oxLDL). Remarkably, Hashizume and Mihara (113) investigated the influence of interleukin-6 (IL-6) and TNF-α on expression of scavenger receptor in human arterial endothelial cells. They observed an increased in the expression of scavenger receptor and they also reported that oxLDL-induced CCL2 was increased by the presence of IL-6 and TNF-α. Therefore IL-6 and TNF-α promote a positive feedback of inflammation and atherogenesis and suggests that both are implicated in atherogenesis process also via oxLDL/CCL2 induction. Moreover, CCL2 is responsible to the rupture and thrombosis of atherosclerotic plaque (114).

On the other side, HDL particles have been shown to have antioxidant (115-117) and anti-inflammatory properties, including the suppression of cytokine-induced endothelial cell adhesion molecules (CAM) (118). Moreover, Apolipoprotein A-I (Apo A-I) in HDL was shown to stabilize PON1 and to significantly promote its lactonase activity (119). Conversely, the presence of Apo AII in HDL is associated with PON1 inactivation (120). In serum, PON1 protein protects both LDL and HDL against lipid peroxidation and preserves its anti-atherogenic effects by inhibition of HDL oxidation (121,

122). Mackness et al. (123) first showed that PON1 inhibits CCL2 production in endothelial cells incubated with oxLDL. They found that HDL, as well as recombinant PON1, abolished CCL2 production in cultured endothelial cells, while HDL from avian was unable to elicit this reaction (avian HDL does not have PON1).

Several studies support the concept of an anti-inflammatory function of PON1. Tward et al. (124) evaluated the overexpression of human PON1 in mice fed with an atherosclerotic diet. These authors observed that the mice developed less atherosclerotic lesions, lower oxidative stress, and about 44% lower CCL2 expression in their aortas than their corresponding control mice. Rozenberg et al. (125) showed that PON1 deficient mice had higher peripheral lipid peroxidation and a higher degree of macrophage oxidative stress. Subsequent investigations observed that the function of PON1 (antioxidant and anti-inflammatory) is multifaceted and may include not only an inhibition of lipid peroxidation but also an increase in macrophage cholesterol efflux via HDL. Rosenblat et al. (126) incubated cultured macrophages with HDL derived from human PON1-trangenic mice. They observed that PON1 improved cholesterol efflux, through an increase in macrophage lysophosphatidylcholine content, which stimulated the action of the ATP-binding cassette transporter A1 (ABCA1), the HDL binding to the cells, and the cholesterol uptake by this lipoprotein. Other study (127) in mice deficient of both Apo A-I and LDL receptor showed lower serum PON1 activities, impaired reverse cholesterol transport, increased atherosclerosis development, and increased CCL2 concentrations.

Taking all these data into account, it seems plausible that PON1 inhibits the monocyte/macrophage transmigration induced by oxidative stress due to its capacity to degrade lipid peroxides and down-regulate CCL2 production by vascular endothelial cells as well as to its capacity to increase macrophage-associated cholesterol efflux via lysophosphatidylcholine synthesis and ABCA1 activation. This rationale offers a clear mechanism connecting lipid peroxidation, inflammation and the protective functions of PON1 and HDL against atherogenesis (Figure7).

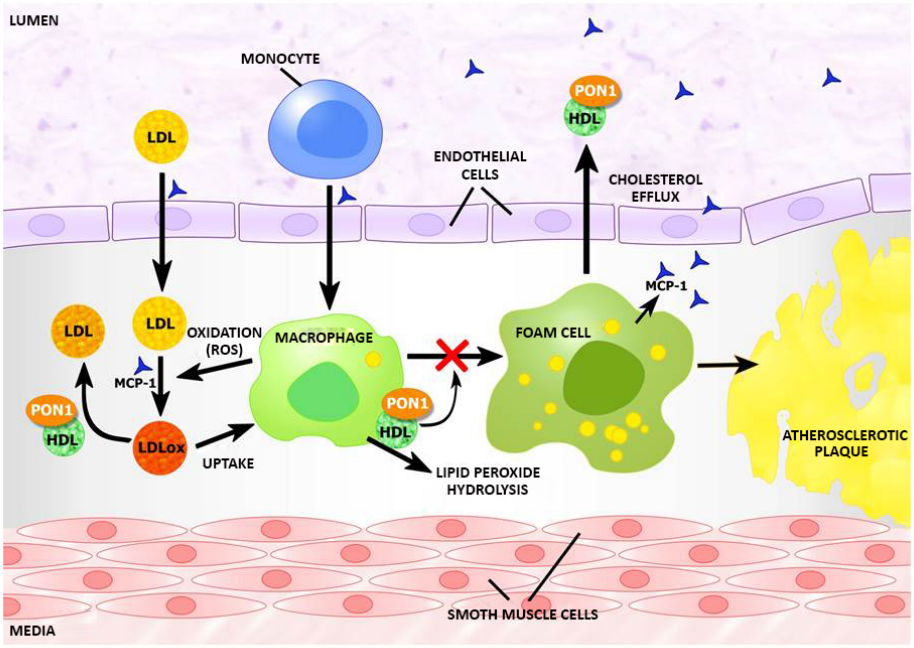


Figure 7. Representation of the role of PON1 in the atherosclerotic process. Circulating monocytes are activated as a consequence of a pro-oxidant and pro-inflammatory environment and become to macrophages. LDL particles enter the vessel as well. Macrophages promote LDL oxidation by the liberation of free radicals. Oxidized LDL go into macrophages by via the scavenger receptor and contributes to their conversion to foam cells. The presence of macrophage oxidations of LDL and foam-cell formation are hallmarks of initial atherogenesis. PON1 hydrolysis of oxidized lipids in LDL reverts this lipoprotein to normal LDL and, as a result, attenuates the development of atherosclerosis. PON1, by inhibiting the production of MCP-1, is anti-inflammatory and favors cholesterol efflux from macrophages. Adapted from Camps et al. (128).

3.2. Diabetes and PON1

Diabetes is a severe NCD that happens either when the pancreas does not produce sufficient insulin or when the organism cannot effectively use it. Diabetes is an important public health problem, and the number of cases and the prevalence of diabetes have been progressively increasing over the past few decades. A statistical study in 2014 showed that 8.5% of adults aged 18 years and older had diabetes. In 2012 this disease was the direct cause of 1.5 million deaths and hyperglycemia was the cause of another 2.2 million deaths (129).

Diabetes is characterized by chronic hyperglycemia, microvascular complications (e.g., renal glomerulus, retina, and peripheral nerve), and macrovascular complications (e.g., atherosclerosis, CADs, stroke) (130).

Diabetes is associated with high levels of oxidative stress (131). There are data showing that ROS formation is a direct consequence of hyperglycemia (132). Antioxidant systems are important mechanisms for defending organism from oxidative stress-induced damage, and disequilibrium in the redox mechanism may trigger to the pathology or complications of diabetes (133, 134).

In the 1990s, various reports showed associations between $PON1_{192}$, $PON1_{55}$, and $PON1_{-108}$ polymorphisms and cardiovascular complications in diabetic patients (63, 65, 135). The presence of RR, LL and TT alleles was significantly associated with higher rates of adverse cardiac events. In addition, Letellier et al. (136) reported also that PON1 activity is decreased in diabetic patients. In addition, diabetic patients have their PON1 in HDL glycated and as a result, have reduced ability to metabolize membrane lipid hydroperoxides (137, 138). Moreover, studies in PON1 deficient mice and PON1 transgenic mice showed the protective role of PON1 against streptozotocin-induced diabetes. Overexpression of PON1 was linked with reduced diabetes-induced macrophage oxidative stress, reduced diabetes development, and reduced mortality, in comparison to control mice, and even more so, when compared with PON1 deficient mice (139).

The increased oxidative stress in diabetic patients triggers in a detriment in PON1 activity. Indeed, the decrease in PON1 activity is inversely correlated with the levels of plasma oxLDL in these patients (140).

Several studies demonstrated that the administration of nutritional antioxidants should be necessary to preserve or maintain PON1 activity in patients that present high oxidative stress. The consumption of pomegranate juice in diabetic patients caused a significant increase in serum PON1 activity, and consequently a reduction in serum oxidative stress (141) and, also increased the PON1 binding to HDL, resulting in PON1 activation (142). *In vitro* studies showed that the presence of natural antioxidants such as quercetin (from red wine), flavonoids glabridin (from licorice root) or punicalagin (from pomegranate), during LDL oxidation, together with PON1, reduced the peroxidation and conserved PON1 activities (143).

One of the common pharmacological treatments of diabetes is the administration of metformin. Metformin is a biguanide and is derived from galegrine, extracted from the plant *Galega officinalis* (144). The drug is widely endorsed as initial therapy by professional organizations because of its low cost, security profile, and potential cardiovascular benefits (145).

Metformin acts firstly at the liver by decreasing glucose output, secondarily, by increasing glucose uptake in the peripheral tissues (mainly muscle) and, finally by increasing β-oxidation in adipose tissue (146-151). These effects are mediated by the activation of a 5´adenosine monophosphate (AMP)-activated protein kinase (AMPK) (148, 150). The AMPK is a heterotrimeric complex that is activated by an increase in the AMP/ATP ratio, and is considered to be a cellular energy sensor that contributes to regulate energy balance and caloric intake. Once activated, AMPK inhibits anabolic processes that require energy and, instead, activates catabolic processes that produce energy. Then, AMPK participates in glycolysis regulation, glucose uptake, lipid oxidation, fatty acid synthesis, cholesterol synthesis and gluconeogenesis and, it has been considered as a possible target enzyme in the treatment of some diseases such as obesity, type 2 diabetes and hepatic steatosis. (150-152). In addition, metformin has antioxidant properties which are not completely characterized. It is able to reduce the ROS by inhibiting mitochondrial respiration (153) and reduction advanced glycosylation end product (AGE) indirectly through diminution of hyperglycemia and directly through an insulin-dependent mechanism (154).

3.3. Cancer and PON1

Cancer is defined as the uncontrolled growth of cells, which can invade and expanded to distant sites of the body. The disease can have severe health consequences, and is a leading cause of death. Actually, 8.2 million people die each year, approximately 13% of all deaths worldwide (155).

Oxidative stress in the cell triggering to DNA damage and, could contribute to neoplastic growth. Known the extensive antioxidants effects of PON1, several studies have investigated the association of the functional *PON1* polymorphism with cancer risk.

A recent meta-analysis (including 25 studies) has reported solid evidence of a link between the *PON1*$_{55}$ polymorphism mutation, leading a reduction of PON1 enzyme activity, and overall cancer risk. In addition, the investigators classified by cancer type, reporting a high risk of prostate and breast cancer for patients carrying the MM phenotype. As regards to *PON1*$_{192}$, they found a reduction risk of cancer for persons of Asian ancestry carrying the RR phenotype, which significantly increases PON1 paraoxonase activity (156). Moreover, other meta-analysis of breast cancer has reported similar results with respect to both *PON1*$_{192}$ and *PON1*$_{55}$ polymorphism (157).

3.4. Neurological disorders and PON1

Neurological disorders are diseases that affect the central and peripheral nervous system. These disorders include epilepsy, Alzheimer diseases and other dementias, cerebrovascular diseases (stroke, migraine and other headache disorders), multiple sclerosis, Parkinson´s disease, neuroinfections, brain tumors, traumatic disorders of the nervous system, and neurological disorders as a result of malnutrition.

The neurological disorders prevalence is around of 100 million people worldwide. Among these, Alzheimer's disease is the most common cause of dementia and may contribute to 60–70% of cases (158).

Oxidative stress plays a key role in many neurodegerative diseases, such as Alzheimer´s and Parkinson diseases, among others.

Alzheimer´s disease is the principal cause of dementia beginning with compromised memory. The pathogenesis of Alzheimer disease comprises diffuse and neuritic extracellular amyloid plaques in brain tissue, which are often encircled by dystrophic neurites and intraneuronal neurofibrillary tangles (159).

Several studies including patients with Alzheimer disease found a reduction in PON1 activity with respect to control subjects (160, 161). In addition, Wehr et al. observed a negative correlation between PON1 activity and homocysteine (lactonase activity is exerted on oxidized phospholipids and homocysteine-thiolactone) levels in patients with Alzheimer disease (161).

Several studies have addressed the association between PON1 polymorphism and Alzheimer´s disease. Nevertheless, there are controversial observations with respect to this subject. Wingo et al. observed no association between both $PON1_{192}$ and $PON1_{55}$ polymorphisms in African Americans or Caucasians (162). However, another study from China showed that $PON1_{192}$ polymorphism is associated with Alzheimer´s disease in 1000 subjects (cases and controls) (163).

A recent meta-analysis conducted in China showed that there was no significant association between both polymorphisms and the disease (164).

On the other side, Parkinson´s disease is a neurodegenerative disorder. It is related to with the progressive degeneration of the dopamine producing neurons in the substantia nigra of the midbrain. The etiology of the disease is not well understood, and interplay of genetic susceptibility with environmental factors is suspected (165).

Organophosphate exposure has been recognized as a risk factor for Parkinson´s disease in some epidemiologic studies (166, 167). Multiple studies have investigated PON1 as a potential candidate gene for Parkinson´s disease risk, but the direct evidence from genetic association studies remains controversial.

A recent meta-analysis of twelve studies (nine involved Caucasians and three involved Asians) suggested that the $PON1_{55}$ and $PON1_{192}$ polymorphisms had no association for Parkinson´s disease (168). Conversely, there is existent solid evidence suggesting that there is a correlation between the presence of certain genotypes and the development of the disease under toxic environment (162, 169, 170).

4. The role of obesity in non-communicable diseases.

The World Health Organization has considered obesity as the “epidemics of the twenty-first century”.

The worldwide prevalence of obesity more than doubled between 1980 and 2014. Currently, 42 million children under the age of 5 were overweight or

obese. In 2014, more than 1.9 billion adults were overweight and, of these, over 600 million were obese. Overweight and obesity are linked to more deaths worldwide than underweight.

Obesity is considered as an important risk factor for the development of atherosclerosis, CVDs, type 2 diabetes mellitus, cancer, non-alcoholic fatty liver disease (NAFLD) and other age-related diseases (171).

One of the consequences to overweight or obesity is the alteration of ER homeostasis and its correct functionality, triggering a state known ER stress. The mechanism by which the cell tries to restore the cellular homeostasis is known as unfolded protein response (UPR). The main role of this cellular response is to restore the normal functioning of the ER, using several strategies. However, if this is insufficient to alleviate the stress, the UPR leads to cell death. ER dysfunction, chronic inflammation, and the consequent UPR play significant roles in the pathogenesis of induced metabolic disturbances. Nutritional excess is commonly stored in the adipose tissue, but its capacity is limited. When this happens, adipocytes exhibit signs of stress that are linked to metabolic dysfunction and disease (172). Moreover, PON2 plays an important role in mitochondrial oxidative stress and ER stress (77). Several studies showed that PON2 is located inside the membranes of ER (82, 83). PON2 expression is induced at both the promoter and protein levels by ER stress inhibiting apoptosis. Moreover, PON2 protects against ER stress by regulating calcium homeostasis (83, 84). Therefore, PON2 is an important endogenous defense mechanism against oxidative stress and UPR-induced cell death.

On the other side, the fast growing prevalence of obesity in both children and adults also triggers an increase in NAFLD, accepted worldwide as the most common cause of chronic liver disease (173, 174).

In addition, NAFLD is associated with insulin resistance and other metabolic risk factors (diabetes mellitus, dyslipidemia, central abdominal obesity and CVD). Its prevalence also increases with the age and it is influenced by genetics. Other risk factors of this disease are gender, ethnicity, and chronic infections (173-175).

NAFLD (also known as hepatic steatosis) is characterized by the presence of a significant quantity of lipid accumulation in the liver parenchyma (affecting ≥5% of hepatocytes), in the absence of excess alcohol consumption. These

hepatic deposits lead to a broad spectrum of liver damage. The hepatic steatosis may progress to become non-alcoholic steatohepatitis (NASH), characterized by the combination of fat in liver parenchyma with inflammation, hepatocyte ballooning and lobular inflammation, and to fibrosis and cirrhosis which can result in hepatocellular carcinoma and liver failure (Figure 8) (176, 177).

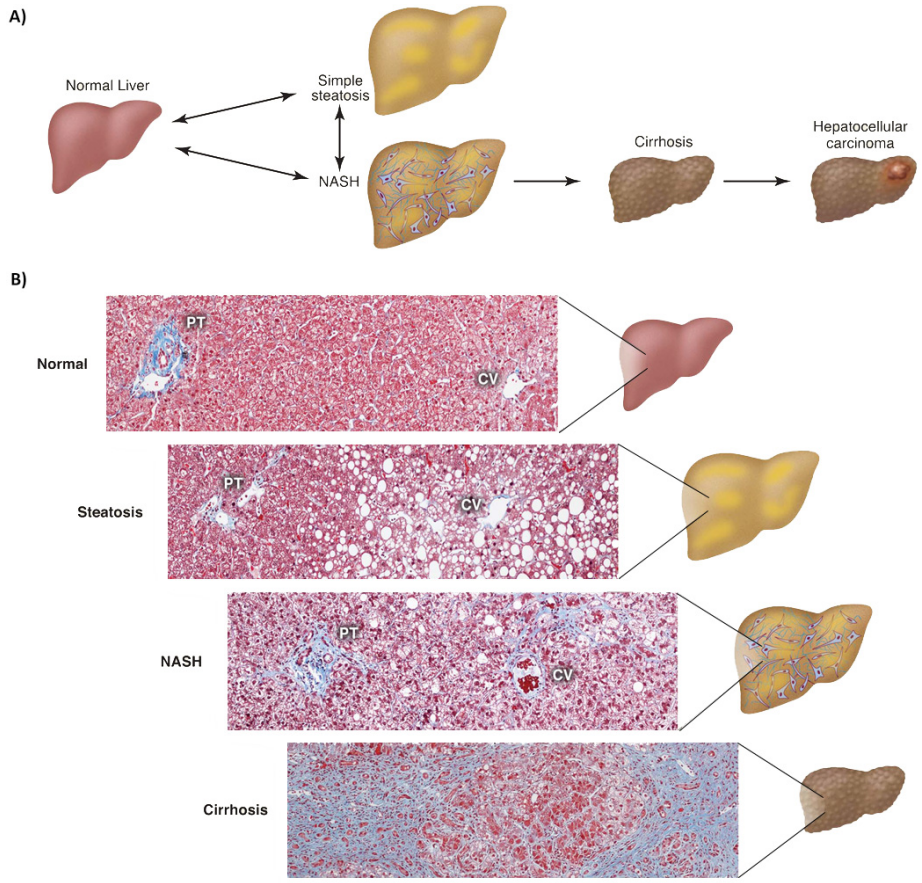


Figure 8. Disease spectrum of non-alcoholic fatty liver disease. A) Overview of NAFLD progression. The deposits of fat in hepatocytes produces steatosis. Association of steatosis and inflammation, cell death, and fibrosis is known as NASH, which can lead to cirrhosis. B) Histological sections of normal liver, steatosis, NASH, and cirrhosis. The Masson trichrome stain shows the collagen fibers in blue. Portal triad and central vein are represented such as PT and CV, respectively. Adapted from Cohen et al. (176).

The liver is a metabolic organ that performs important biochemical functions essential for metabolic homeostasis and it is one of the principal regulators of glucose and lipid metabolism. In the case of NAFLD, several disorders can be found in the liver´s capacity to process lipids and this has been associated to multifactorial alterations in diet, genetics, hormone regulation, adipose tissue, the immune system and gut microbiota.

The quantity of lipids existing in hepatocytes represents a multifaceted interaction between: a) uptake of circulating plasma triglycerides or free fatty acids (coming from lipolysis in adipose tissue), b) de novo lipogenesis in the liver, c) fatty acid oxidation and d) fatty acid exportation within very low density lipoproteins (VLDL) (175, 178-180).

The intrahepatic accumulation of fatty acids in the liver is a source of inflammation and oxidative stress, which may be responsible for the progression from NAFLD to NASH and may predispose to further severe lesions. Several enzymatic antioxidant mechanisms protect the liver from oxidative injury. One of them is PON1, through its protective role against oxidative stress. In addition, the liver plays a key role in the synthesis of serum PON1 (40, 181-183). Investigations from Ferré et al. (184) showed a reduced microsomal activity of PON1 and an increase of lipid peroxidation in rats with CCl_4-induced cirrhosis. Moreover, they studied the effect of zinc dietary supplementation, which is a metal that possesses antioxidant and antifibrogenetic properties and they observed a normalization of lipid peroxidation and an enhance of PON1 activity in rats treated with zinc dietary supplementation to respect to control rats.

Initial studies had observed a significant reduction in the serum arylesterase activity of PON1 in patients with liver cirrhosis (185-187). These reports were confirmed by Ferré et al. (181, 188). They analyzed the PON1 activity in patients with various degrees of chronic liver damage and, their studies showed a significant diminution of serum PON1 activity in patients with chronic hepatitis, and an even greater diminution in cirrhotic patients, compared to a control group.

On the other side, NAFLD is usually an asymptomatic disease. The tests for liver dysfunction are insufficiently sensitive for a reliable indication of absence or presence of liver disease, and it is often diagnosed fortuitously following a routine blood tests or an imaging study done for other reasons.

Currently, liver biopsy still represents the gold standard for the diagnosis of NAFLD. However, some doctors and patients are indisposed to carry out this invasive method. In addition to the sampling error, diagnosis is dependent on the experience and subjectivity of the pathologist and its cost and morbidity contributes to the search for additional modalities of diagnosis and staging of the disease. In the last decade, many non-invasive methods have been developed to decrease the number of liver biopsies and to overcome their problems (189, 190).

A growing number of potential biomarkers have been proposed for the diagnosis of NAFLD. Some studies reported that the measurement of serum PON1 activity may add valuable information in the assessment of the extent of liver damage, which may obviate the need for biopsy material for histological assessment. Serum PON1 activity has a great diagnostic accuracy when differentiating patients with liver disease from control subjects and, when added to a standard battery of liver functions testes, increases the overall sensitivity without damaging the specificity (69, 181, 191).

5. Animal models in scientific research

Animal research is imperative to understand the pathophysiology of human diseases, contributing to the development of effective medical treatments.

Research animals share many characteristics with humans such as being complex living systems, with striking similarity in physiology, anatomy, a genome that is over 95% similar to people, and vulnerability to the same health problems.

The cost, space, and time required to perform research are optimized using mice due to their short generation time and overall lifespan, as well as their small size (192).

In addition, our ability to directly manipulate its genome provides an incredible powerful tool to model specific diseases. Depending on the target in question, there are a number of models that can be applied. Perhaps, this is the most important advantage to use mice for biomedical research (193).

During the last 20 years, scientists developed techniques that allowed them to target genes within the mouse genome. Mice with an inactivated gene (knockout mice) are a resource to understand the genetic basis of different metabolic diseases such as atherosclerosis, obesity and fatty liver, among others.

With these animal studies scientists are able to expand their ability to accurately develop models of disease to directly test their theories and novel therapeutic approaches.

HYPOTHESIS & AIMS

Hypothesis

Non-communicable diseases (NCD) share common molecular mechanisms including oxidative stress, inflammation and metabolic alterations. Chronic inflammation is linked to oxidation, anti-inflammatory cascades are associated to reduced oxidation, increased oxidative stress produces inflammation, and redox balance inhibits the inflammatory cellular response. PON1 is an important enzyme in the defense of the organism against oxidative stress. We hypothesize that PON1 deficiency is associated to severe metabolic disturbances that may be related to inflammation and the comorbidities of some NCD, such as NAFLD and atherosclerosis.

Aims

- To study the metabolic and histological effects of PON1 deficiency in the liver of mice fed a high-fat and high-cholesterol diet.

- To investigate the influence of PON1 on metabolic alterations when oxidized LDL is incubated with endothelial cells.

- To evaluate whether metformin elicits toxic effects in the livers of PON1 deficient mice fed a standard chow diet or a high-fat diet.

RESULTS

STUDY 1

Paraoxonase-1 Deficiency is Associated with Severe Liver Steatosis in Mice Fed a High-fat High-cholesterol Diet: A Metabolomic Approach

J Proteome Res 2013; 12(4):1946-55

Abstract

Oxidative stress is a determinant of liver steatosis and the progression to more severe forms of disease. The present study investigated the effect of paraoxonase-1 (PON1) deficiency on histological alterations and hepatic metabolism in mice fed a high-fat high-cholesterol diet. We performed non-targeted metabolomics on liver tissues from 8 male PON1-deficient mice and 8 wild-type animals fed a high-fat, high-cholesterol diet for 22 weeks. We also measured 8-oxo-20-deoxyguanosine, reduced and oxidized glutathione, malondialdehyde, 8-isoprostanes and protein carbonyl concentrations. Results indicated lipid droplets in 14.5% of the hepatocytes of wild-type mice and in 83.3% of the PON1-deficient animals ($P < 0.001$). The metabolomic assay included 322 biochemical compounds, 169 of which were significantly decreased and 16 increased in PON1-deficient mice. There were significant increases in lipid peroxide concentrations and oxidative stress markers. We also found decreased glycolysis and the Krebs cycle. The urea cycle was decreased, and the pyrimidine cycle had a significant increase in orotate. The pathways of triglyceride and phospholipid synthesis were significantly increased. We conclude that PON1 deficiency is associated with oxidative stress and metabolic alterations leading to steatosis in the livers of mice receiving a high-fat high-cholesterol diet.

Introduction

Paraoxonase-1 (PON1) is an enzyme synthesized mainly by the liver, and found in the circulation bound to high-density lipoproteins (1,2). The original function attributed to PON1 was that of a lactonase; lipophylic lactones constituting its primary substrates (3). PON1 also degrades oxidized phospholipids and, as such, plays a role in the organism's antioxidant system (2). Alterations in circulating PON1 levels are associated with a variety of diseases involving oxidative stress (2).

Hepatic steatosis represents the most common form of liver disease in Western societies (4). In addition to being a precursor of fibrosis, cirrhosis, and hepatoma, hepatic steatosis is linked to diabetes, obesity, and cardiovascular disease (5). It is also an important feature of the metabolic

syndrome (6). Oxidative stress plays a determinant role in the onset of steatosis and its progression to more severe forms of liver disease (7). Also, it plays an important role in the development of inflammation, (8) and fibrogenesis (9).

Since oxidative stress influences the changes leading to fatty liver and cirrhosis and, since PON1 exerts a protective effect against oxidative stress, it would be logical to infer an association between this enzyme and liver-function impairment. We had observed, in rats with experimental fibrosis, decreased hepatic PON1 activity related to enhanced lipid peroxidation and liver damage (10). Moreover, serum PON1 activity was found to be decreased in patients with chronic hepatitis or cirrhosis, and the magnitude of the alteration was related to the extent of liver damage (11, 12). Evidence also indicated that PON1 over-expression provided strong protection against the development of experimental liver disease (13).

Despite these potentially important pointers, there is a dearth of experimental data on the biochemical mechanisms underlying the putative protective role of PON1 in liver disease. The present study sought to investigate the effect of PON1 deficiency in the livers of mice fed a high-fat high-cholesterol diet.

Materials and Methods

1.1. Experimental animals and dietary intervention

Male PON1-deficient animals of the C57BL/6J genetic background (14) were the progeny of mice provided by the Division of Cardiology of the University of California in Los Angeles. Wild-type animals were from the C57BL/6J strain (Charles River Labs., Wilmington, MA, USA). At 10 weeks of age, eight mice of each strain were fed a high-fat high-cholesterol diet (w/w 20% fat and 1.00% cholesterol; Harlan, Barcelona, Spain). At 32 weeks of age, animals were sacrificed after an overnight fast. Livers were removed and stored at −80 °C until standard analyses of oxidative stress markers, or metabolomics analyses were performed. A portion of liver was fixed for 24 h in 10% neutral-buffered formalin for histological evaluation. Wild-type (n = 8) and PON1-deficient mice (n = 8) fed with a standard mouse chow (Charles River Labs.) were used as controls. All procedures followed those set by the Ethics

Committee on Animal Experimentation of the Faculty of Medicine of Reus which, in turn, reflected the Helsinki requirements.

Metabolomic analyses of liver tissue

The metabolomics platform employed in the present study has been described in detail (15). Briefly, small-molecule metabolites from slivers of liver tissue were extracted with methanol. The resulting extract was divided into aliquots for analysis by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; separately under positive as well as negative mode) and gas chromatography-mass spectrometry (GC-MS). Metabolites were identified relative to ion data of a reference library of approximately 2,800 standard chemical entries that included retention times, mass (m/z), and MS or MS/MS spectra. Results of metabolomic measurements are expressed as the means of areas under the peaks of the PON1-deficient mice divided by the corresponding peaks of the wild-type mice.

Standard biochemical analyses of oxidative stress markers

Hepatic concentrations of malondialdehyde (MDA), oxidized and reduced glutathione (GSSG and GSH, respectively), and 8-oxo-20-deoxyguanosine (8-oxo-dG) were measured by HPLC, as previously described (16-18). Tissue levels of 8-isoprostanes and protein carbonyls were determined using commercial ELISA assays (Cayman Chemical Co., Ann Arbor, MI, USA).

Histological analyses

Liver sections of 2 µm thickness were stained with hematoxylin and eosin to evaluate histological alterations. The degree of steatosis was evaluated by image analysis software (AnalySISTM image software system, Soft Imaging System, Munster, Germany) together with a semi-quantitative score reflecting the percentage of hepatocytes containing lipid droplets. The scores were arbitrarily dichotomized as 1: <33%; 2: 33-66%; 3: >66% (19). Monocyte chemoattractant protein-1 (MCP-1) expression as a marker of inflammation was measured by immunohistochemistry using specific antibodies (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA). 4-hydroxy-2-nonenal (4-HNE) protein adducts as an index of lipid peroxidation were analyzed with a specific antibody purchased from the Japan Institute for the Control of Ageing (Shizuoka, Japan). All immunohistochemical methods had negative

controls which were treated similarly to test samples, but with the primary antibody omitted from the incubations

Statistical analyses

Differences between any two groups were assessed with the Mann-Whitney U test. Spearman correlation coefficients were used to evaluate the degree of association between variables. Welch's t-test for group comparisons was used for metabolomic analyses. Statistical software employed was either the program "R" http://cran.r-project.org/ (for metabolomic analyses) or the SPSS 18.0 package (standard biochemical analyses).

Results

Histological analyses

Histological examination showed a marked steatosis in the liver tissue of PON1-deficient mice fed with a high-fat high-cholesterol diet (Figure 1 A); the steatosis score being significantly increased ($p < 0.001$) in these animals compared to the wild-type mice (Figure 1 B). Lipid droplets were present in 14.5% (on average) of the hepatocytes of wild-type mice and in 83.3% of the PON1-deficient animals. Immunohistochemical analyses showed an increased expression of 4-HNE and MCP-1 (markers of oxidative stress and inflammation, respectively) in PON1-deficient mice, compared to their wild-type counterparts (Figure 1 C to F). On the contrary, PON1-deficient mice fed with a standard mice diet did not show any evidence of histological hepatic alterations, increased inflammation or oxidative stress compared to wild type animals (Figure 2) and, for this reason, the metabolomic and biochemical study was only continued in animals fed with a high-fat high-cholesterol diet.

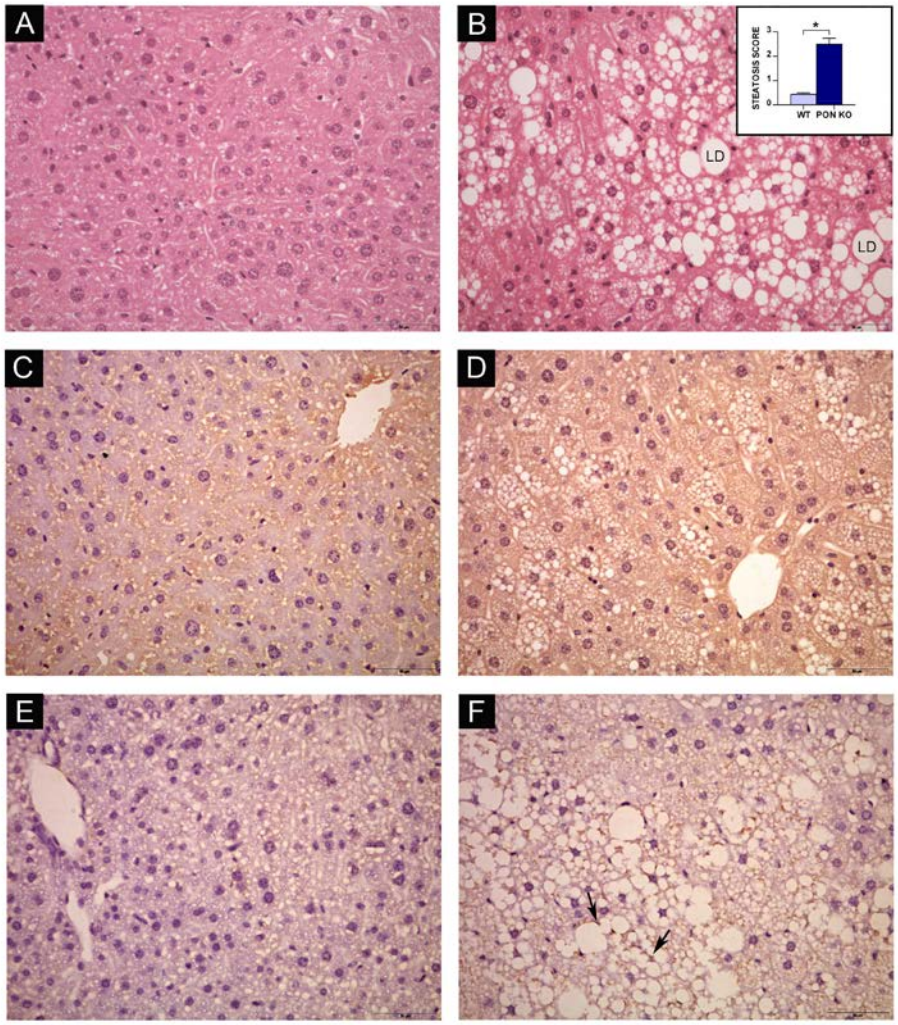


Figure 1. Histological analyses of liver tissue sections of PON1-deficient mice (right panels) and wild-type animals (left panels) fed with a high-fat high-cholesterol diet. A and B: Hematoxylin-eosin. C and D: 4-OH-nonenal immunohistochemistry. E and F: MCP-1 immunohistochemistry. The insert in B is the steatosis score measurement in both types of mice. LD: lipid droplets. The arrows in F show positive MCP-1 immunostaining around l ipid droplets.

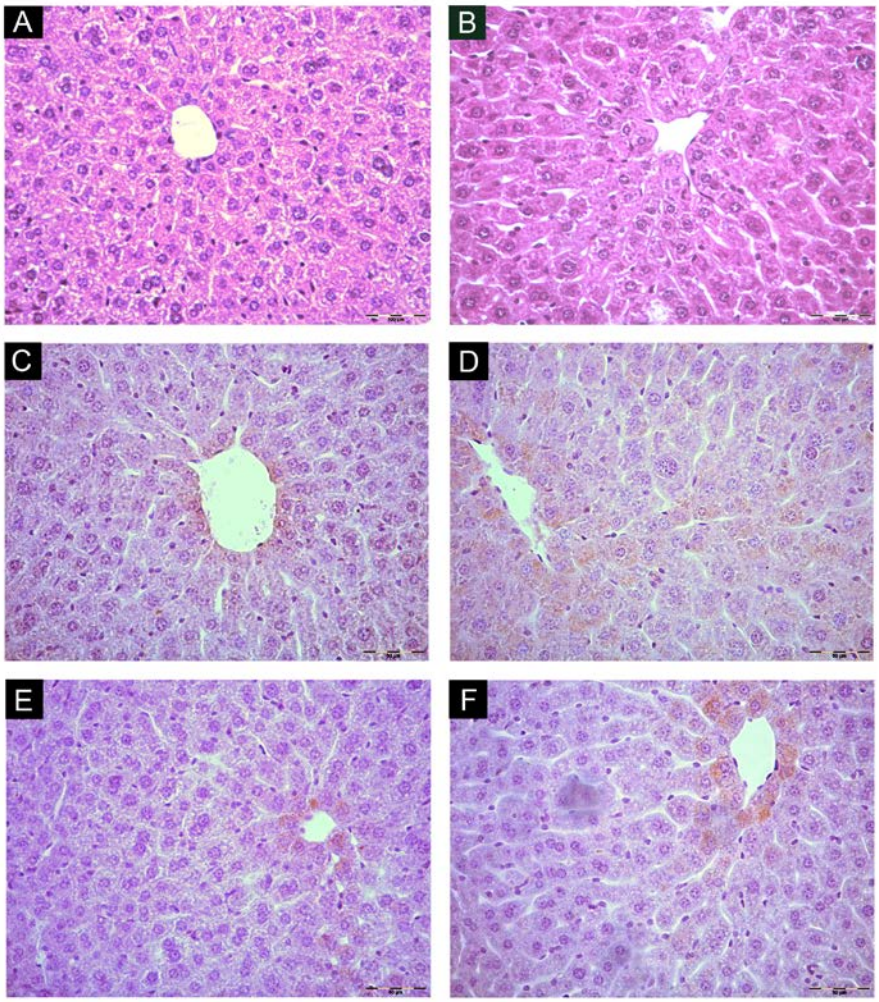


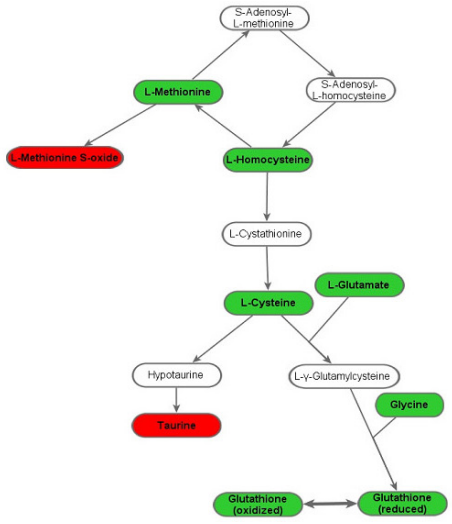
Figure 2. Histological analyses of liver tissue sections of PON1-deficient mice (right panels) and wild-type animals (left panels) fed with a standard mouse chow. A and B: Hematoxylin-eosin. C and D: 4-OH-nonenal immunohistochemistry. E and F: MCP-1 immunohistochemistry.

Metabolomic profiling

Results of the global metabolomic analyses, including an exhaustive list of the measured metabolites, unadjusted data, and heat map, are shown in Supplementary Table 1. We analyzed 322 biochemical compounds and, relative to the wild-type animals, 169 were significantly decreased and 16

were increased in PON1-deficient mice. The main findings are highlighted below.

Glutathione metabolism. GSH reduces peroxides and free radicals in a non-enzymatic process, to produce GSSG. Normally, GSH levels are modulated to meet oxidative demands by regulated rates of synthesis as well as significant recycling via the gamma-glutamyl cycle. Liver tissue PON1(-/-) showed significant GSH and GSSG depletion (Figure 3). GSSG levels decreased moderately (to approximately 80% of control mice values) while hepatic GSH content showed a considerable depletion (to approximately 27% of control mice values). As such, the ratio of GSSG/GSH was increased in PON1-deficient liver tissue; strongly indicative of exposure to oxidative stress. We also observed significantly lower levels of GSH precursors, and recycling pathway metabolites, in PON1-deficient mice. Methionine, an essential amino acid, was significantly reduced in the PON1-deficient liver tissue, as were most metabolites involved in the biosynthetic pathway between methionine and the GSH biosynthetic precursor cysteine. Also affected were the metabolites reflecting the alternate pathways of methionine salvage. Comparable changes in the parallel metabolic pathway of α-ketobutyrate to ophthalmate are in agreement with these precursor-limiting influences (Figure 3).



SUPER PATHWAY	BIOCHEMICAL NAME	PON1 KO-32wk / WT-32wk
Glutathione	glycine	0.49
	glutamate	0.71
	cysteine	0.50
	methionine sulfoxide	1.61
	hypotaurine	0.40
	taurine	1.84
	S-adenosylhomocysteine	1.15
	methionine	0.42
	homocysteine	0.44
	glutathione, reduced	0.27
	glutathione, oxidized	0.81

Figure 3. Alterations in the glutathione pathway in PON1-deficient mice compared to wild-type animals. The data on the right show the quotients of the areas-under-the-peak of the PON1-deficient mice relative to those of the wild-type animals. Decreased and increased metabolites that achieve statistical significance are shown in green and red, respectively.

Other oxidative stress markers. The hepatic concentrations of α-tocopherol and ascorbate (and their biosynthetic precursor in rodents, gulono-1,4-lactone) were significantly lower in PON1-deficient mice. Significant increases in levels of 13-hydroxy-octadecadienoate (13-HODE) and 9-hydroxy-octadecadienoate (9-HODE) are indicators of elevated lipid peroxidation, and provide evidence of an oxidizing environment in liver tissue with PON1 deficiency. Likewise, an elevated level of the oxidized amino acid methionine sulfoxide provides additional support for this concept (Supplementary Table 1).

Alterations in hepatic lipid metabolism. Liver metabolism is assessed here following an overnight fast, thus the predominant metabolic activity under normal conditions would encompass gluconeogenesis from lactate, glycerol, and amino acids to generate glucose for release to the circulation and use of fatty acids released from adipose tissue lipid stores for ketogenesis. Glycerol, mono- and di-acylglycerol levels showed a significant decrease in PON1-deficient mice, suggesting decreased triacylglyceride lipolysis or increased synthesis (Supplementary Table 1). Free fatty acid levels were altered with PON1 deficiency. Seven polyunsaturated fatty acids (PUFA) were decreased, while three monounsaturated fatty acids (MUFA) were increased i.e. the ratio of PUFA/MUFA was lower in PON1-deficient mice than in wild-type animals. In addition, we observed a decrease in carnitine levels. Carnitine is a quaternary ammonium compound necessary for the transport of long-chain fatty acids into the mitochondria. Carnitine can be diet-derived or synthesized from lysine and proline; amino acids that are decreased in PON1-deficient mice. The overall outcome would be a depressed fatty acid oxidation, which is supported by significantly lower levels of the ketone body 3-hydroxybutyrate. Phospholipid precursors, lysolipid intermediates, and breakdown products showed complex alterations in livers of PON1-deficient mice. Lysolipid levels, for example, can reflect relative rates of membrane remodeling. Levels of multiple lysolipids were reduced by PON1 deficiency, which indicates relatively reduced membrane remodeling and/or breakdown under these conditions. Metabolism of bile acids is also reduced, with a decrease in the levels of squalene, which is the precursor of cholesterol and bile acid synthesis and which is necessary for the absorption of dietary lipids and hydrophobic vitamins A, D, E and K.

Glucose metabolism. Glucose metabolism in liver is impacted upon by the reciprocally regulated pathways of glycolysis and gluconeogenesis. We observed, in PON1-deficient mice, a significant alteration in intermediates

that are shared by these opposing glucose metabolism pathways including lover levels of 3-phosphoglycerate and phosphoenolpyruvate, but elevated fructose 1,6-diphosphate (observed as a isobar with glucose 1,6-diphosphate because they are indistinguishable on the metabolomics platform). Together, ketogenesis is reduced which is consistent with relatively low acetyl-CoA and thus relatively low activation of pyuvate carboxylase (gluconeogenesis) and the fructose 1,6-diphosphate is elevated, which is an allosteric activator of the enzyme pyruvate kinase (glycolysis). These findings indicate aberrant regulation of liver glucose metabolism in PON1-deficient mice in the fasted condition. Low levels of the 3-carbon intermediates as well as lactate suggest that despite high fructose 1,6-diphosphate, glycolysis is not activated. Moreover, these changes were accompanied by a reduction in Krebs cycle activity, as indicated by significantly lower levels of several intermediates. In addition, increased levels of several intermediates in the pentose phosphate pathway (PPP) including ribulose 5-phosphate/xylulose 5-phosphate (isobars) and ribose 5-phosphate were observed in PON1-deficient mice relative to wild-type, which suggest a shift from glycolysis to the PPP. Because the early steps in the PPP are important for generating reducing equivalents in the form of NADPH, this elevation may reflect higher requirements to regenerate reduced glutathione. Overall, livers from PON1-deficient mice showed an impaired ability to obtain energy from sugar (Figure 4) or fat.

Amino acid and nucleotide metabolism. Amino acid levels were significantly lower in PON1-deficient liver tissue (Figure 4). The values reflect the combined influence of uptake from the circulation, de novo synthesis, protein synthesis and degradation rates, as well as amino acid catabolism. The urea cycle, a key aspect of the nitrogen biochemical pathway, can serve as a marker of amino acid catabolic rates. Intermediates in this cycle were also reduced in livers of PON1-deficient mice suggesting that the low amino acid levels are present even in the circumstance of reduced catabolism. Although liver can extract amino acids from the circulation via specific amino acid transporters, one hypothesis to explain this impact of PON1 deficiency on amino acid levels in the liver is that the severe depletion of glutathione, as a result of high oxidative demands, reduces the γ-glutamyl cycle activity. This not only serves to recycle glutathione but also to transfer amino acids across the plasma membrane.

With regard to nucleotide metabolism, we observed significant increases in orotate and inosine in PON1-deficient mice, relative to their wild-type

counterparts. Orotate is known to accumulate under conditions in which ornithine, as a substrate for ornithine transcarbamoylase, is limited and, as such, this suggests that the two impacts of PON1 deficiency in the liver may be related (Supplementary Table 1).

SUPER PATHWAY	BIOCHEMICAL NAME	PON1 KO-32wk / WT-32wk
Amino acid	threonine	0.60
	aspartate	0.44
	asparagine	0.19
	glutamate	0.71
	glutamine	0.66
	histidine	0.45
	leucine	0.45
	valine	0.48
	methionine	0.42
	isoleucine	0.49
	arginine	0.14
	alanine	0.45
	proline	0.42
Glycolisis	glucose-6-phosphate	1.06
	glucose	1.02
	fructose-6-phosphate	0.96
	Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate	2.02
	3-phosphoglycerate	0.72
	1,3-dihydroxyacetone	2.14
	phosphoenolpyruvate	0.63
	lactate	0.66
Energy	citrate	0.27
	cis-aconitate	0.31
	succinate	0.89
	succinylcarnitine	0.68
	succinyl CoA	0.79
	fumarate	0.54
	malate	0.69
	phosphate	0.75

Figure 4. Alterations in the glycolytic pathway, Krebs cycle, and amino acid pathways in PON1-deficient mice compared to wild-type animals. The data on the right show the quotient of the areas-under-the-peak of the PON1-deficient mice relative to those of the wild-type animals. Decreased and increased metabolites that achieve statistical significance are shown in green and red, respectively.

Cofactors and vitamins. Coenzyme A is synthesized via a multi-step, ATP-dependent pathway from the vitamin pantothenate. This precursor was significantly reduced in the livers of PON1-deficient mice. Similar changes were also observed for several other vitamins and cofactors, including several B-vitamins and the cofactors flavin adenine dinucleotide and flavin mononucleotide (Supplementary Table 1). Cofactor and vitamin limiting levels in the PON1-deficient animals are likely to have profound impacts on multiple biochemical pathways.

Standard biochemical analysis of oxidative stress markers

Metabolomic analyses of oxidative stress markers were confirmed by standard biochemical methods. We observed that PON1-deficient mice had significant increases in the hepatic content of MDA, GSSG and 8-isoprostanes (indices of lipid peroxidation), protein carbonyls (indices of protein oxidation), and 8-oxo-dG (an index of DNA oxidation) (Figure 5).

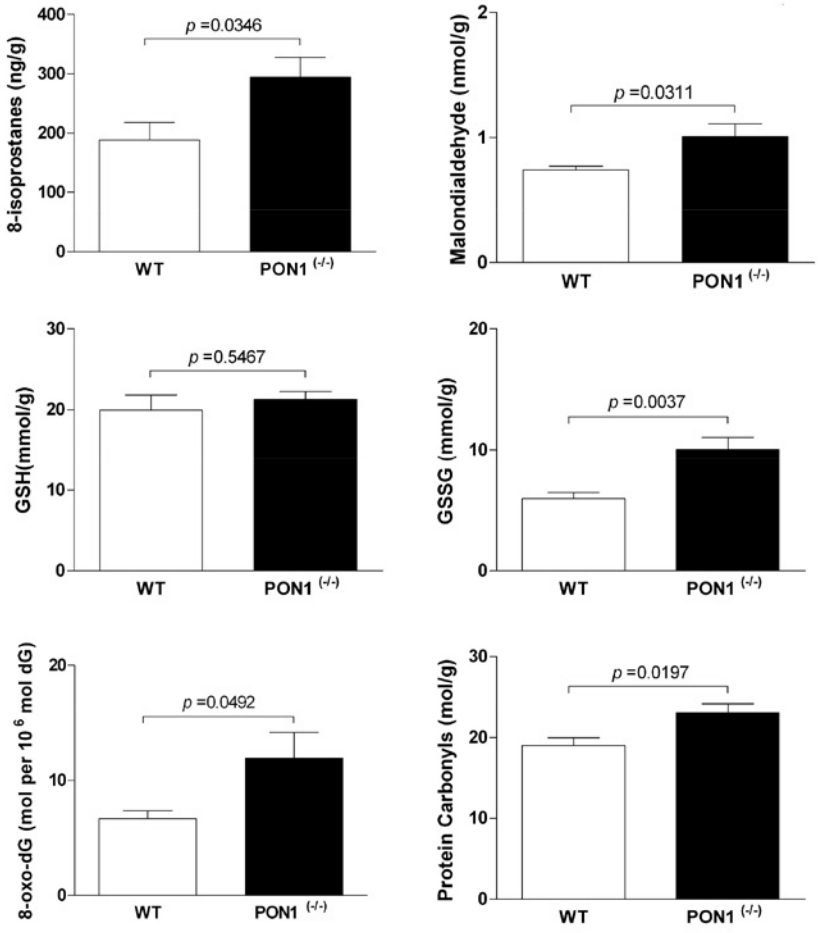


Figure 5. Results of oxidative stress markers in PON1-deficient mice compared to wild-type animals.

Discussion

Non-alcoholic fatty liver disease (NAFLD) is defined, in humans, as the accumulation of triglycerides within hepatocytes that exceeds 5% of liver weight. This alteration is gradually becoming one of the most common observations in liver diseases, and is identified using imaging techniques in about 30% of adults (20). Excessive food intake is perceived as one of the main causes of NAFLD (21). Recent studies show that a high cholesterol intake is a major stimulant in the development of NAFLD (22). The present study shows dramatic metabolic and histological alterations in the livers of PON1-deficient mice fed a high-fat high-cholesterol diet, suggesting that this enzyme plays a major role in the protection against diet-induced fatty liver. Enhanced hepatic oxidative stress is demonstrated in our PON1-deficient mice by the increased concentrations of several biochemical markers of lipid, protein and DNA oxidation, and by the increased GSSG/GSH ratio. A notable consequence of oxidative stress is the increased concentration of the peroxidized lipids 9-HODE and 13-HODE. These compounds inhibit the incorporation of triglycerides into lipoproteins (23) and, as such, may contribute to the development of steatosis. They also stimulate extracellular matrix synthesis, (24) and thus provide a link between benign steatosis and fibrosis.

Several studies indicate a strong association between oxidative stress and lipid alterations in steatosis and steatohepatitis (25). The present study showed PON1 deficiency to be associated with decreased carnitine levels which, in turn, may be explained by altered amino acid metabolism. Carnitine is a key factor in fatty acid oxidation i.e. the transport of free fatty acids into the mitochondrial matrix is regulated by the carnitine-dependent enzyme shuttle (26). A decreased hepatic carnitine concentration could result in inhibition of free fatty acid oxidation, and this derangement is associated with increased fat content (27). Our model may differ, perhaps, from human steatosis, since it is not clearly evident whether downregulation of fatty acid oxidation is involved in the onset of this derangement (22) Kotronen et al. (27) had not found any alterations in hepatic fatty acid oxidation in patients with NAFLD, both in the basal state and after exogenously-induced hyperinsulinemia.

Data are scarce regarding the pattern of hepatic fatty acid composition in NAFLD. We observed decreased concentrations of most free fatty acids in PON1-deficient mice, and a decreased PUFA/MUFA ratio. These results are similar to those of De Almeida et al. (28) showing that patients with steatohepatitis had higher MUFA concentrations than control subjects. In addition, Wang et al. (29) observed a decrease in PUFA and in PUFA and saturated fatty acids in mice receiving a high-fat, high-cholesterol diet supplemented with 0.5% bile. PUFA are known to play an important role in stimulating the expression of PPARα, and they play an anti-inflammatory and hepatoprotective role as well (30). Hepatic lipid metabolism is closely linked to glucose metabolism. Our results show that PON1 deficiency is associated with a general decrease in the glycolytic and Krebs cycle pathways, indicating a decreased ability to obtain energy. The mechanisms underlying these alterations cannot be fully ascertained from the present study, but we also observed significant decreases in the hepatic concentrations of cofactors that play key roles in these pathways. We also observed increased concentrations of ribose 5-phosphate, ribulose 5-phosphate/xylulose 5-phosphate (isobars) and xylonate. These data suggest a shift of glucose metabolism from the glycolytic to the pentose phosphate pathway. This concept is supported by the observed increased concentrations of mannose and fructose. Alterations in glycolysis and Krebs cycle may influence lipid metabolism in several ways. For example, decreased Krebs cycle may decrease acetyl-CoA carboxylase and, subsequently, fatty acid synthase (FAS) leading to an inhibition of fatty acid synthesis. This mechanism would explain the general decrease in fatty acid concentrations observed in our study. However, this effect could be partially counteracted by the increased xylulose 5-phosphate, which stimulates the carbohydrate responsive element binding protein, and stimulates FAS activity (31).

Hepatic amino acid concentrations were notably decreased in PON1-deficient mice, with the exceptions of methionine sulfoxide and taurine, which were increased. Methionine sulfoxide is the oxidized form of methionine and cannot be utilized by tissues. An increase in the concentration of this metabolite could result in a decreased methionine availability. Methionine, as a key methyl group donor for choline biosynthesis, is a precursor for phospholipid synthesis. Hence, a decrease would imply impairment in the synthesis and secretion of very-low density lipoproteins which, in turn, would contribute to the development of steatosis

(32). Indeed, the administration of a choline- and methionine-deficient diet to mice is widely employed as an experimental model of stetatohepatitis (33). Taurine plays an important role in several metabolic functions such as detoxification, membrane stabilization, and antioxidation, suggesting that the observed increase is a compensatory defense mechanism. This hypothesis is supported by data from Chang et al.(34) who observed that the administration of taurine produced a decrease in the hepatic accumulation of triglycerides in hamsters receiving a high-fat diet. They also observed that taurine increased the cytochrome 7A1 levels, which intervenes in the catabolism and secretion of cholesterol. Further, Chen et al. (35) reported that the administration of taurine protected against the development of steatosis in rats fed ethanol, by reducing oxidative stress and downregulating the expression of adiponectin and tumor necrosis factor.

Our results identified intense MCP-1 immunostaining around lipid droplets in hepatic tissue sections of PON1-deficient mice, which were not observed in wild-type animals. We previously reported similar findings (including high plasma MCP-1 concentrations) in low-density lipoprotein receptor-deficient mice fed a high-fat high-cholesterol diet (36). In both models, MCP-1 hepatic expression is detected around lipid droplets, suggesting a close link between steatosis and the inflammatory response. Taken together, these data suggest that the liver is a significant contributor to the organism's MCP-1 pool. This is a novel concept, since it is generally accepted that the hepatic inflammation in NAFLD and NASH is related to adipose tissue MCP-1 overexpression which would indirectly influence hepatic inflammation (21). We suggest that, on the contrary, it is the hepatic MCP-1 synthesis that plays the significant role in this process. The finding that PON1 deficiency is associated with increased MCP-1 expression is not surprising since, as we had demonstrated several years ago, PON1 inhibits MCP-1 production in endothelial cells incubated with oxidized low density lipoproteins (37).

Finally, the present study provides new data on the relationships between steatosis and hepatocellular carcinoma (HCC). This type of cancer can occur in livers without underlying cirrhosis (38,39). The present study has identified two pro-oncogenic molecules: orotate and 8-oxo-dG, the concentrations of which are increased in the livers of PON1-deficient mice. Hepatic concentrations of orotate in PON1-deficient mice are 2-fold that in control animals. This compound is a precursor of pyrimidine nucleotides, and its excess has been shown to alter DNA synthesis (40) and to promote liver

carcinogenesis (40,41). Conversely, 8-oxo-dG-adducts are produced as a consequence of oxidative DNA damage (18); the adducts being mutagenic and the cause of G-to-T transversions (42). Concentrations of 8-oxo-dG have been reported to be notably increased in the livers of rats treated with the powerful hepatocarcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (43). The association between NAFLD and HCC represents a growing area of study, albeit the specific sequence of events leading to HCC in the setting of NAFLD is still unresolved. We present novel data indicating that steatosis induced by PON1 deficiency is associated with increased concentrations of at least two pro-oncogenic molecules which could explain, at least in part, the increased susceptibility of fatty liver towards cancer. Our study also suggests that the measurement of orotate and 8-oxo-dG could be useful biomarkers in estimating the probability of HCC development in patients with NAFLD. However, we had not specifically investigated HCC in the present work, and further studies are warranted in appropriate patients to explore this hypothesis.

Nutritional investigations in humans have suggested that high-fat high-cholesterol diets are important determinants in NAFLD, independently of the concomitant development of insulin resistance or metabolic syndrome (22). High cholesterol intake and increased serum cholesterol concentrations have been reported to be among the strongest risk factors in the development of NAFLD (44,45). Cholesterol overload can upregulate LXRα-SREBP-1c pathway in the liver, and activate fatty acid synthesis which, in turn, would lead to steatosis (22). Previous studies have shown that NAFLD is associated with oxidative stress and low serum and hepatic PON1 levels in patients and in rats with fatty liver induced by a methionine-choline-deficient diet (46-48). The finding of reduced hepatic PON1 activity in rats with experimental steatosis is interesting and, together with the present investigation, suggest that intracellular PON1 is more important than circulating PON1 in protecting liver tissue from dietary-induced changes leading to NAFLD.

A caveat to the present results is that, since NAFLD is not a monogenic disorder in humans, studies in animals with merely a single gene deletion may not mimic the etiology of the human disease at the molecular level. In addition, the small number of animals in the present study would suggest that our findings be considered preliminary. However, the phenotypic alterations observed in our experimental model are essentially consistent with the current knowledge of human NAFLD.

Conclusion

The main goal of the present investigation has been to demonstrate that PON1 plays a protective role against hepatic derangements, secondary to fat and cholesterol overnutrition. We highlight, as well, some biochemical pathways that could explain the observed relationships between the "benign" steatosis and more severe forms of liver disease, such as fibrosis or HCC. Our findings could have considerable clinical relevance since decreased serum and liver PON1 activity is an early alteration in patients with liver impairment (10-13).

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STUDY 2

Paraoxonase-1 Inhibits Oxidized Low-Density Lipoprotein-Induced Metabolic Alterations and Apoptosis in Endothelial Cells: A Nondirected Metabolomic Study

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Abstract

We studied the influence of PON1 on metabolic alterations induced by oxidized LDL when incubated with endothelial cells. HUVEC cells were incubated with native LDL, oxidized LDL, oxidized LDL plus HDL from wild type mice, and oxidized LDL plus HDL from PON1-deficient mice. Results showed alterations in carbohydrate and phospholipid metabolism and increased apoptosis in cells incubated with oxidized LDL. These changes were partially prevented by wild type mouse HDL, but the effects were less effective with HDL from PON1-deficient mice. Our results suggest that PON1 may play a significant role in endothelial cell survival by protecting cells from alterations in the respiratory chain induced by oxidized LDL. These results extend current knowledge on the protective role of HDL and PON1 against oxidation and apoptosis in endothelial cells.

Introduction

Atherosclerosis, one of the major causes of morbidity and mortality in the Western World involves complex interactions among endothelial cells of the arterial wall, blood cells, and circulating lipoproteins (1). Oxidative stress, which is mainly derived from mitochondrial dysfunction, decreases nitrous oxide (NO) synthesis, up-regulates the secretion of adhesion molecules and inflammatory cytokines, and is responsible for the oxidation of low-density lipoproteins (LDL) (2, 3). These events play a key role in the pathogenesis of atherosclerosis (4, 5).

Paraoxonase-1 (PON1) is an enzyme found in the circulation associated with high-density lipoproteins (HDL) (6, 7). The original function attributed to PON1 was that of a lactonase, and lipophylic lactones constitute its primary substrates (8). PON1 also degrades oxidized phospholipids and, as such, plays a role in an organism's antioxidant system (7). In the atherosclerosis process, PON1 accumulates in the artery wall (9), and $PON1^{(-/-)}$ mice have been shown to have greater levels of oxidized LDL and larger atheromatous plaques when fed a pro-atherogenic diet (10). PON1 also inhibits the production of the pro-

inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1), induced by oxidized LDL in endothelial cells (11).

Despite its potential clinical and biochemical relevance, there is a paucity of studies investigating the influence of PON1 on metabolic alterations when oxidized LDL is incubated with endothelial cells. We reasoned that metabolomics might be a useful tool to evaluate the effects of this enzyme. The study was complemented with an evaluation of oxidative stress and apoptosis in this cell line.

Materials and methods

Experimental design

We employed primary cultures of human umbilical vein endothelial cells (HUVEC), cultured according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). HUVEC were grown in medium 200 supplemented with low serum growth, 10 mg/L gentamicin and 0.25 mg/L amphotericin (all these reagents were from Invitrogen), and maintained in a humidified incubator at 37°C, with 5% CO_2. Cells were sub-cultured when 80%-90% confluent. In all the experiments, cells were plated in 10 cm Petri dishes at a density of 2.5 x 10^3 cells per dish, and at passage 3. Petri dishes at 70% confluence were incubated over 24h with isolated human LDL (50 mg/L), oxidized LDL (50 mg/L), oxidized LDL (50 mg/L) + HDL (40 mg/L) from wild type mice, oxidized LDL (50 mg/L) + HDL (40 mg/L) from PON1 $^{(-/-)}$ mice, or with serum-free media as controls. All incubations were performed in serum-free media.

Normal human sera were obtained from healthy individuals participating in a population-based study being conducted in our institution. The study was approved by the Ethics Committee (Institutional Review Board) of the *Hospital Universitari Sant Joan de Reus.* Sera were pooled and used for lipoprotein fractionation and LDL isolation by sequential preparative ultracentrifugation (12, 13). Human oxidized LDL was prepared by incubation of native LDL with 5 μM $CuSO_4$, as described previously (11). Increased thiobarbituric acid-reactive substances levels were detected in LDL after oxidation (45 *vs.* <0.5 mmol/g protein).

Normal mice were from the C57BL/6J strain (Charles River Labs., Wilmington, MA, USA), and PON1 $^{(-/-)}$ mice were the progeny of those provided by the Division of Cardiology of the University of California in Los Angeles and were of a C57BL/6J genetic background (10). Animals were housed under standard conditions and given a commercial mouse diet (14% Protein Rodent Maintenance diet, Harlan, Barcelona, Spain) in accordance with our institutional guidelines. At 16 weeks of age they were sacrificed and approximately 30 mL of sera were pooled for HDL isolation (12,13).

Metabolomics analyses.

The metabolomics platform employed in the present study has been previously described in detail (14). Briefly, small molecule metabolites from an equivalent amount of cell cytoplasm homogenates were extracted with methanol, and the resulting extract divided into equal fractions for analysis by ultra high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; separately under positive mode and negative mode) and gas chromatography-mass spectrometry (GC-MS). Metabolites were identified by comparing the ion data obtained to a reference library of ~2,800 chemical standard entries. Comparisons included retention times, mass (m/z), and MS or MS/MS spectra. Results of metabolomics measurements are expressed as the mean quotients between the areas under the peak of the different experimental conditions.

Differences between groups were assessed with Welch's *t*-test for group comparisons. Statistical analyses were performed with the program "R" http://cran.r-project.org/.

Caspase 9 western blot.

We analyzed caspase 9 expression in endothelial cell homogenates as a marker of apoptosis pathways. The cytoplasmic homogenates were prepared with a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) [15]. Denaturing electrophoresis was performed in polyacrylamide gels (4-12%) from Invitrogen (Carlsbad, CA, USA). Transfer was performed with the iBlot Gel Transfer Device (Invitrogen). Blotting was performed with the ECL Advanced Western Blotting Detection kit (GE

Healthcare, Fairfield, CT, USA) using a rabbit anti-caspase 9 antibody at 1:2000 dilution (Abcam, Cambridge, UK) (13).

Measurement of apoptosis by flow cytometry.

Cells (300 μL of cell suspension at approximately 10^9 cells/L) were stained with annexin V conjugated with fluorescein isothiocianate in the presence of propidium iodide. This enables the detection of phosphatidylserine on the surface of apoptotic cells. We used the annexin-FITC kit (Beckman-Coulter®, Fullerton, CA, USA) according to the manufacturer's instructions, in a Coulter® Epics XL-MLC™ flow cytometer (Beckman-Coulter®).

Measurement of PON1 activities and total peroxide concentrations.

PON1 lactonase activity in the culture's supernatant was measured as the hydrolysis of 5-thiobutyl butyrolactone (TBBL), as described (16). The assay reagent contained 1 mmol/L $CaCl_2$, 0.25 mmol/L TBBL and 0.5 mmol/L 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.05 mmol/L Tris-HCl buffer (pH = 8.0). The change in absorbance was monitored at 412 nm. Activities were expressed as U/L (1 U = 1 mmol of TBBL hydrolyzed per minute). The concentration of total peroxides in the supernatant was determined by a colorimetric enzymatic assay (Immun-Diagnostik, AG, Benshein, Germany).

Results and Discussion

PON1 lactonase activity remained relatively low in supernatants of those cultures not containing added HDL. PON1 lactonase activity significantly increased in those cultures with normal HDL, and returned to low levels in those cultures with HDL from $PON1^{(-/-)}$ mice. These results were as expected, and provide a quality control of the HDL preparations obtained (Fig. 1A). Total peroxide concentrations in the supernatants were maximal in the cultures with added oxidized LDL, and showed a significant decrease following the addition of normal HDL. This decrease was not as marked following the addition of HDL from $PON1^{(-/-)}$ mice (Fig. 1B).

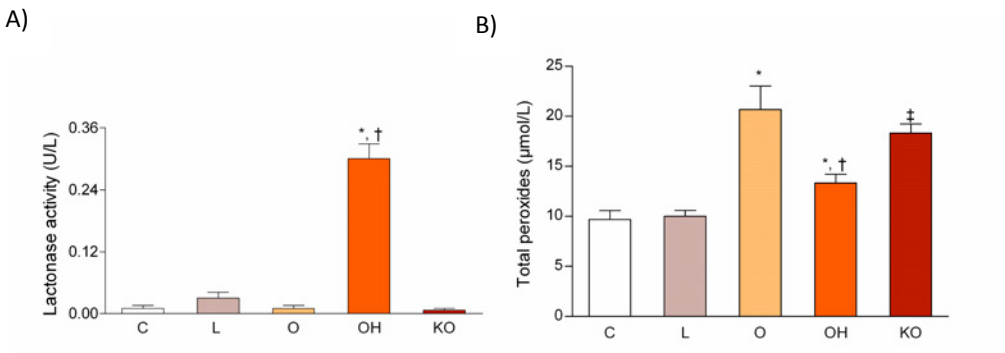

Figure 1. PON1 lactonase activity (A) and total peroxide concentrations (B) in the supernatant of the HUVEC cell culture (n = 3, for each experiment). Endothelial cells were incubated over 24h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1 $^{(-/-)}$ mice (KO); or with serum-free media as controls (C). *: $P < 0.05$; †: $P < 0.05$, with respect to O; ‡: $P < 0.01$, with respect to C.

We analyzed 124 biochemical compounds by non-directed metabolomics, corresponding to carbohydrate, lipid, amino acid, and nucleotide metabolism, as well as vitamins and xenobiotics. We obtained statistically significant variations in 37 metabolites (Table 1). The main findings corresponded to carbohydrate and phospholipid metabolism, and are summarized in the following sections (below).

Table 1. Heat map of metabolites showing statistically significant differences between groups.

Pathway	Metabolite	L/C*	O/C*	O/L*	OH/O*	KO/O*	K0/OH*
Glycine, serine, and threonine metabolism	Threonine	**1.40**	0.80	*0.57*	0.96	1.10	1.16
Glutamate metabolism	N-acetylglutamate	1.14	0.84	0.73	1.10	0.92	*0.83*
Phenylalanine and tyrosine metabolism	Phenylalanine	1.12	**0.63**	*0.56*	***1.41***	**1.19**	0.84
	Tyrosine	1.11	0.62	*0.56*	**1.57**	1.37	0.87
Valine, leucine, and isoleucine metabolism	Isoleucine	1.36	0.65	*0.48*	**1.57**	**1.38**	0.88
	Leucine	1.09	**0.70**	*0.64*	1.25	1.07	0.85
	Valine	1.26	**0.72**	*0.57*	1.18	1.08	0.91
Urea cycle; arginine-, proline-, metabolism	Praline	1.18	0.89	*0.75*	0.92	0.97	1.05
Gamma-glutamyl peptides	Gamma-glutamyl-leucine	*0.73*	0.87	1.19	1.09	1.48	1.36
Amino-sugar metabolism	Fucose	*0.72*	**0.76**	1.05	1.25	1.06	0.85
	Galactose	*0.37*	0.94	***2.58***	1.04	0.73	0.70
	Mannose-6-phosphate	0.64	2.21	***3.44***	1.01	0.87	0.87
	Glucose-6-phosphate	0.33	2.06	***6.20***	1.22	1.17	0.96
	Fructose-6-phosphate	0.50	**2.50**	***5.01***	1.13	0.94	0.83
	2-phosphoglycerate	**2.28**	0.67	*0.29*	***2.17***	1.05	*0.48*
	3-phosphoglycerate	1.62	**0.42**	*0.26*	***2.92***	***1.94***	*0.67*
	1,3-dihydroxyacetone	0.85	0.98	1.15	0.80	*0.60*	0.75
	Phosphoenolpyruvate	1.06	*0.23*	*0.22*	***4.66***	***3.05***	0.66
Nucleotide sugars, pentose metabolism	Gluconate	*0.43*	0.89	***2.07***	1.06	0.86	0.81
TCA cycle	Fumarate	1.35	0.84	*0.62*	1.10	1.12	1.02
	Malate	1.31	0.89	*0.68*	**1.21**	1.07	0.88
Oxidative phophorylation	Acetyl phosphate	1.00	1.12	1.12	0.84	*0.59*	0.70

	Phosphate	0.96	1.45	***1.52***	0.89	*0.70*	0.78
Medium chain fatty acid	Laurate (12:0)	0.98	1.15	**1.17**	0.92	*0.74*	*0.81*
Fatty acid, dicarboxylate	Undecanedioate	1.28	**1.52**	1.19	2.55	*0.70*	0.28
Glycerolipid metabolism	Ethanolamine	1.00	0.68	*0.68*	1.28	***1.55***	1.21
	Choline	1.07	0.84	*0.78*	***1.29***	1.25	0.96
	Glycerol 3-phosphate	1.54	0.31	*0.20*	***4.13***	***2.87***	*0.69*
	Glycerophosphorylcholine	*0.60*	1.17	***1.97***	0.80	**0.78**	0.97
Purine metabolism, adenine containing	Adenosine 3´-monophosphate	**2.38**	0.73	*0.31*	***1.70***	1.09	*0.64*
Pyrimidine metabolism, uracil containing	Uracil	1.22	0.48	*0.40*	***1.94***	***1.91***	0.99
	Uridine 5´-monophosphate	*0.50*	1.19	***2.38***	0.84	0.92	1.10
Pantothenate and CoA metabolism	Pantothenate	0.98	*0.87*	0.89	***1.19***	1.13	0.95
Rivoflavin metabolism	Riboflavin (Vitamin B2)	*0.68*	0.76	1.11	1.15	1.11	0.97
Benzoate metabolism	4-hydroxy catechol	1.23	1.37	1.11	0.79	*0.43*	*0.54*
Chemicals	Glycolate (hydroxyacetate)	1.12	**1.55**	**1.38**	*0.47*	0.77	**1.64**
	Glycerol 2-phosphate	0.98	0.65	*0.67*	1.96	1.11	0.57

Endothelial cells were incubated over 24h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from $PON^{-/-}$ mice (KO); or with serum-free media as controls (C). Bold italic and italic cells in the Table indicate $P \leq 0.05$. Bold italic indicates that the mean values are significantly higher; italic indicates significantly lower. Bold text indicates $0.05 < P < 0.10$. *Results are expressed as the mean quotients of the areas under the peak of the different experimental conditions. For example, galactose values are, on average, 2.58 times higher when endothelial cells are incubated with oxidized LDL than when incubated with native LDL. All measurements were performed in triplicate.

Hexose metabolism.

The addition of LDL to cultured endothelial cells decreased the levels of gluconate, galactose, and phosphorylated hexose intermediates. These molecules are important entrance intermediates in energy- and biomass-generating pathways such as glycolysis, pentose phosphate, and protein glycosylation. Their decreases suggest that these pathways were activated to a greater extent in endothelial cells treated with LDL, compared to control-treated cells. In contrast, increased levels of gluconate, galactose, and phosphorylated hexose intermediates were seen in all cells that were treated with oxidized LDL, relative to LDL alone, and regardless of whether HDL was also added to the cultures (Fig. 2).

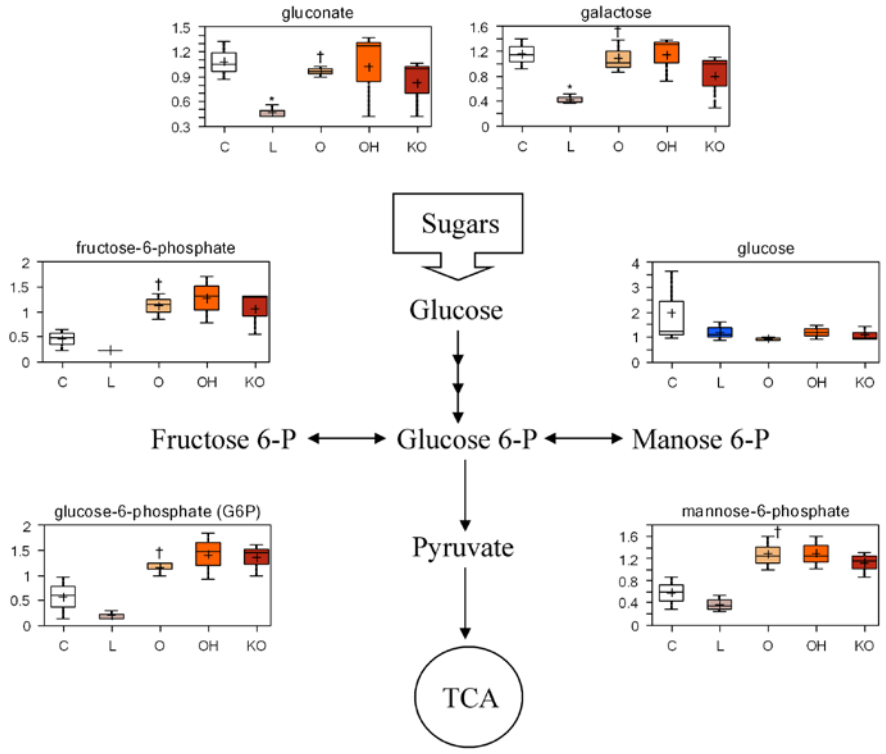

Figure 2. Variations in the hexose metabolites in HUVEC cell homogenates (n = 3, for each experiment). Endothelial cells were incubated over 24h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1 $^{(-/-)}$ mice (KO); or with serum-free media as controls (C). *: $P < 0.05$ with respect to C; †: $P < 0.05$ with respect to L.

Glycolysis and tricarboxylic acid (TCA) cycle.

Relative to control cultures, the addition of LDL resulted in increased levels of 3-phosphoglycerate and 2-phosphoglycerate (which are 3-carbon glycolytic intermediates). This same treatment also increased the levels of the TCA cycle intermediates (fumarate and malate) relative to control cultures. An interpretation of these data is that uptake of LDL by endothelial cells results in the generation of acetyl-CoA, which drives flux through the TCA cycle. Increased levels of LDL-generated acetyl-CoA may have relieved the need for carbohydrate-derived precursors, thereby inhibiting glycolytic flux into the TCA cycle and elevating the 3-carbon intermediates.

By comparison, treatment of endothelial cells with oxidized LDL may have induced levels of oxidative stress that were sufficient to impair normal energy pathways. For example, in cells treated with oxidized LDL, 6-carbon glycolytic intermediates accumulated, whereas the 3-carbon intermediates were reduced. This may be due to changes in glyceraldehyde-3-phosphate dehydrogenase (GADPH; levels or activity) in response to oxidized LDL, since superoxide overproduction inhibits GADPH through a mechanism that involves poly (ADP-ribose) polymerase (PARP) activation (16). Likewise, TCA cycle intermediates were lower in oxidized LDL-treated cells due, most likely, to the attenuated conversion of 6-carbon glycolytic intermediates to 3-carbon compounds that feed into this cycle through pyruvate and acetyl-CoA. These changes suggest that energy production through glycolysis is impaired, since ATP generation occurs downstream of GADPH activity.

The addition of normal HDL to oxidized LDL-treated cells partially reverses its impact on energy metabolism pathways, since levels of the 3-carbon glycolytic intermediates as well as TCA cycle intermediates are more similar to levels observed after LDL treatment alone. It is of note that the impact of addition of HDL from PON1$^{(-/-)}$ mice on these molecules was intermediate between the effects produced by treatment with PON1-containing HDL and of no HDL (Fig. 3). This observation is of considerable importance because PARP activation and its consequent metabolic changes have been associated with endothelial dysfunction in diseases such as atherosclerosis and diabetes (17, 18). Indeed, the levels of circulating endothelial cells are increased in patients with diabetes mellitus (19), and PON1 has been shown to attenuate diabetes development in mice (20, 21). Our results suggest that the

beneficial role of PON1 may involve, at least in part, a protection against the biochemical changes leading to endothelial dysfunction.

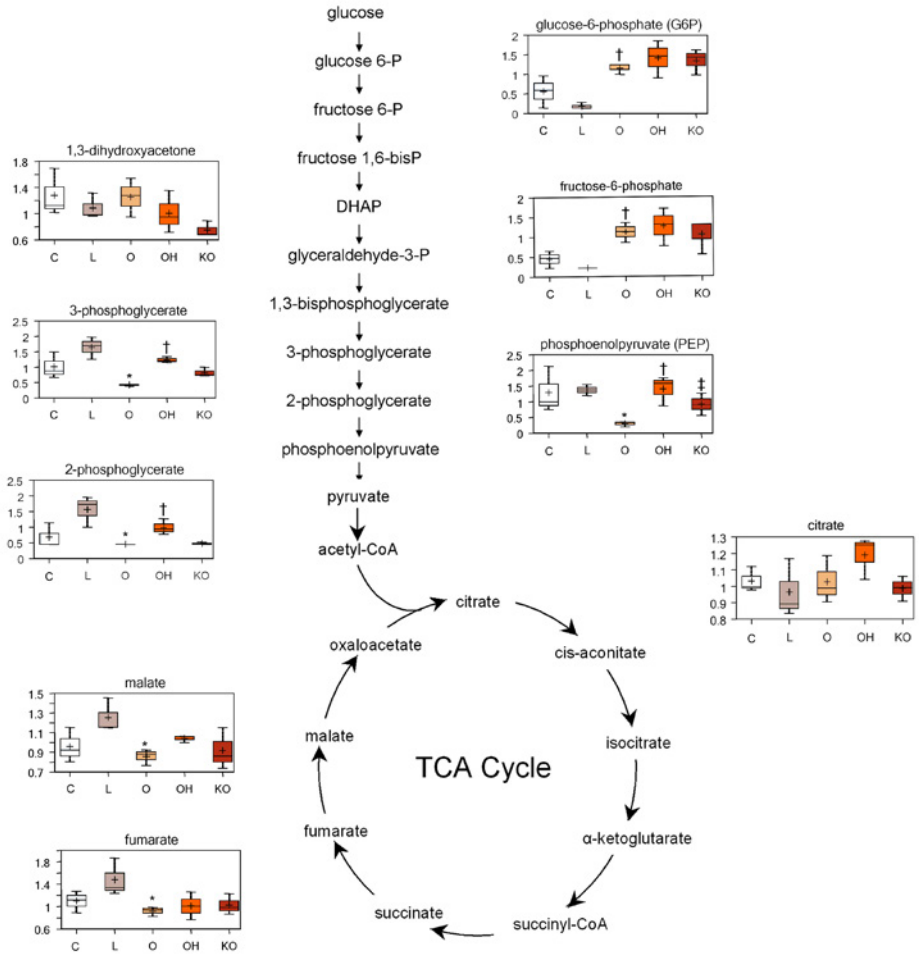

Figure 3. Variations in the metabolites of the glycolytic pathway and tricarboxylic acid cycle in HUVEC cell homogenates (n = 3, for each experiment). Endothelial cells were incubated over 24h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1 $^{(-/-)}$ mice (KO); or with serum-free media as controls (C). *: $P < 0.05$ with respect to L; †: $P < 0.05$ with respect to O; ‡: $P < 0.05$ with respect to OH.

Phospholipid metabolism.

Levels of choline, ethanolamine and glycerol-3-phosphate – key building blocks for phospholipids – were similar in endothelial cells following

treatment with LDL, when compared to levels in control cells. By comparison, oxidized LDL reduced levels of phospholipid precursors and increased the levels of at least one phospholipid breakdown product. This could indicate that oxidized LDL induces membrane damage, breakdown, or remodeling. As was observed for the energy metabolism pathways, co-administration of normal HDL to oxidized LDL-treated cells reversed, or partially reversed, these deleterious effects. However, the addition of HDL from PON1$^{(-/-)}$ mice only generated subtle changes in phospholipid-related compounds, when compared to treatment with normal HDL (Fig. 4).

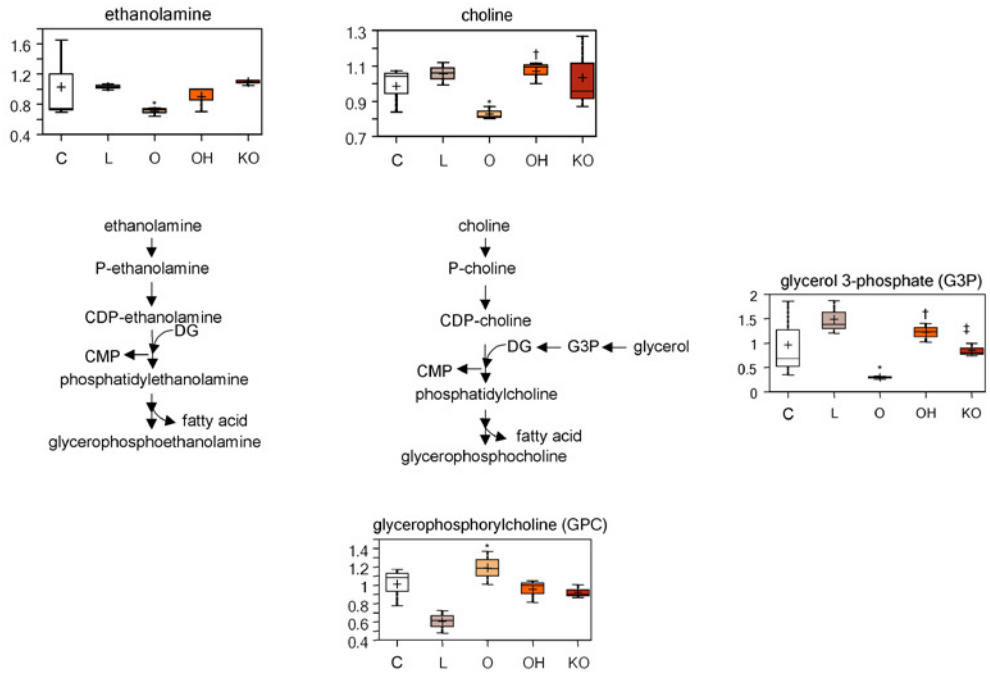

Figure 4. Variations in phospholipid metabolites in HUVEC cell homogenates (n = 3, for each experiment). Endothelial cells were incubated over 24h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1 $^{(-/-)}$ mice (KO); or with serum-free media as controls (C). *: $P < 0.05$ with respect to L; †: $P < 0.05$ with respect to O; ‡: $P < 0.05$ with respect to OH.

Apoptosis.

The observation of alterations in phospholipids levels and the suggested membrane damage channeled us towards investigating the possibility of an increased apoptosis in endothelial cells incubated with oxidized LDL, and a possible protection by introducing HDL as co-incubation. Hence, we analyzed caspase 9 protein expression. The activation of this enzyme is a good indicator of apoptosis induction, since caspase 9 plays a determinant role in apoptosome formation (22), Also, we measured the numbers of apoptotic cells by flow cytometry. We observed that oxidized LDL addition increased caspase 9 expression and the percentage of apoptotic endothelial cells, when compared to control cells and cells treated with normal LDL. Co-incubation with normal HDL completely pre-empted this effect. However, the influence of HDL from PON1$^{(-/-)}$ animals was much lower (Fig. 5 A and B). We observed a strong direct correlation (r = 0.91; P < 0.001) between total peroxides concentrations and the percentage of apoptotic cells (Fig. 5C). Previous studies had shown that increased lipid peroxidation in HDL particles from coronary artery disease patients was associated with an impaired capacity of this particle to stimulate endothelial NO production (23). Notably, PON1 has been reported to prevent lipid peroxidation in HDL particles, and to promote HDL-mediated inactivation of oxidized lipids in LDL. Its activity was shown to be decreased in patients with coronary disease (7). Further, HDL and PON1 decreased the formation of malondialdehyde-like epitopes and the formation of apoptotic particles in monocytes (24). A very recent study showed that HDL from healthy people induced the expression of endothelial anti-apoptotic protein Bcl-xL and reduced endothelial cell apoptosis *in vitro* as well as *in vivo* in apoE-deficient mice. In contrast, HDL from coronary artery disease patients did not inhibit endothelial apoptosis, failed to activate endothelial Bcl-xL, and stimulated endothelial pro-apoptotic pathways (25). Our findings of a decreased oxidized LDL-induced apoptosis by normal HDL, but not by HDL from PON1$^{(-/-)}$ mice, together with a significant association between lipid peroxidation (as measured by total peroxides concentrations) and the percentage of the apoptotic cells would tend to confirm this very recent information.

Our results suggest that PON1 may play a significant role in cell survival by improving mitochondrial function. Indeed, mitochondria regulate apoptosis in response to cellular stress signals and, hence, determine whether cells live

or die. As such, it is probable that peroxides constitute important candidates in the regulation of cell death, and that mitochondria act as both sensor and effector sites (26). This could explain the influence of apoptosis-related proteins on mitochondrial respiration. Whether or not this finding has any impact on the atherosclerosis process warrants further exploration.

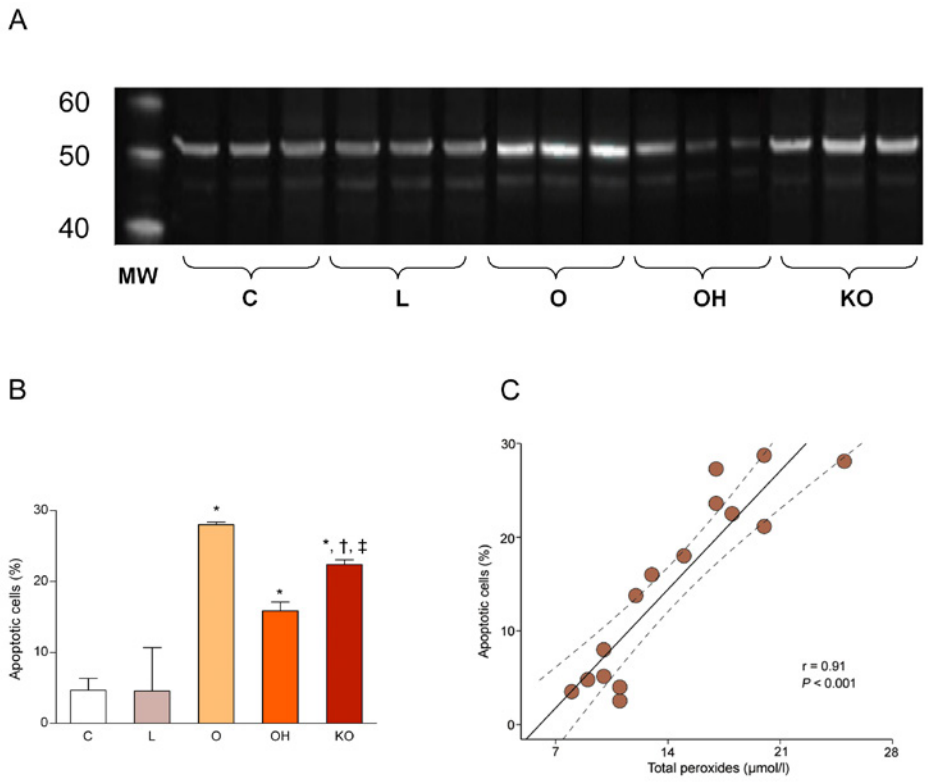

Figure 5. (A) Western blot analyses for caspase 9; (B) percentage of apoptotic cells; (C) relationship between total peroxide concentrations and the percentage of apoptotic cells in HUVEC cell homogenates (n = 3, for each experiment). Endothelial cells were incubated over 24h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1 $^{(-/-)}$ mice (KO); or with serum-free media as controls (C). MW: Molecular weight marker. *: $P < 0.01$ with respect to C; †: $P < 0.05$ with respect to OH; ‡: $P < 0.01$ with respect to O.

Conclusion

Epidemiological studies have shown that the risk of atherosclerosis is inversely associated with HDL concentrations. The protective effect of this lipoprotein has been attributed, in part, to the antioxidant and anti-inflammatory action of PON1 (27). We have showed, previously, that PON1 inhibits MCP-1 induction in endothelial cells (11), and which suggested a protective role against liver inflammation mediated by MCP-1 (28). More recent studies indicated that the anti-inflammatory effect of PON1 depends on its association with HDL (29), and that PON1 stimulates HDL anti-atherogenicity (30), and macrophage response (31), and increases the duration over which HDL is able to prevent LDL oxidation (32).

The present study is novel in using a metabolomic approach to investigate the protective effect of PON1 on endothelial cells incubated with oxidized LDL. We observed important metabolic alterations in human endothelial cells incubated with oxidized LDL. These include an impaired glycolysis, TCA cycle, phospholipids, and activation of apoptotic pathways. These changes were ameliorated by incubation with normal HDL, while HDL isolated from PON1$^{(-/-)}$ mice showed an impaired efficiency to protect against the oxLDL-induced changes. These results extend the current knowledge on the protective role of HDL and PON1 against oxidation in endothelial cells.

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STUDY 3

Metformin administration induces hepatotoxic effects in paraoxonase-1 deficient mice

Chem Biol Interact 2016; 249:56-63

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.

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.

Methods

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).

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).

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.

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).

Statistical Analysis

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

Results

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).

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).

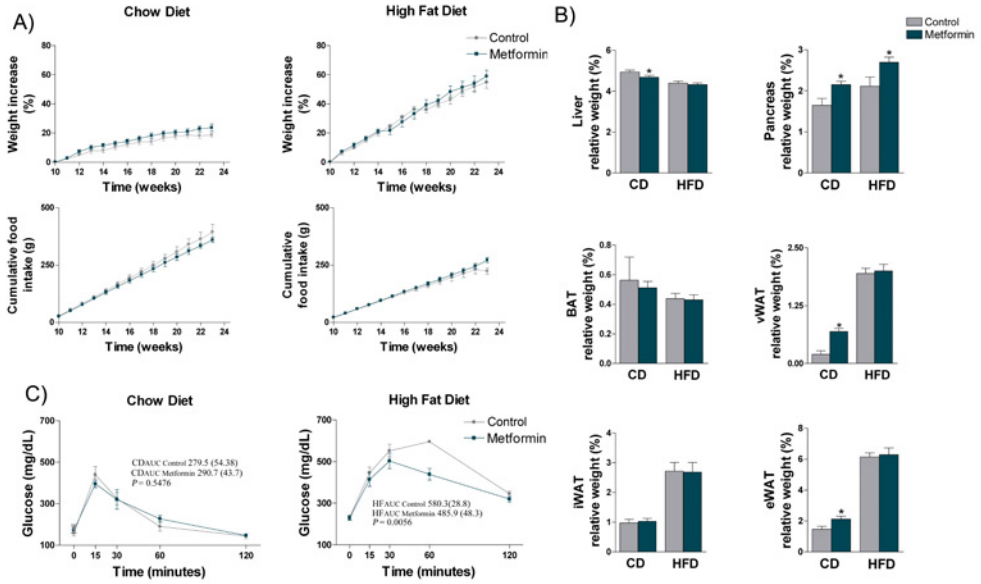

Figure 1. Effects of metformin administration in PON1-deficient mice fed a chow diet (CD) and a high fat diet (HFD). A) Cumulative food intake and weight increase in mice having metformin administered and fed CD (left panel) and HFD (right panel) from 10 to 24 weeks of age. There were significant differences 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

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).

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

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).

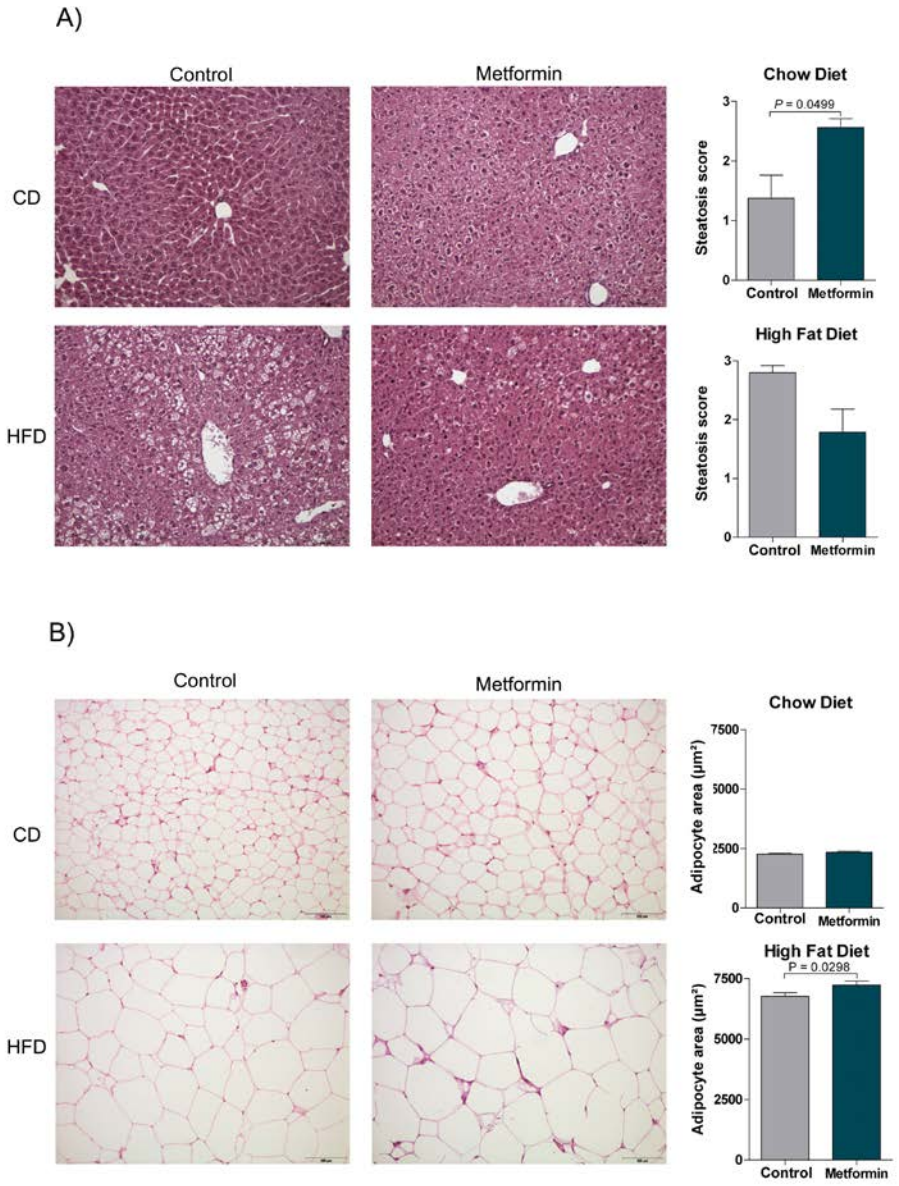

Figure 2. Hematoxylin-eosin staining of the liver and eWAT of PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). The arrows show ballooning hepatocytes. Magnification x10

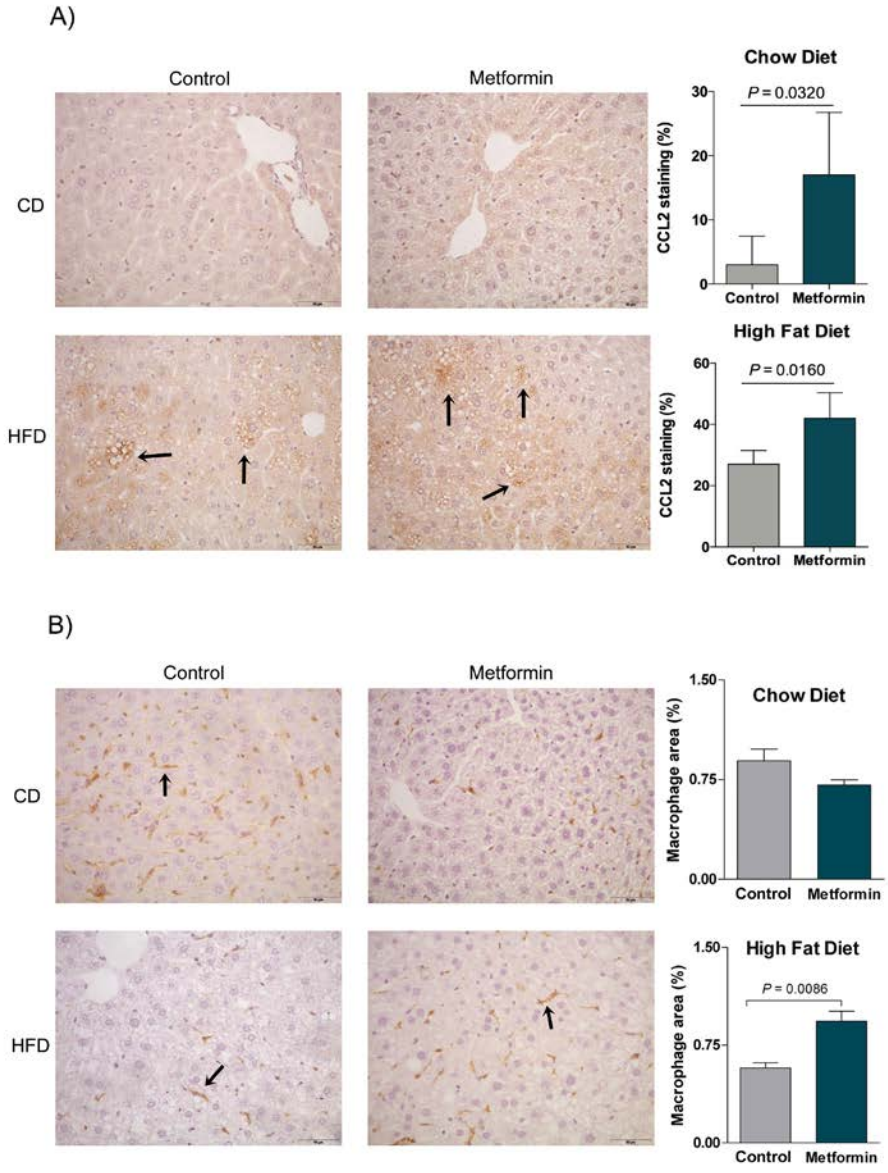

Figure 3. Immunohistochemical analyses of liver tissues of PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). A) Immunochemical staining for CCL2. The arrows show positively-stained areas. B) Immunochemical staining for F4/80 and macrophage area quantification. The arrows show positive staining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

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).

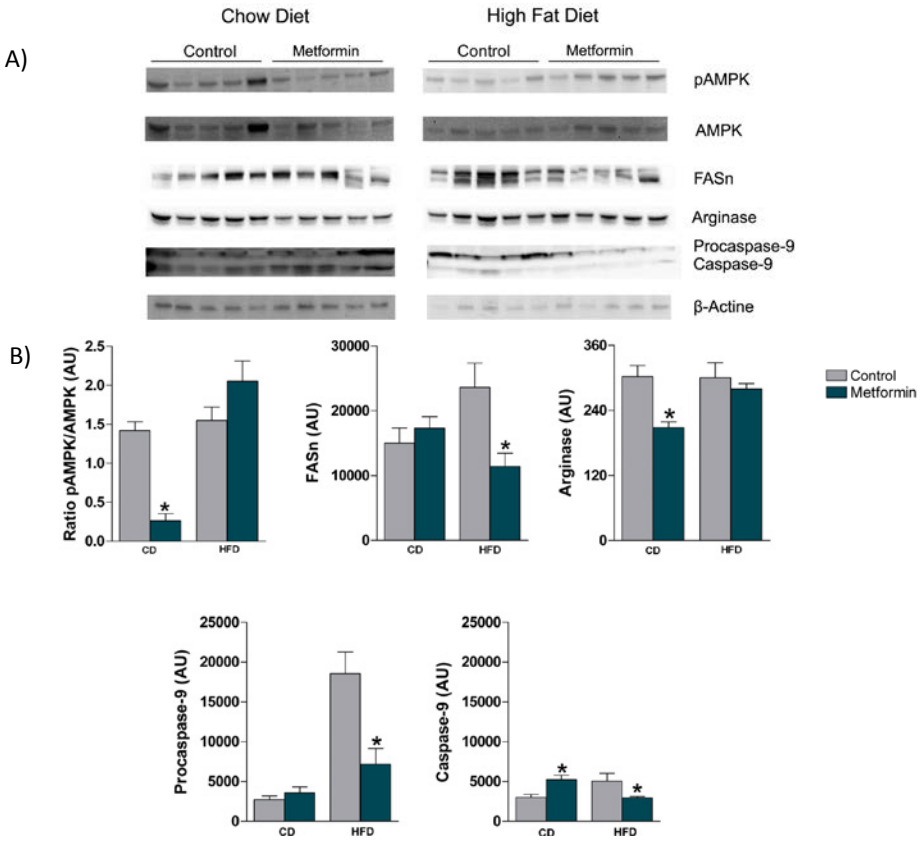

Figure 4. Western blot analyses of liver in PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). A) Immunoblots for pAMPK, AMPK, FASn, arginase, procaspase-9 and caspase-9. B) Quantification of these immunoblots. Results are shown as arbitrary units (AU). a $P < 0.05$ with respect to the control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

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).

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. 832) 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.

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DISCUSSION

As stated in the Introduction, the most important NCD are diabetes, cancer, cardiovascular disease, chronic respiratory disease and neurological diseases (1, 2). Their most common and preventable risk factors are smoking, sedentary lifestyle, unhealthy diet and the damaging use of alcohol. The combination of these factors triggers to key metabolic/physiological changes such as high blood pressure, overweight/obesity, and high blood glucose and cholesterol concentration (194).

NCD share common molecular mechanisms including oxidative stress, inflammation and mitochondrial alterations (3-8). The PON family of enzymes is an important endogenous antioxidant system implicated in several biochemical pathways: protection against oxidative damage and lipid peroxidation, contribution to innate immunity, bioactivation of drugs, detoxification of reactive molecules, modulation of endoplasmic reticulum stress and regulation of cell proliferation/apoptosis (27).

NAFLD, an important comorbidity of obesity, is accepted worldwide as the most common cause of chronic liver disease. Its incidence and prevalence are constantly increasing (173, 174). This disease is closely associated with the metabolic syndrome (MS), with approximately 90% of NAFLD patients having more than one of the following disorders: type 2 diabetes mellitus, obesity, dyslipidemia or hypertension (195). Previous studies from our group reported that serum PON1 activity may add relevant clinical information to the evaluation of chronic liver diseases. We also previously reported that patients with chronic liver diseases showed a significant increase of both serum PON1 concentration and hepatic PON1 expression, and reduced PON1 enzymatic activity. We also found that there were significant relationships between PON1, liver damage, liver fibrosis, fatty acid synthase (FAS) concentration and FAS expression in these patients (181, 196). The pathogenic mechanisms responsible for fatty liver disease are still not fully clarified.

Metabolomics is commonly employed to the study of part or the complete set of small molecules in biological samples. It has emerged as a powerful tool to discover novel biomarkers. One of its most important advantages is that this method can be used to identify an exclusive “metabolomic signature” of disease through the detection of changes in metabolite levels (197, 198). Several studies have identified new biomarkers for Parkinson´s

disease (199), prostate cancer (200) and type 2 diabetes mellitus among other important NCD (201, 202).

On the other side, when PON1 deficient mice are fed high-fat high-cholesterol diet, are more susceptible to present with inflammation, lipoprotein oxidation, atherosclerosis and hepatic steatosis when compared to wild type littermates (203, 204). The first objective of the present Thesis was to better understand how the deficiency of PON1 influences to the pathogenesis of NAFLD in mice.

The results showed dramatic metabolic and histological alterations in the livers of PON1 deficient mice fed a high-fat and high-cholesterol diet. PON1 deficient mice showed an increase in oxidative stress. Increased concentrations of peroxides 9-HODE and 13-HODE (which participate in the inhibition of triglyceride incorporation into lipoproteins and also stimulate extracellular matrix synthesis), provided a link between steatosis and fibrosis (205, 206). In addition, we observed a reduction in carnitine levels, which is an important factor in fatty acids transport to the mitochondrial matrix for their oxidation. Moreover, the amino acid methionine sulfoxide was increased. Methionine is a precursor for phospholipid synthesis. Hence, its reduction would imply impairment in the synthesis and secretion of very-low density lipoproteins. Therefore, these alterations would contribute to the development of steatosis in PON1 deficient mice.

We also observed a possible compensatory defense mechanism by the increase of the amino acid taurine in those mice. Studies reported that, in animals, the administration of taurine produces a decrease in the hepatic accumulation of triglycerides (207, 208). On the contrary, a decreased PUFA/MUFA ratio is observed in PON1 deficient mice. It is well known that PUFA play an important role in stimulating the expression of PPARα, and they play an anti-inflammatory and hepatoprotective role (209).

Several years ago Mackness et al. (123) demonstrated that PON1 inhibits CCL2 production in endothelial cells incubated with oxidized low density lipoproteins. We found an intense CCL2 immunostaining around lipid droplets in hepatic tissue sections of PON1 deficient mice, suggesting a close link between steatosis and the inflammatory response.

When steatosis is associated with inflammation, cell death, and fibrosis, it is called NASH. It can progress to cirrhosis, and then the risk of hepatocellular carcinoma is increased. Interestingly, the present study identified two pro-

oncogenic molecules (oratate and 8-oxo-dG) that were increased in PON1 deficient mice. Orotate is a precursor of pyrimidine nucleotides, and its excess is related to alterations in DNA synthesis (210) and the promotion of liver carcinogenesis (210, 211). Moreover, concentrations of 8-oxo-dG have been reported to be notably increased in the livers of rats treated with the powerful hepatocarcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (212). Hence, our study also suggests that the measurement of orotate and 8-oxo-dG could be useful biomarkers in estimating the probability of hepatocarcinoma development in patients with NAFLD.

Recent studies report that NAFLD is associated to increased cardiovascular risk. Accumulated evidence suggests that the clinical burden of NAFLD is not restricted to liver associated mortality, but also with a large amount of deaths associated to cancer (213, 214) and CVD (214, 215). Moreover, there is growing evidence that NAFLD is a risk factor by itself contributing to the development of CVD, independently of classical known risk factors (216). NAFLD has been demonstrated to be related with subclinical manifestations of atherosclerosis (216-219).

Atherosclerosis involves a complex interaction among endothelial cells of the arterial wall, blood cells, and circulating lipoproteins (100). Previous studies showed that in the atherosclerotic process, PON1 is accumulated in the artery wall (220). PON1 deficient mice fed with a proatherogenic diet have greater levels of oxLDL and larger atheromatous plaques (203). Hence, we decided to investigate the metabolic alterations in endothelial cells, when they were incubated with oxLDL and treated with HDL from PON1 deficient mice and wild type mice. Our study reported alterations in carbohydrate and phospholipid metabolism and increased apoptosis in cells incubated with oxLDL. These changes were partially prevented by wild type mouse HDL, but the effects were less effective with HDL from PON1 deficient mice. The risk of atherosclerosis is inversely associated with HDL concentration. The antioxidant and anti-inflammatory action of this lipoprotein is attributed, in part, to PON1 (221). Our results suggest that PON1 plays a significant role in endothelial cell survival by protecting cells from modifications induced by oxLDL in the respiratory chain. These results amplify current knowledge on the protective role of HDL and PON1 against oxidation and apoptosis in endothelial cells.

The fact that many NAFLD patients develop CVD (214, 215), suggests that there is potential association between NAFLD and cardiometabolic disorders. Oxidative stress and insulin resistance have been assumed to be driving factors in the progression of NAFLD to NASH, and both are recognized contributors to type 2 diabetes (222). In addition, the accumulation of fatty acid in the liver triggers to hepatic insulin resistance characterized by a higher production of endogenous liver glucose and also acts as a stimulus for further increased whole-body insulin resistance and dyslipidemia, leading to accelerated atherosclerosis (223).

Beneficial lifestyle changes led to ameliorate insulin resistance and hepatic steatosis (224). Metformin, an AMPK activator, regulates hepatic lipid metabolism by inducing adipose triglyceride lipase in patients with diabetes (150). Several studies supported the beneficial role of metformin in patients with NAFLD, showing an improvement on liver biochemistry, histology, and metabolic syndrome features (225-227). However, some recent studies have found no benefit of metformin treatment on liver steatosis, aminotransferase levels, and insulin resistance (228-230). In addition, several cases of metformin-induced aggravation of liver injury have been reported in patients with liver disease (228, 229, 231, 232); 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.

For this reason, we wanted to evaluate how the treatment of metformin affects the liver of PON1 deficient mice. Our results showed that the administration of metformin produced an aggravation of hepatic steatosis in the livers of PON1 deficient mice receiving a chow diet, and a general increased presence of CCL2 in the liver of animals fed either chow diet or high fat diet. This chemokine is responsible for the recruitment of monocytes to sites of inflammation, followed by their differentiation to macrophages (172) and is considered pathognomonic of the onset of the inflammatory reaction. Previous studies from our group reported that CCL2 is a good marker of the severity of inflammation in patients with liver disease (233). Moreover, several studies demonstrated that there is an inverse close relationship between PON1 and CCL2 (123, 234). We found that metformin administration under high fat diet was associated to an increase in the total

number of macrophages, and, although this number did not change in chow diet-fed animals, they had a significant reduction 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 (235). Unexpectedly, metformin did not produce an activation of AMPK in the liver of the mice fed either of the diets. These results could be due to our mice being PON1 deficient and having a certain degree of spontaneous steatosis. The effects of metformin in livers with steatosis remain unclear (148, 236, 237). In our model, AMPK inactivation in mice receiving chow diet and metformin could explain the accumulation of fat, resulting in an increase in hepatic steatosis. Nevertheless, in mice receiving high fat diet, metformin administration produces the opposite effect i.e. a reduction in the accumulation of fat in the liver. We propose a possible channeling of fat towards and accumulation in epididimal white adipose tissue, which is perhaps, a defense mechanism to protect the liver, due to a reduction observed in fatty acid synthase (FASn) protein expression in mice fed with high fat diet together its lower grade of steatosis. This reduction of FASn was also observed by Kita et al. (148) in metformin-treated mice.

Taking all these data into account and since patients with liver impairment have low hepatic and serum PON1 activities, our results suggest that metformin should be administered with caution in these patients. With respect to patients with type 2 diabetes, it would be interesting to monitor their liver function in for a possible hepatic dysfunction. We propose that PON1 is an important enzyme necessary for the beneficial effects of metformin in the liver.

CONCLUSIONS

Conclusions

- PON1 deficiency is associated to hepatic steatosis in mice fed a high fat and cholesterol intake. These changes are accompanied by severe metabolic alterations, and with increased oxidative stress and inflammation. Our results highlight the protective role of PON1 as an intracellular antioxidant in the liver. These findings could have clinical relevance since decreased serum and liver PON1 activity is an early alteration in patients with liver impairment.

- HDL from PON1 deficient mice has an impaired capacity to protect endothelial cells from oxLDL. We observed important metabolic disturbances when cells are incubated with HDL from PON1 deficient animals. These alterations include an impaired glycolysis, TCA cycle, phospholipid metabolism, and activation of apoptotic pathways.

- Metformin administration produces undesirable effects in the liver of PON1 deficient mice. An increase in the severity of steatosis was observed in animals fed a chow diet together with an aggravation of inflammation irrespective of the diet administered. Since individuals with liver impairment have low hepatic and serum PON1 activities, our results suggest caution on the administration of metformin in these patients.

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SUPPLEMENTARY MATERIAL

Journal of proteome research

Article

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Paraoxonase-1 Deficiency Is Associated with Severe Liver Steatosis in Mice Fed a High-fat High-cholesterol Diet: A Metabolomic Approach

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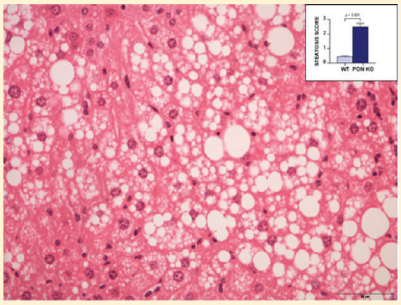
Supporting Information

ABSTRACT: Oxidative stress is a determinant of liver steatosis and the progression to more severe forms of disease. The present study investigated the effect of paraoxonase-1 (PON1) deficiency on histological alterations and hepatic metabolism in mice fed a high-fat high-cholesterol diet. We performed nontargeted metabolomics on liver tissues from 8 male PON1-deficient mice and 8 wild-type animals fed a high-fat, high-cholesterol diet for 22 weeks. We also measured 8-oxo-20-deoxyguanosine, reduced and oxidized glutathione, malondialdehyde, 8-isoprostanes and protein carbonyl concentrations. Results indicated lipid droplets in 14.5% of the hepatocytes of wild-type mice and in 83.3% of the PON1-deficient animals ($P < 0.001$). The metabolomic assay included 322 biochemical compounds, 169 of which were significantly decreased and 16 increased in PON1-deficient mice. There were significant increases in lipid peroxide concentrations and oxidative stress markers. We also found decreased glycolysis and the Krebs cycle. The urea cycle was decreased, and the pyrimidine cycle had a significant increase in orotate. The pathways of triglyceride and phospholipid synthesis were significantly increased. We conclude that PON1 deficiency is associated with oxidative stress and metabolic alterations leading to steatosis in the livers of mice receiving a high-fat high-cholesterol diet.



KEYWORDS: *metabolomics, nonalcoholic fatty liver disease, oxidative stress, paraoxonase-1, steatosis*

INTRODUCTION

Paraoxonase-1 (PON1) is an enzyme synthesized mainly by the liver and found in the circulation bound to high-density lipoproteins.[1,2] The original function attributed to PON1 was that of a lactonase, lipophilic lactones constituting its primary substrates.[3] PON1 also degrades oxidized phospholipids and, as such, plays a role in the organism's antioxidant system.[2] Alterations in circulating PON1 levels are associated with a variety of diseases involving oxidative stress.[2]

Hepatic steatosis represents the most common form of liver disease in Western societies.[4] In addition to being a precursor of fibrosis, cirrhosis, and hepatoma, hepatic steatosis is linked to diabetes, obesity, and cardiovascular disease.[5] It is also an important feature of the metabolic syndrome.[6] Oxidative stress plays a determinant role in the onset of steatosis and its progression to more severe forms of liver disease.[7] Also, it plays an important role in the development of inflammation,[8] and fibrogenesis.[9]

Since oxidative stress influences the changes leading to fatty liver and cirrhosis and, since PON1 exerts a protective effect against oxidative stress, it would be logical to infer an association between this enzyme and liver-function impairment. We had observed, in rats with experimental fibrosis, decreased hepatic PON1 activity related to enhanced lipid peroxidation and liver damage.[10] Moreover, serum PON1 activity was found to be decreased in patients with chronic hepatitis or cirrhosis, and the magnitude of the alteration was related to the extent of

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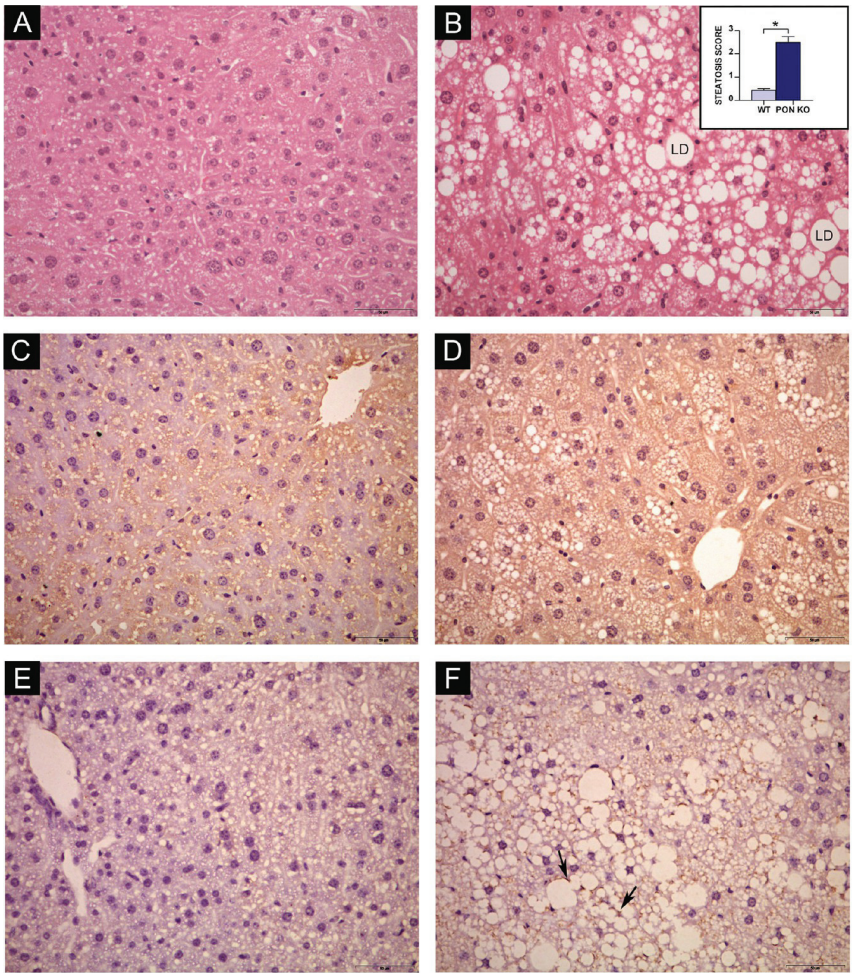


Figure 1. Histological analyses of liver tissue sections of PON1-deficient mice (right panels) and wild-type animals (left panels) fed with a high-fat high-cholesterol diet. (A and B) Hematoxylin-eosin. (C and D) 4-OH-nonenal immunohistochemistry. (E and F) MCP-1 immunohistochemistry. The insert in B is the steatosis score measurement in both types of mice. LD, lipid droplets. The arrows in F show positive MCP-1 immunostaining around lipid droplets.

liver damage.[11,12] Evidence also indicated that PON1 overexpression provided strong protection against the development of experimental liver disease.[13]

Despite these potentially important pointers, there is a dearth of experimental data on the biochemical mechanisms underlying the putative protective role of PON1 in liver disease. The present study sought to investigate the effect of PON1 deficiency in the livers of mice fed a high-fat high-cholesterol diet.

MATERIALS AND METHODS

Experimental Animals and Dietary Intervention

Male PON1-deficient animals of the C57BL/6J genetic background[14] were the progeny of mice provided by the Division of Cardiology of the University of California in Los Angeles. Wild-type animals were from the C57BL/6J strain (Charles River Laboratories, Wilmington, MA). At 10 weeks of age, eight mice of each strain were fed a high-fat high-cholesterol diet (w/w 20% fat and 1.00% cholesterol; Harlan, Barcelona, Spain). At 32 weeks of age, animals were sacrificed after an overnight fast. Livers were removed and stored at −80 °C until standard analyses of oxidative stress markers, or metabolomics analyses were performed. A portion of liver was fixed for 24 h in 10% neutral-buffered formalin for histological evaluation. Wild-type (n = 8) and PON1-deficient mice (n = 8) fed with a standard mouse chow (Charles River Laboratories) were used as controls. All procedures followed those set by the Ethics Committee on Animal Experimentation of the Faculty of Medicine of Reus which, in turn, reflected the Helsinki requirements.

Metabolomic Analyses of Liver Tissue

The metabolomics platform employed in the present study has been described in detail.[15] Briefly, small-molecule metabolites from slivers of liver tissue were extracted with methanol. The

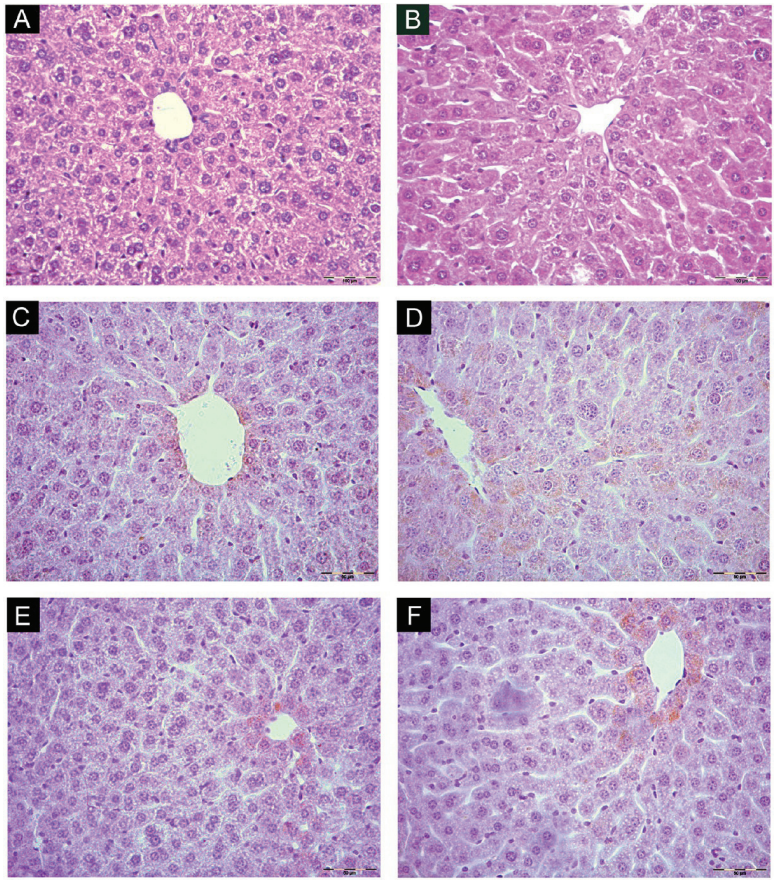


Figure 2. Histological analyses of liver tissue sections of PON1-deficient mice (right panels) and wild-type animals (left panels) fed with a standard mouse chow. (A and B) Hematoxylin-eosin. (C and D) 4-OH-nonenal immunohistochemistry. (E and F) MCP-1 immunohistochemistry.

resulting extract was divided into aliquots for analysis by ultra high performance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS; separately under positive as well as negative mode) and gas chromatography−mass spectrometry (GC−MS). Metabolites were identified relative to ion data of a reference library of approximately 2800 standard chemical entries that included retention times, mass (m/z), and MS or MS/MS spectra. Results of metabolomic measurements are expressed as the means of areas under the peaks of the PON1-deficient mice divided by the corresponding peaks of the wild-type mice.

Standard Biochemical Analyses of Oxidative Stress Markers

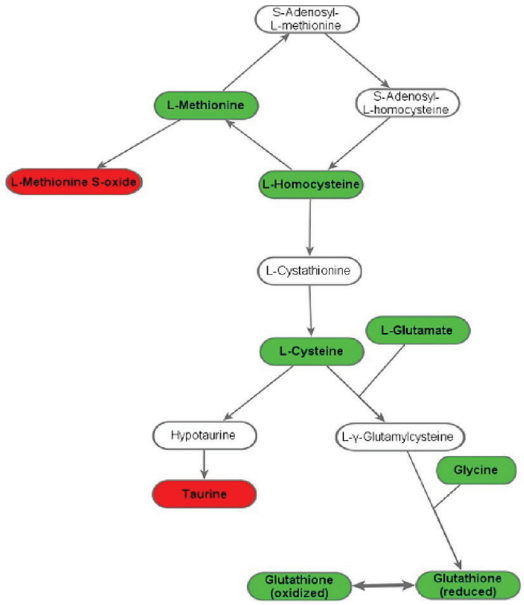
Hepatic concentrations of malondialdehyde (MDA), oxidized and reduced glutathione (GSSG and GSH, respectively), and 8-oxo-20-deoxyguanosine (8-oxo-dG) were measured by HPLC, as previously described.[16−18] Tissue levels of 8-isoprostanes and protein carbonyls were determined using commercial ELISA assays (Cayman Chemical Co., Ann Arbor, MI).

Histological Analyses

Liver sections of 2 μm thickness were stained with hematoxylin and eosin to evaluate histological alterations. The degree of steatosis was evaluated by image analysis software (AnalySIS image software system, Soft Imaging System, Munster, Germany) together with a semiquantitative score reflecting the percentage of hepatocytes containing lipid droplets. The scores were arbitrarily dichotomized as 1: <33%; 2: 33−66%; 3: >66%.[19] Monocyte chemoattractant protein-1 (MCP-1) expression as a marker of inflammation was measured by immunohistochemistry using specific antibodies (Santa Cruz Biotechnology Inc. Santa Cruz, CA). 4-hydroxy-2-nonenal (4-HNE) protein adducts as an index of lipid peroxidation were analyzed with a specific antibody purchased from the Japan Institute for the Control of Aging (Shizuoka, Japan). All immunohistochemical methods had negative controls which were treated similarly to test samples, but with the primary antibody omitted from the incubations.

Statistical Analyses

Differences between any two groups were assessed with the Mann−Whitney U test. Spearman correlation coefficients were used to evaluate the degree of association between variables. Welch's t-test for group comparisons was used for metabolomic analyses. Statistical software employed was either the program



SUPER PATHWAY	BIOCHEMICAL NAME	PON1 KO-32wk / WT-32wk
Glutathione	glycine	0.49
	glutamate	0.71
	cysteine	0.50
	methionine sulfoxide	1.61
	hypotaurine	0.40
	taurine	1.84
	S-adenosylhomocysteine	1.15
	methionine	0.42
	homocysteine	0.44
	glutathione, reduced	0.27
	glutathione, oxidized	0.81

Figure 3. Alterations in the glutathione pathway in PON1-deficient mice compared to wild-type animals. The data on the right show the quotients of the areas-under-the-peak of the PON1-deficient mice relative to those of the wild-type animals. Decreased and increased metabolites that achieve statistical significance are shown in green and red, respectively.

"R" http://cran.r-project.org/ (for metabolomic analyses) or the SPSS 18.0 package (standard biochemical analyses).

RESULTS

Histological Analyses

Histological examination showed a marked steatosis in the liver tissue of PON1-deficient mice fed with a high-fat high-cholesterol diet; the steatosis score being significantly increased ($p < 0.001$) in these animals compared to the wild-type mice (Figure 1A-B). Lipid droplets were present in 14.5% (on average) of the hepatocytes of wild-type mice and in 83.3% of the PON1-deficient animals. Immunohistochemical analyses showed an increased expression of 4-HNE and MCP-1 (markers of oxidative stress and inflammation, respectively) in PON1-deficient mice, compared to their wild-type counterparts (Figure 1C–F). On the contrary, PON1-deficient mice fed with a standard mice diet did not show any evidence of histological hepatic alterations, increased inflammation or oxidative stress compared to wild type animals (Figure 2), and for this reason, the metabolomic and biochemical study was only continued in animals fed with a high-fat high-cholesterol diet.

Metabolomic Profiling

Results of the global metabolomic analyses, including an exhaustive list of the measured metabolites, unadjusted data, and heat map, are shown in Supplementary Table 1 (Supporting Information). We analyzed 322 biochemical compounds and, relative to the wild-type animals, 169 were significantly decreased and 16 were increased in PON1-deficient mice. The main findings are highlighted below.

Glutathione Metabolism. GSH reduces peroxides and free radicals in a nonenzymatic process, to produce GSSG. Normally, GSH levels are modulated to meet oxidative demands by regulated rates of synthesis as well as significant recycling via the gamma-glutamyl cycle. Liver tissue PON1$^{(-/-)}$ showed significant GSH and GSSG depletion (Figure 3). GSSG levels decreased moderately (to approximately 80% of control mice values) while hepatic GSH content showed a considerable depletion (to approximately 27% of control mice values). As such, the ratio of GSSG/GSH was increased in PON1-deficient liver tissue; strongly indicative of exposure to oxidative stress. We also observed significantly lower levels of GSH precursors, and recycling pathway metabolites, in PON1-deficient mice. Methionine, an essential amino acid, was significantly reduced in the PON1-deficient liver tissue, as were most metabolites involved in the biosynthetic pathway between methionine and the GSH biosynthetic precursor cysteine. Also affected were the metabolites reflecting the alternate pathways of methionine salvage. Comparable changes in the parallel metabolic pathway of α-ketobutyrate to ophthalmate are in agreement with these precursor-limiting influences (Figure 3).

Other Oxidative Stress Markers. The hepatic concentrations of α-tocopherol and ascorbate (and their biosynthetic precursor in rodents, gulono-1,4-lactone) were significantly lower in PON1-deficient mice. Significant increases in levels of 13-hydroxy-octadecadienoate (13-HODE) and 9-hydroxy-octadecadienoate (9-HODE) are indicators of elevated lipid peroxidation, and provide evidence of an oxidizing environment in liver tissue with PON1 deficiency. Likewise, an elevated level of the oxidized amino acid methionine sulfoxide provides additional support for this concept (Supplementary Table 1, Supporting Information).

Alterations in Hepatic Lipid Metabolism. Liver metabolism is assessed here following an overnight fast, thus the predominant metabolic activity under normal conditions would encompass gluconeogenesis from lactate, glycerol, and

SUPER PATHWAY	BIOCHEMICAL NAME	PON1 KO-32wk: WT-32wk
Amino acid	threonine	0.60
	aspartate	0.44
	asparagine	0.19
	glutamate	0.71
	glutamine	0.66
	histidine	0.45
	leucine	0.45
	valine	0.48
	methionine	0.42
	isoleucine	0.49
	arginine	0.14
	alanine	0.45
	proline	0.42
Glycolisis	glucose-6-phosphate	1.06
	glucose	1.02
	fructose-6-phosphate	0.96
	isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate	2.02
	3-phosphoglycerate	0.72
	1,3-dihydroxyacetone	2.14
	phosphoenolpyruvate	0.63
	lactate	0.66
Energy	citrate	0.27
	cis-aconitate	0.31
	succinate	0.89
	succinylcarnitine	0.68
	succinyl CoA	0.79
	fumarate	0.54
	malate	0.69
	phosphate	0.75

Figure 4. Alterations in the glycolytic pathway, Krebs cycle, and amino acid pathways in PON1-deficient mice compared to wild-type animals. The data on the right show the quotient of the areas-under-the-peak of the PON1-deficient mice relative to those of the wild-type animals. Decreased and increased metabolites that achieve statistical significance are shown in green and red, respectively.

amino acids to generate glucose for release to the circulation and use of fatty acids released from adipose tissue lipid stores for ketogenesis. Glycerol, mono- and diacylglycerol levels showed a significant decrease in PON1-deficient mice, suggesting decreased triacylglyceride lipolysis or increased synthesis (Supplementary Table 1, Supporting Information). Free fatty acid levels were altered with PON1 deficiency. Seven polyunsaturated fatty acids (PUFA) were decreased, while three monounsaturated fatty acids (MUFA) were increased, that is, the ratio of PUFA/MUFA was lower in PON1-deficient mice than in wild-type animals. In addition, we observed a decrease in carnitine levels. Carnitine is a quaternary ammonium compound necessary for the transport of long-chain fatty acids into the mitochondria. Carnitine can be diet-derived or synthesized from lysine and proline; amino acids that are decreased in PON1-deficient mice. The overall outcome would be a depressed fatty acid oxidation, which is supported by significantly lower levels of the ketone body 3-hydroxybutyrate. Phospholipid precursors, lysolipid intermediates, and breakdown products showed complex alterations in livers of PON1-deficient mice. Lysolipid levels, for example, can reflect relative rates of membrane remodeling. Levels of multiple lysolipids were reduced by PON1 deficiency, which indicates relatively reduced membrane remodeling and/or breakdown under these conditions. Metabolism of bile acids is also reduced, with a decrease in the levels of squalene, which is the precursor of cholesterol and bile acid synthesis and which is necessary for the absorption of dietary lipids and hydrophobic vitamins A, D, E and K.

Glucose Metabolism. Glucose metabolism in liver is impacted upon by the reciprocally regulated pathways of glycolysis and gluconeogenesis. We observed, in PON1-deficient mice, a significant alteration in intermediates that are shared by these opposing glucose metabolism pathways including lover levels of 3-phosphoglycerate and phosphoenolpyruvate, but elevated fructose 1,6-diphosphate (observed as a isobar with glucose 1,6-diphosphate because they are indistinguishable on the metabolomics platform). Together, ketogenesis is reduced which is consistent with relatively low acetyl-CoA and thus relatively low activation of pyuvate carboxylase (gluconeogenesis) and the fructose 1,6-diphosphate is elevated, which is an allosteric activator of the enzyme pyruvate kinase (glycolysis). These findings indicate aberrant regulation of liver glucose metabolism in PON1-deficient mice in the fasted condition. Low levels of the 3-carbon intermediates as well as lactate suggest that despite high fructose 1,6-diphosphate, glycolysis is not activated. Moreover, these changes were accompanied by a reduction in Krebs cycle activity, as indicated by significantly lower levels of several intermediates. In addition, increased levels of several intermediates in the pentose phosphate pathway (PPP) including ribulose 5-phosphate/xylulose 5-phosphate (isobars) and ribose 5-phosphate were observed in PON1-deficient mice relative to wild-type, which suggest a shift from glycolysis to the PPP. Because the early steps in the PPP are important for

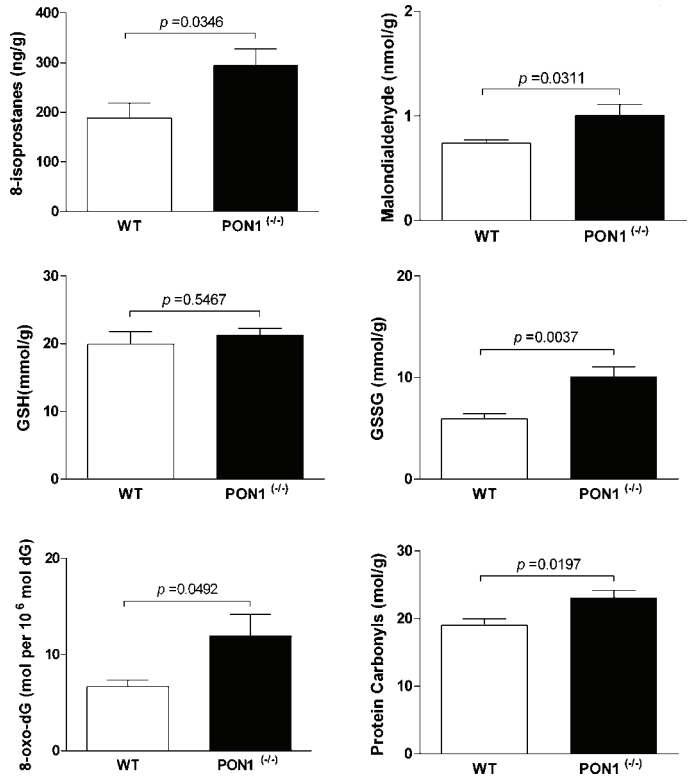


Figure 5. Results of oxidative stress markers in PON1-deficient mice compared to wild-type animals.

generating reducing equivalents in the form of NADPH, this elevation may reflect higher requirements to regenerate reduced glutathione. Overall, livers from PON1-deficient mice showed an impaired ability to obtain energy from sugar (Figure 4) or fat.

Amino Acid and Nucleotide Metabolism. Amino acid levels were significantly lower in PON1-deficient liver tissue (Figure 4). The values reflect the combined influence of uptake from the circulation, *de novo* synthesis, protein synthesis and degradation rates, as well as amino acid catabolism. The urea cycle, a key aspect of the nitrogen biochemical pathway, can serve as a marker of amino acid catabolic rates. Intermediates in this cycle were also reduced in livers of PON1-deficient mice suggesting that the low amino acid levels are present even in the circumstance of reduced catabolism. Although liver can extract amino acids from the circulation via specific amino acid transporters, one hypothesis to explain this impact of PON1 deficiency on amino acid levels in the liver is that the severe depletion of glutathione, as a result of high oxidative demands, reduces the γ-glutamyl cycle activity. This not only serves to recycle glutathione but also to transfer amino acids across the plasma membrane.

With regard to nucleotide metabolism, we observed significant increases in orotate and inosine in PON1-deficient mice, relative to their wild-type counterparts. Orotate is known to accumulate under conditions in which ornithine, as a substrate for ornithine transcarbamoylase, is limited, and as such, this suggests that the two impacts of PON1 deficiency in the liver may be related (Supplementary Table 1, Supporting Information).

Cofactors and Vitamins. Coenzyme A is synthesized via a multistep, ATP-dependent pathway from the vitamin pantothenate. This precursor was significantly reduced in the livers of PON1-deficient mice. Similar changes were also observed for several other vitamins and cofactors, including several B-vitamins and the cofactors flavin adenine dinucleotide and flavin mononucleotide (Supplementary Table 1, Supporting Information). Cofactor and vitamin limiting levels in the PON1-deficient animals are likely to have profound impacts on multiple biochemical pathways.

Standard Biochemical Analysis of Oxidative Stress Markers

Metabolomic analyses of oxidative stress markers were confirmed by standard biochemical methods. We observed that PON1-deficient mice had significant increases in the hepatic content of MDA, GSSG and 8-isoprostanes (indices of lipid peroxidation), protein carbonyls (indices of protein oxidation), and 8-oxo-dG (an index of DNA oxidation) (Figure 5).

■ DISCUSSION

Nonalcoholic fatty liver disease (NAFLD) is defined, in humans, as the accumulation of triglycerides within hepatocytes that exceeds 5% of liver weight. This alteration is gradually becoming one of the most common observations in liver diseases, and is identified using imaging techniques in about 30% of adults.[20] Excessive food intake is perceived as one of the

main causes of NAFLD.[21] Recent studies show that a high cholesterol intake is a major stimulant in the development of NAFLD.[22] The present study shows dramatic metabolic and histological alterations in the livers of PON1-deficient mice fed a high-fat high-cholesterol diet, suggesting that this enzyme plays a major role in the protection against diet-induced fatty liver. Enhanced hepatic oxidative stress is demonstrated in our PON1-deficient mice by the increased concentrations of several biochemical markers of lipid, protein and DNA oxidation, and by the increased GSSG/GSH ratio. A notable consequence of oxidative stress is the increased concentration of the peroxidized lipids 9-HODE and 13-HODE. These compounds inhibit the incorporation of triglycerides into lipoproteins[23] and, as such, may contribute to the development of steatosis. They also stimulate extracellular matrix synthesis,[24] and thus provide a link between benign steatosis and fibrosis.

Several studies indicate a strong association between oxidative stress and lipid alterations in steatosis and steatohepatitis.[25] The present study showed PON1 deficiency to be associated with decreased carnitine levels which, in turn, may be explained by altered amino acid metabolism. Carnitine is a key factor in fatty acid oxidation, that is, the transport of free fatty acids into the mitochondrial matrix is regulated by the carnitine-dependent enzyme shuttle.[26] A decreased hepatic carnitine concentration could result in inhibition of free fatty acid oxidation, and this derangement is associated with increased fat content.[27] Our model may differ, perhaps, from human steatosis, since it is not clearly evident whether downregulation of fatty acid oxidation is involved in the onset of this derangement.[22] Kotronen et al.[27] had not found any alterations in hepatic fatty acid oxidation in patients with NAFLD, both in the basal state and after exogenously induced hyperinsulinemia.

Data are scarce regarding the pattern of hepatic fatty acid composition in NAFLD. We observed decreased concentrations of most free fatty acids in PON1-deficient mice, and a decreased PUFA/MUFA ratio. These results are similar to those of De Almeida et al.[28] showing that patients with steatohepatitis had higher MUFA concentrations than control subjects. In addition, Wang et al.[29] observed a decrease in PUFA and in PUFA and saturated fatty acids in mice receiving a high-fat, high-cholesterol diet supplemented with 0.5% bile. PUFA are known to play an important role in stimulating the expression of PPARα, and they play an anti-inflammatory and hepatoprotective role as well.[30]

Hepatic lipid metabolism is closely linked to glucose metabolism. Our results show that PON1 deficiency is associated with a general decrease in the glycolytic and Krebs cycle pathways, indicating a decreased ability to obtain energy. The mechanisms underlying these alterations cannot be fully ascertained from the present study, but we also observed significant decreases in the hepatic concentrations of cofactors that play key roles in these pathways. We also observed increased concentrations of ribose 5-phosphate, ribulose 5-phosphate/xylulose 5-phosphate (isobars) and xylonate. These data suggest a shift of glucose metabolism from the glycolytic to the pentose phosphate pathway. This concept is supported by the observed increased concentrations of mannose and fructose. Alterations in glycolysis and Krebs cycle may influence lipid metabolism in several ways. For example, decreased Krebs cycle may decrease acetyl-CoA carboxylase and, subsequently, fatty acid synthase (FAS) leading to an inhibition of fatty acid synthesis. This mechanism would explain the general decrease in fatty acid concentrations observed in our study. However, this effect could be partially counteracted by the increased xylulose 5-phosphate, which stimulates the carbohydrate responsive element binding protein, and stimulates FAS activity.[31]

Hepatic amino acid concentrations were notably decreased in PON1-deficient mice, with the exceptions of methionine sulfoxide and taurine, which were increased. Methionine sulfoxide is the oxidized form of methionine and cannot be utilized by tissues. An increase in the concentration of this metabolite could result in a decreased methionine availability. Methionine, as a key methyl group donor for choline biosynthesis, is a precursor for phospholipid synthesis. Hence, a decrease would imply impairment in the synthesis and secretion of very-low density lipoproteins which, in turn, would contribute to the development of steatosis.[32] Indeed, the administration of a choline- and methionine-deficient diet to mice is widely employed as an experimental model of stetatohepatitis.[33] Taurine plays an important role in several metabolic functions such as detoxification, membrane stabilization, and antioxidation, suggesting that the observed increase is a compensatory defense mechanism. This hypothesis is supported by data from Chang et al.[34] who observed that the administration of taurine produced a decrease in the hepatic accumulation of triglycerides in hamsters receiving a high-fat diet. They also observed that taurine increased the cytochrome 7A1 levels, which intervenes in the catabolism and secretion of cholesterol. Further, Chen et al.[35] reported that the administration of taurine protected against the development of steatosis in rats fed ethanol, by reducing oxidative stress and downregulating the expression of adiponectin and tumor necrosis factor.

Our results identified intense MCP-1 immunostaining around lipid droplets in hepatic tissue sections of PON1-deficient mice, which were not observed in wild-type animals. We previously reported similar findings (including high plasma MCP-1 concentrations) in low-density lipoprotein receptor-deficient mice fed a high-fat high-cholesterol diet.[36] In both models, MCP-1 hepatic expression is detected around lipid droplets, suggesting a close link between steatosis and the inflammatory response. Taken together, these data suggest that the liver is a significant contributor to the organism's MCP-1 pool. This is a novel concept, since it is generally accepted that the hepatic inflammation in NAFLD and NASH is related to adipose tissue MCP-1 overexpression which would indirectly influence hepatic inflammation.[21] We suggest that, on the contrary, it is the hepatic MCP-1 synthesis that plays the significant role in this process. The finding that PON1 deficiency is associated with increased MCP-1 expression is not surprising since, as we had demonstrated several years ago, PON1 inhibits MCP-1 production in endothelial cells incubated with oxidized low density lipoproteins.[37]

Finally, the present study provides new data on the relationships between steatosis and hepatocellular carcinoma (HCC). This type of cancer can occur in livers without underlying cirrhosis.[38,39] The present study has identified two pro-oncogenic molecules: orotate and 8-oxo-dG, the concentrations of which are increased in the livers of PON1-deficient mice. Hepatic concentrations of orotate in PON1-deficient mice are 2-fold that in control animals. This compound is a precursor of pyrimidine nucleotides, and its excess has been shown to alter DNA synthesis[40] and to promote liver carcinogenesis.[40,41] Conversely, 8-oxo-dG-adducts are produced

as a consequence of oxidative DNA damage;[18] the adducts being mutagenic and the cause of G-to-T transversions.[42] Concentrations of 8-oxo-dG have been reported to be notably increased in the livers of rats treated with the powerful hepatocarcinogen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.[43] The association between NAFLD and HCC represents a growing area of study, albeit the specific sequence of events leading to HCC in the setting of NAFLD is still unresolved. We present novel data indicating that steatosis induced by PON1 deficiency is associated with increased concentrations of at least two pro-oncogenic molecules which could explain, at least in part, the increased susceptibility of fatty liver toward cancer. Our study also suggests that the measurement of orotate and 8-oxo-dG could be useful biomarkers in estimating the probability of HCC development in patients with NAFLD. However, we did not specifically investigate HCC in the present work, and further studies are warranted in appropriate patients to explore this hypothesis.

Nutritional investigations in humans have suggested that high-fat high-cholesterol diets are important determinants in NAFLD, independently of the concomitant development of insulin resistance or metabolic syndrome.[22] High cholesterol intake and increased serum cholesterol concentrations have been reported to be among the strongest risk factors in the development of NAFLD.[44,45] Cholesterol overload can upregulate the LXRα-SREBP-1c pathway in the liver and activate fatty acid synthesis which, in turn, would lead to steatosis.[22] Previous studies have shown that NAFLD is associated with oxidative stress and low serum and hepatic PON1 levels in patients and in rats with fatty liver induced by a methionine-choline-deficient diet.[46−48] The finding of reduced hepatic PON1 activity in rats with experimental steatosis is interesting and, together with the present investigation, suggest that intracellular PON1 is more important than circulating PON1 in protecting liver tissue from dietary-induced changes leading to NAFLD.

A caveat to the present results is that, since NAFLD is not a monogenic disorder in humans, studies in animals with merely a single gene deletion may not mimic the etiology of the human disease at the molecular level. In addition, the small number of animals in the present study would suggest that our findings be considered preliminary. However, the phenotypic alterations observed in our experimental model are essentially consistent with the current knowledge of human NAFLD.

CONCLUSION

The main goal of the present investigation was to demonstrate that PON1 plays a protective role against hepatic derangements, secondary to fat and cholesterol overnutrition. We highlight, as well, some biochemical pathways that could explain the observed relationships between the "benign" steatosis and more severe forms of liver disease, such as fibrosis or HCC. Our findings could have considerable clinical relevance since decreased serum and liver PON1 activity is an early alteration in patients with liver impairment.[10−13]

ASSOCIATED CONTENT

Supporting Information

Supplementary table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): E.K. and R.P.M. are employees of Metabolon, Inc. and, as such, have affiliations with or financial involvement with Metabolon, Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed..

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Supplementary information corresponding to the manuscript:

Paraoxonase-1 Deficiency is Associated with Severe Liver Steatosis in mice Fed a High-fat High-cholesterol Diet: A Metabolomic Approach.

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Research Article

Paraoxonase-1 Inhibits Oxidized Low-Density Lipoprotein-Induced Metabolic Alterations and Apoptosis in Endothelial Cells: A Nondirected Metabolomic Study

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We studied the influence of PON1 on metabolic alterations induced by oxidized LDL when incubated with endothelial cells. HUVEC cells were incubated with native LDL, oxidized LDL, oxidized LDL plus HDL from wild type mice, and oxidized LDL plus HDL from PON1-deficient mice. Results showed alterations in carbohydrate and phospholipid metabolism and increased apoptosis in cells incubated with oxidized LDL. These changes were partially prevented by wild type mouse HDL, but the effects were less effective with HDL from PON1-deficient mice. Our results suggest that PON1 may play a significant role in endothelial cell survival by protecting cells from alterations in the respiratory chain induced by oxidized LDL. These results extend current knowledge on the protective role of HDL and PON1 against oxidation and apoptosis in endothelial cells.

1. Introduction

Atherosclerosis, one of the major causes of morbidity and mortality in the Western world, involves complex interactions among endothelial cells of the arterial wall, blood cells, and circulating lipoproteins [1]. Oxidative stress, which is mainly derived from mitochondrial dysfunction, decreases nitrous oxide (NO) synthesis, upregulates the secretion of adhesion molecules and inflammatory cytokines, and is responsible for the oxidation of low-density lipoproteins (LDLs) [2, 3]. These events play a key role in the pathogenesis of atherosclerosis [4, 5].

Paraoxonase-1 (PON1) is an enzyme found in the circulation associated with high-density lipoproteins (HDLs) [6, 7]. The original function attributed to PON1 was that of a lactonase, and lipophylic lactones constitute its primary substrates [8]. PON1 also degrades oxidized phospholipids and, as such, plays a role in an organism's antioxidant system [7]. In the atherosclerosis process, PON1 accumulates in the artery wall [9], and PON1$^{(-/-)}$ mice have been shown to have greater levels of oxidized LDL and larger atheromatous plaques when fed a proatherogenic diet [10]. PON1 also inhibits the production of the proinflammatory chemokine monocyte chemoattractant protein-1 (MCP-1), induced by oxidized LDL in endothelial cells [11].

Despite its potential clinical and biochemical relevance, there is a paucity of studies investigating the influence of PON1 on metabolic alterations when oxidized LDL is incubated with endothelial cells. We reasoned that metabolomics might be a useful tool to evaluate the effects of this enzyme. The study was complemented with an evaluation of oxidative stress and apoptosis in this cell line.

2. Materials and Methods

2.1. Experimental Design. We employed primary cultures of human umbilical vein endothelial cells (HUVECs), cultured according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). HUVECs were grown in medium 200 supplemented with low serum growth, 10 mg/L gentamicin and 0.25 mg/L amphotericin (all these reagents were from Invitrogen), and maintained in a humidified incubator at 37°C, with 5% CO_2. Cells were subcultured when 80%–90% confluent. In all the experiments, cells were plated in 10 cm Petri dishes at a density of 2.5×10^3 cells per dish, and at passage 3. Petri dishes at 70% confluence were incubated over 24 h with isolated human LDL (50 mg/L), oxidized LDL (50 mg/L), oxidized LDL (50 mg/L) + HDL (40 mg/L) from wild type mice, oxidized LDL (50 mg/L) + HDL (40 mg/L) from PON1$^{(-/-)}$ mice, or with serum-free media as controls. All incubations were performed in serum-free media.

Normal human sera were obtained from healthy individuals participating in a population-based study being conducted in our institution. The study was approved by the Ethics Committee (Institutional Review Board) of the Hospital Universitari Sant Joan de Reus. Sera were pooled and used for lipoprotein fractionation and LDL isolation by sequential preparative ultracentrifugation [12, 13]. Human oxidized LDL was prepared by incubation of native LDL with 5 μM $CuSO_4$, as described previously [11]. Increased thiobarbituric acid-reactive substances levels were detected in LDL after oxidation (45 versus <0.5 mmol/g protein).

Normal mice were from the C57BL/6J strain (Charles River Labs., Wilmington, MA, USA), and PON1$^{(-/-)}$ mice were the progeny of those provided by the Division of Cardiology of the University of California in Los Angeles and were of a C57BL/6J genetic background [10]. Animals were housed under standard conditions and given a commercial mouse diet (14% Protein Rodent Maintenance Diet, Harlan, Barcelona, Spain) in accordance with our institutional guidelines. At 16 weeks of age, they were sacrificed and approximately 30 mL of sera were pooled for HDL isolation [12, 13].

2.2. Metabolomics Analyses. The metabolomics platform employed in the present study has been previously described in detail [14]. Briefly, small molecule metabolites from an equivalent amount of cell cytoplasm homogenates were extracted with methanol, and the resulting extract divided into equal fractions for analysis by ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; separately under positive mode and negative mode) and gas chromatography-mass spectrometry (GC-MS). Metabolites were identified by comparing the ion data obtained to a reference library of ~2,800 chemical standard entries. Comparisons included retention times, mass (m/z), and MS or MS/MS spectra. Results of metabolomics measurements are expressed as the mean quotients between the areas under the peak of the different experimental conditions.

Differences between groups were assessed with Welch's t-test for group comparisons. Statistical analyses were performed with the program "R" http://cran.r-project.org/.

2.3. Caspase 9 Western Blot. We analyzed caspase 9 expression in endothelial cell homogenates as a marker of apoptosis pathways. The cytoplasmic homogenates were prepared with a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) [15]. Denaturing electrophoresis was performed in polyacrylamide gels (4–12%) from Invitrogen (Carlsbad, CA, USA). Transfer was performed with the iBlot Gel Transfer Device (Invitrogen). Blotting was performed with the ECL Advanced Western Blotting Detection Kit (GE Healthcare, Fairfield, CT, USA) using a rabbit anticaspase 9 antibody at 1 : 2000 dilution (Abcam, Cambridge, UK) [13].

2.4. Measurement of Apoptosis by Flow Cytometry. Cells (300 μL of cell suspension at approximately 10^9 cells/L) were stained with annexin V conjugated with fluorescein isothiocyanate in the presence of propidium iodide. This enables the detection of phosphatidylserine on the surface of apoptotic cells. We used the Annexin-FITC Kit (Beckman-Coulter, Fullerton, CA, USA) according to the manufacturer's instructions, in a Coulter Epics XL-MLC flow cytometer (Beckman-Coulter).

2.5. Measurement of PON1 Activities and Total Peroxide Concentrations. PON1 lactonase activity in the culture's supernatant was measured as the hydrolysis of 5-thiobutyl butyrolactone (TBBL), as described [16]. The assay reagent contained 1 mmol/L $CaCl_2$, 0.25 mmol/L TBBL, and 0.5 mmol/L 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.05 mmol/L Tris-HCl buffer (pH = 8.0). The change in absorbance was monitored at 412 nm. Activities were expressed as U/L (1 U = 1 mmol of TBBL hydrolyzed per minute). The concentration of total peroxides in the supernatant was determined by a colorimetric enzymatic assay (Immundiagnostik, AG, Bensheim, Germany).

3. Results and Discussion

PON1 lactonase activity remained relatively low in supernatants of those cultures not containing added HDL. PON1 lactonase activity significantly increased in those cultures with normal HDL and returned to low levels in those cultures with HDL from PON1$^{(-/-)}$ mice. These results were as expected and provide a quality control of the HDL preparations obtained (Figure 1(a)). Total peroxide concentrations in the supernatants were maximal in the cultures with added oxidized LDL and showed a significant decrease following the addition of normal HDL. This decrease was not as marked following the addition of HDL from PON1$^{(-/-)}$ mice (Figure 1(b)).

We analyzed 124 biochemical compounds by nondirected metabolomics, corresponding to carbohydrate, lipid, amino acid, and nucleotide metabolism, as well as vitamins and xenobiotics. We obtained statistically significant variations in 37 metabolites (Table 1). The main findings corresponded to

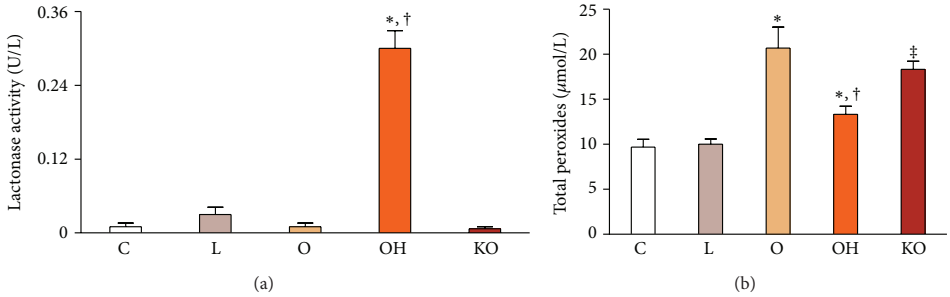

FIGURE 1: PON1 lactonase activity (a) and total peroxide concentrations (b) in the supernatant of the HUVEC cell culture ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). $^{*}P < 0.05$, with respect to C; $^{\dagger}P < 0.05$, with respect to O; $^{\ddagger}P < 0.01$, with respect to C.

carbohydrate and phospholipid metabolism and are summarized in the following sections.

3.1. Hexose Metabolism. The addition of LDL to cultured endothelial cells decreased the levels of gluconate, galactose, and phosphorylated hexose intermediates. These molecules are important entrance intermediates in energy- and biomass-generating pathways such as glycolysis, pentose phosphate, and protein glycosylation. Their decreases suggest that these pathways were activated to a greater extent in endothelial cells treated with LDL, compared to control-treated cells. In contrast, increased levels of gluconate, galactose, and phosphorylated hexose intermediates were seen in all cells that were treated with oxidized LDL, relative to LDL alone, and regardless of whether HDL was also added to the cultures (Figure 2).

3.2. Glycolysis and Tricarboxylic Acid (TCA) Cycle. Relative to control cultures, the addition of LDL resulted in increased levels of 3-phosphoglycerate and 2-phosphoglycerate (which are 3-carbon glycolytic intermediates). This same treatment also increased the levels of the TCA cycle intermediates (fumarate and malate) relative to control cultures. An interpretation of these data is that uptake of LDL by endothelial cells results in the generation of acetyl-CoA, which drives flux through the TCA cycle. Increased levels of LDL-generated acetyl-CoA may have relieved the need for carbohydrate-derived precursors, thereby inhibiting glycolytic flux into the TCA cycle and elevating the 3-carbon intermediates.

By comparison, treatment of endothelial cells with oxidized LDL may have induced levels of oxidative stress that were sufficient to impair normal energy pathways. For example, in cells treated with oxidized LDL, 6-carbon glycolytic intermediates accumulated, whereas the 3-carbon intermediates were reduced. This may be due to changes in glyceraldehyde-3-phosphate dehydrogenase (GADPH; levels or activity) in response to oxidized LDL, since superoxide overproduction inhibits GADPH through a mechanism that involves poly (ADP-ribose) polymerase (PARP) activation [16]. Likewise, TCA cycle intermediates were lower in oxidized LDL-treated cells due, most likely, to the attenuated conversion of 6-carbon glycolytic intermediates to 3-carbon compounds that feed into this cycle through pyruvate and acetyl-CoA. These changes suggest that energy production through glycolysis is impaired, since ATP generation occurs downstream of GADPH activity.

The addition of normal HDL to oxidized LDL-treated cells partially reverses its impact on energy metabolism pathways, since levels of the 3-carbon glycolytic intermediates as well as TCA cycle intermediates are more similar to levels observed after LDL treatment alone. It is of note that the impact of addition of HDL from PON1$^{(-/-)}$ mice on these molecules was intermediate between the effects produced by treatment with PON1-containing HDL and of no HDL (Figure 3). This observation is of considerable importance, because PARP activation and its consequent metabolic changes have been associated with endothelial dysfunction in diseases such as atherosclerosis and diabetes [17, 18]. Indeed, the levels of circulating endothelial cells are increased in patients with diabetes mellitus [19], and PON1 has been shown to attenuate diabetes development in mice [20, 21]. Our results suggest that the beneficial role of PON1 may involve, at least in part, a protection against the biochemical changes leading to endothelial dysfunction.

3.3. Phospholipid Metabolism. Levels of choline, ethanolamine, and glycerol-3-phosphate—key building blocks for phospholipids—were similar in endothelial cells following treatment with LDL, when compared to levels in control cells. By comparison, oxidized LDL reduced levels of phospholipid precursors and increased the levels of at least one phospholipid breakdown product. This could indicate that oxidized LDL induces membrane damage, breakdown, or remodeling. As was observed for the energy metabolism pathways, coadministration of normal HDL to oxidized LDL-treated cells reversed, or partially reversed, these deleterious effects. However, the addition of HDL from PON1$^{(-/-)}$ mice only generated subtle changes in phospholipid-related

Table 1: Heat map of metabolites showing statistically significant differences between groups.

Pathway	Metabolite	L/C*	O/C*	O/L*	OH/O*	KO/O*	KO/OH*
Glycine, serine, and threonine metabolism	Threonine	**1.40**	0.80	*0.57*	0.96	1.10	1.16
Glutamate metabolism	N-acetylglutamate	1.14	0.84	0.73	1.10	0.92	*0.83*
Phenylalanine and tyrosine metabolism	Phenylalanine	1.12	**0.63**	*0.56*	***1.41***	**1.19**	0.84
	Tyrosine	1.11	0.62	*0.56*	**1.57**	1.37	0.87
Valine, leucine, and isoleucine metabolism	Isoleucine	1.36	0.65	*0.48*	**1.57**	**1.38**	0.88
	Leucine	1.09	**0.70**	*0.64*	1.25	1.07	0.85
	Valine	1.26	**0.72**	*0.57*	1.18	1.08	0.91
Urea cycle; arginine-, proline-, metabolism	Praline	1.18	0.89	*0.75*	0.92	0.97	1.05
Gamma-glutamyl peptides	Gamma-glutamyl-leucine	*0.73*	0.87	1.19	1.09	1.48	1.36
Amino-sugar metabolism	Fucose	*0.72*	**0.76**	1.05	1.25	1.06	0.85
Fructose, mannose, galactose, starch, and sucrose metabolism	Galactose	*0.37*	0.94	***2.58***	1.04	0.73	0.7
	Mannose-6-phosphate	0.64	2.21	***3.44***	1.01	0.87	0.87
	Glucose-6-phosphate	0.33	2.06	***6.20***	1.22	1.17	0.96
	Fructose-6-phosphate	0.50	**2.50**	***5.01***	1.13	0.94	0.83
	2-phosphoglycerate	**2.28**	0.67	*0.29*	***2.17***	1.05	*0.48*
	3-phosphoglycerate	1.62	**0.42**	*0.26*	***2.92***	***1.94***	*0.67*
	1,3-dihydroxyacetone	0.85	0.98	1.15	0.80	*0.60*	0.75
	Phosphoenolpyruvate	1.06	*0.23*	*0.22*	***4.66***	***3.05***	0.66
Nucleotide sugars, pentose metabolism	Gluconate	*0.43*	0.89	***2.07***	1.06	0.86	0.81
TCA cycle	Fumarate	1.35	0.84	*0.62*	1.10	1.12	1.02
	Malate	1.31	0.89	*0.68*	**1.21**	1.07	0.88
Oxidative phosphorylation	Acetyl phosphate	1.00	1.12	1.12	0.84	*0.59*	0.70
	Phosphate	0.96	1.45	***1.52***	0.89	*0.70*	*0.78*
Medium chain fatty acid	Laurate (12 : 0)	0.98	1.15	**1.17**	0.92	*0.74*	*0.81*
Fatty acid, dicarboxylate	Undecanedioate	1.28	**1.52**	1.19	2.55	*0.70*	0.28
Glycerolipid metabolism	Ethanolamine	1.00	0.68	*0.68*	1.28	***1.55***	1.21
	Choline	1.07	0.84	*0.78*	***1.29***	1.25	0.96
	Glycerol 3-phosphate	1.54	0.31	*0.20*	***4.13***	***2.87***	*0.69*
	Glycerophosphorylcholine	*0.60*	1.17	***1.97***	0.80	**0.78**	0.97
Purine metabolism, adenine containing	Adenosine 3′-monophosphate	**2.38**	0.73	*0.31*	***1.70***	1.09	*0.64*
Pyrimidine metabolism, uracil containing	Uracil	1.22	0.48	*0.40*	***1.94***	***1.91***	0.99
	Uridine 5′-monophosphate	*0.50*	1.19	***2.38***	0.84	0.92	1.10
Pantothenate and CoA metabolism	Pantothenate	0.98	*0.87*	0.89	***1.19***	1.13	0.95
Riboflavin metabolism	Riboflavin (Vitamin B2)	*0.68*	0.76	1.11	1.15	1.11	0.97
Benzoate metabolism	4-hydroxy catechol	1.23	1.37	1.11	0.79	*0.43*	*0.54*
Chemicals	Glycolate (hydroxyacetate)	1.12	**1.55**	**1.38**	*0.47*	0.77	**1.64**
	Glycerol 2-phosphate	0.98	0.65	*0.67*	1.96	1.11	0.57

Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). Bold italic and italic cells in the Table indicate $P \leq 0.05$. Bold italic indicates that the mean values are significantly higher; italic indicates significantly lower. Bold text indicates $0.05 < P < 0.10$.
*Results are expressed as the mean quotients of the areas under the peak of the different experimental conditions. For example, galactose values are, on average, 2.58 times higher when endothelial cells are incubated with oxidized LDL than when incubated with native LDL. All measurements were performed in triplicate.

compounds, when compared to treatment with normal HDL (Figure 4).

3.4. Apoptosis. The observation of alterationsin phospholipids levels and the suggested membrane damage channeled us towards investigating the possibility of an increased apoptosis in endothelial cells incubated with oxidized LDL, and a possible protection by introducing HDL as coincubation. Hence, we analyzed caspase 9 protein expression. The activation of this enzyme is a good indicator of apoptosis induction, since caspase 9 plays a determinant role in

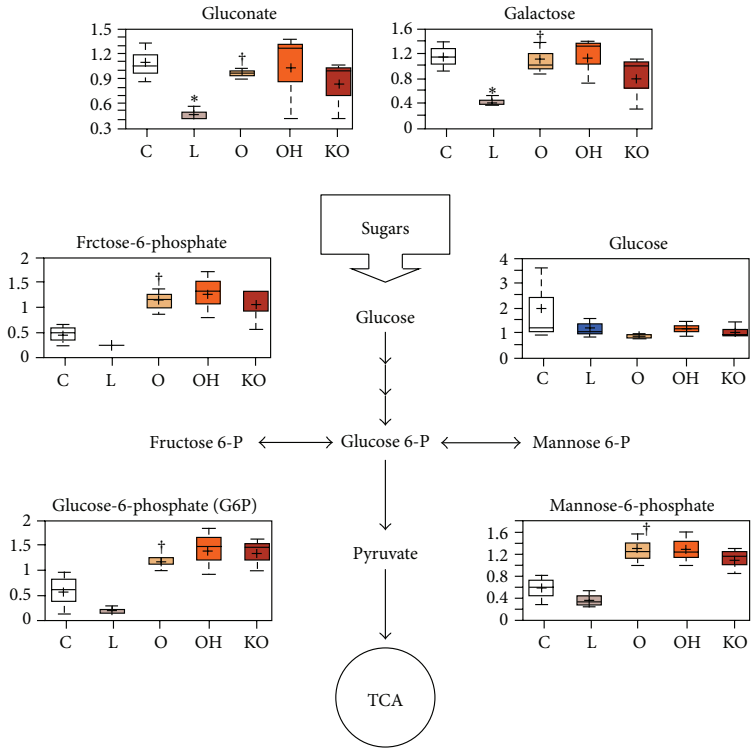

Figure 2: Variations in the hexose metabolites in HUVEC cell homogenates ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). $^{*}P < 0.05$ with respect to C; $^{\dagger}P < 0.05$ with respect to L.

apoptosome formation [22]. Also, we measured the numbers of apoptotic cells by flow cytometry. We observed that oxidized LDL addition increased caspase 9 expression and the percentage of apoptotic endothelial cells, when compared to control cells and cells treated with normal LDL. Coincubation with normal HDL completely preempted this effect. However, the influence of HDL from PON1$^{(-/-)}$ animals was much lower (Figures 5(a) and 5(b)). We observed a strong direct correlation ($r = 0.91$; $P < 0.001$) between total peroxides concentrations and the percentage of apoptotic cells (Figure 5(c)). Previous studies had shown that increased lipid peroxidation in HDL particles from coronary artery disease patients was associated with an impaired capacity of this particle to stimulate endothelial NO production [23]. Notably, PON1 has been reported to prevent lipid peroxidation in HDL particles and to promote HDL-mediated inactivation of oxidized lipids in LDL. Its activity was shown to be decreased in patients with coronary disease [7]. Further, HDL and PON1 decreased the formation of malondialdehyde-like epitopes and the formation of apoptotic particles in monocytes [24]. A very recent study showed that HDL from healthy people induced the expression of endothelial antiapoptotic protein Bcl-xL and reduced endothelial cell apoptosis *in vitro* as well as *in vivo* in apoE-deficient mice. In contrast, HDL from coronary artery disease patients did not inhibit endothelial apoptosis, failed to activate endothelial Bcl-xL, and stimulated endothelial proapoptotic pathways [25]. Our findings of a decreased oxidized LDL-induced apoptosis by normal HDL, but not by HDL from PON1$^{(-/-)}$ mice, together with a significant association between lipid peroxidation (as measured by total peroxides concentrations) and the percentage of the apoptotic cells would tend to confirm this very recent information.

Our results suggest that PON1 may play a significant role in cell survival by improving mitochondrial function. Indeed, mitochondria regulate apoptosis in response to cellular stress signals and, hence, determine whether cells live or die. As such, it is probable that peroxides constitute important candidates in the regulation of cell death, and that mitochondria act as both sensor and effector sites [26]. This could explain the influence of apoptosis-related proteins on mitochondrial respiration. Whether or not this finding has any impact on the atherosclerosis process warrants further exploration.

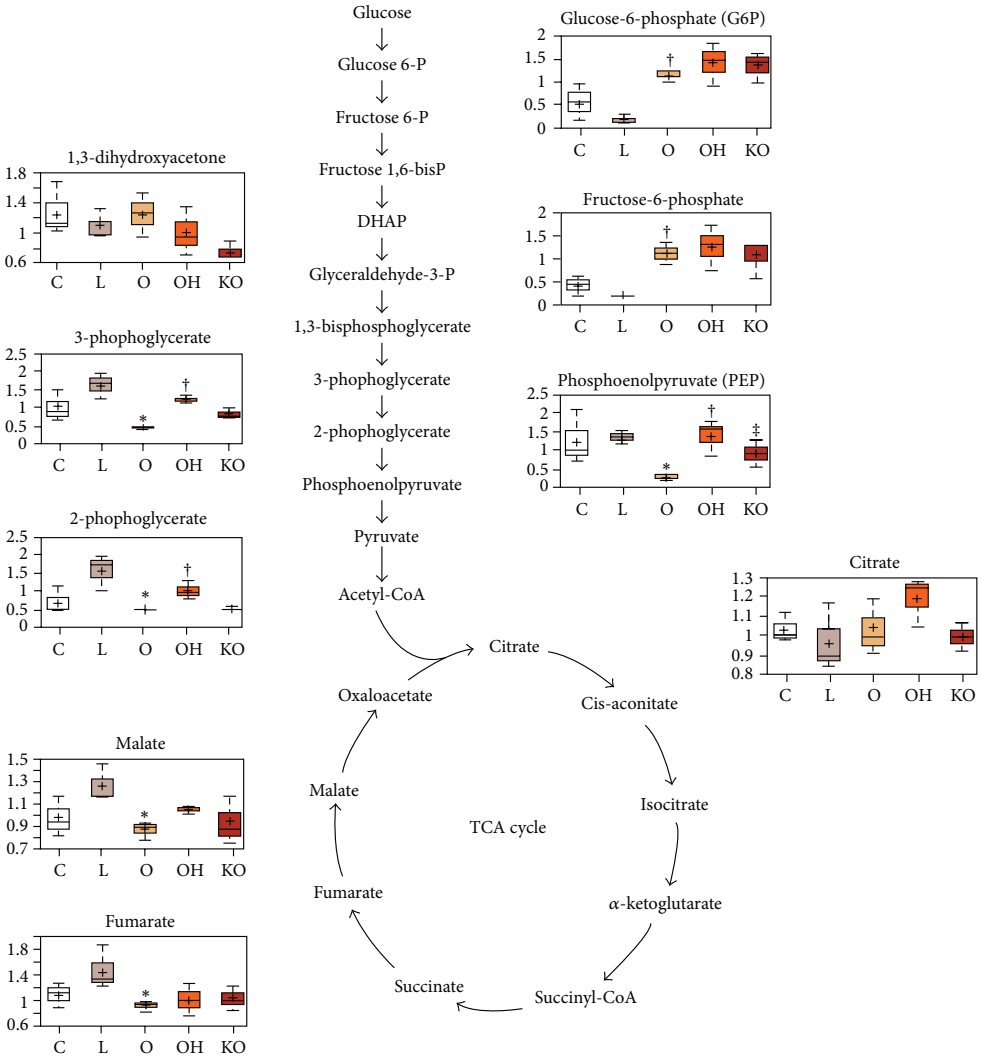

Figure 3: Variations in the metabolites of the glycolytic pathway and tricarboxylic acid cycle in HUVEC cell homogenates ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). $^{*}P < 0.05$ with respect to L; $^{\dagger}P < 0.05$ with respect to O; $^{\ddagger}P < 0.05$ with respect to OH.

4. Conclusion

Epidemiological studies have shown that the risk of atherosclerosis is inversely associated with HDL concentrations. The protective effect of this lipoprotein has been attributed, in part, to the antioxidant and anti-inflammatory action of PON1 [27]. We have showed, previously, that PON1 inhibits MCP-1 induction in endothelial cells [11], which suggested a protective role against liver inflammation mediated by MCP-1 [28]. More recent studies indicated that the anti-inflammatory effect of PON1 depends on its association with HDL [29], and that PON1 stimulates HDL antiatherogenicity [30], and macrophage response [31], and increases the duration over which HDL is able to prevent LDL oxidation [32].

The present study is novel in using a metabolomic approach to investigate the protective effect of PON1 on endothelial cells incubated with oxidized LDL. We observed important metabolic alterations in human endothelial cells incubated with oxidized LDL. These include an impaired glycolysis, TCA cycle, phospholipids, and activation of apoptotic pathways. These changes were ameliorated by incubation

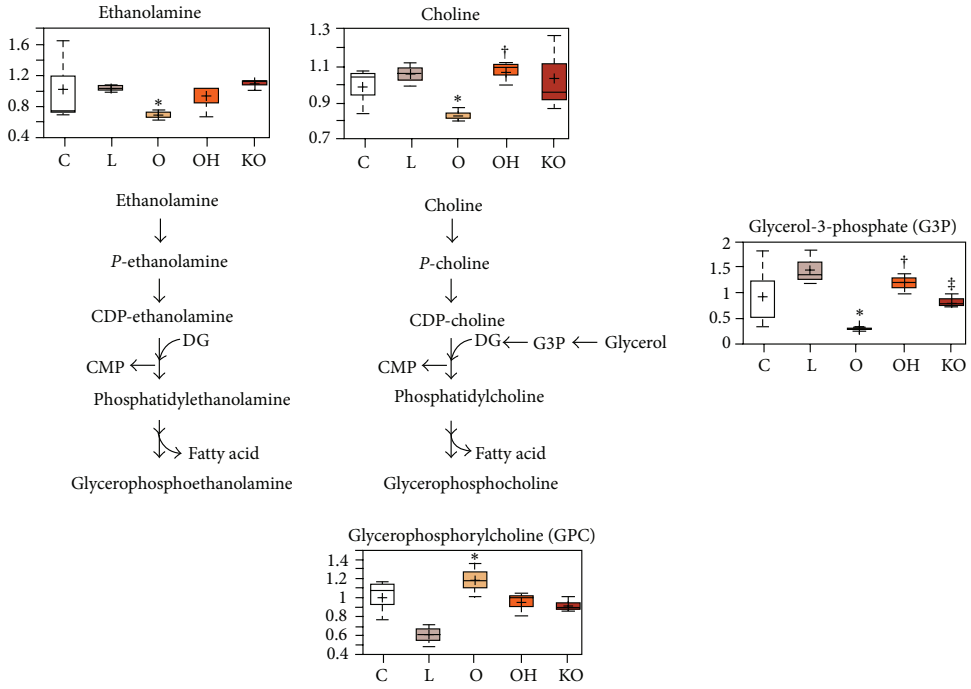

FIGURE 4: Variations in phospholipid metabolites in HUVEC cell homogenates ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). $^{*}P < 0.05$ with respect to L; $^{\dagger}P < 0.05$ with respect to O; $^{\ddagger}P < 0.05$ with respect to OH.

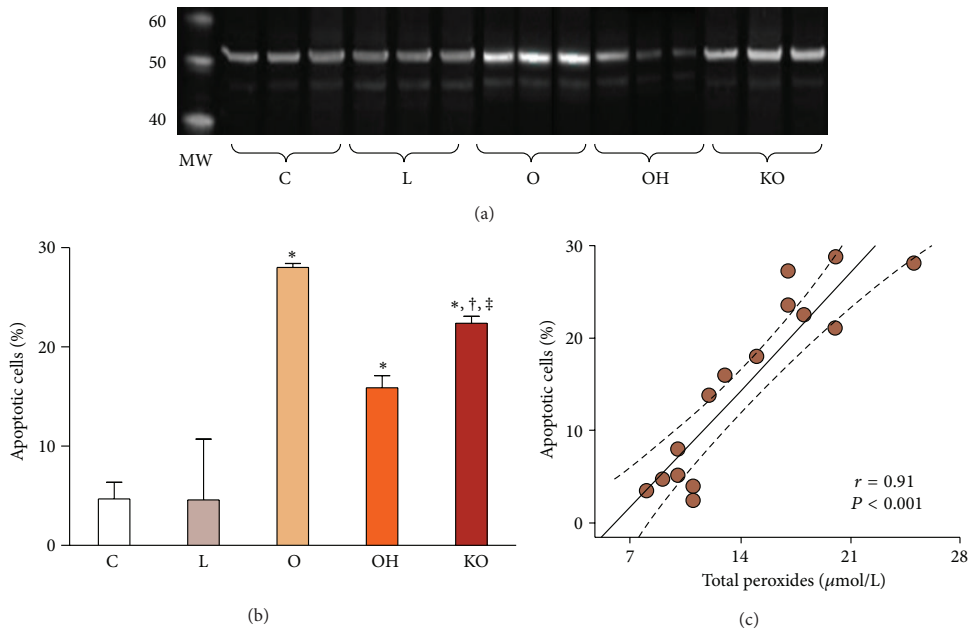

FIGURE 5: (a) Western blot analyses for caspase 9; (b) percentage of apoptotic cells; (c) relationship between total peroxide concentrations and the percentage of apoptotic cells in HUVEC cell homogenates ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). MW: molecular weight marker. $^{*}P < 0.01$ with respect to C; $^{\dagger}P < 0.05$ with respect to OH; $^{\ddagger}P < 0.01$ with respect to O.

with normal HDL, while HDL isolated from PON1$^{(-/-)}$ mice showed an impaired efficiency to protect against the oxLDL-induced changes. These results extend the current knowledge on the protective role of HDL and PON1 against oxidation in endothelial cells.

Abbreviations

DTNB:	5,5′-Dithio-bis-2-nitrobenzoic acid
GADPH:	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS:	Gas chromatography-mass spectrometry
HDL:	High-density lipoproteins
HUVEC:	Human umbilical vein endothelial cells
LDL:	Low-density lipoproteins
PARP:	Poly (ADP-ribose) polymerase
PON1:	Paraoxonase-1
TBBL:	5-thiobutyl butyrolactone
UPLC-MS/MS:	Ultrahigh performance liquid chromatography-tandem mass spectrometry.

Conflict of Interests

The authors declare that they do not have conflict of interests.

Acknowledgments

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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.

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

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.

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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

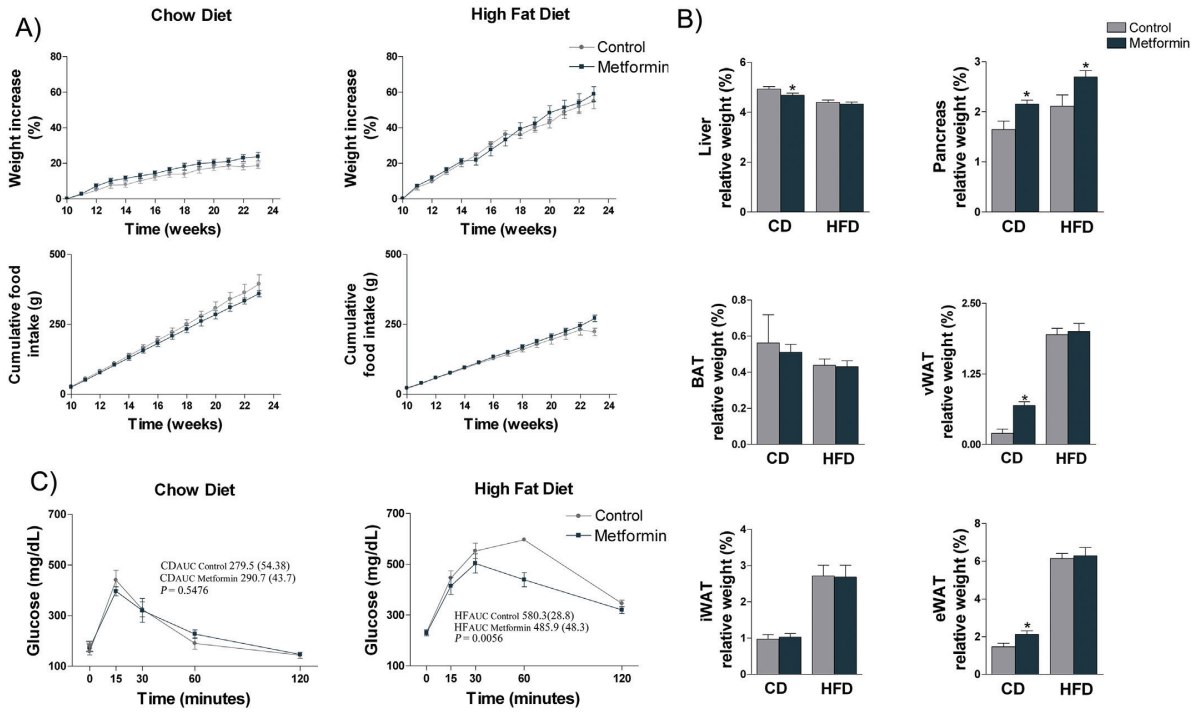

Fig. 1. Effects of metformin administration in PON1-deficient mice fed a chow diet (CD) and a high fat diet (HFD). A) Cumulative food intake and weight increase in mice having metformin administered and fed CD (left panel) and HFD (right panel) from 10 to 24 weeks of age. There were significant differences 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.

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).

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

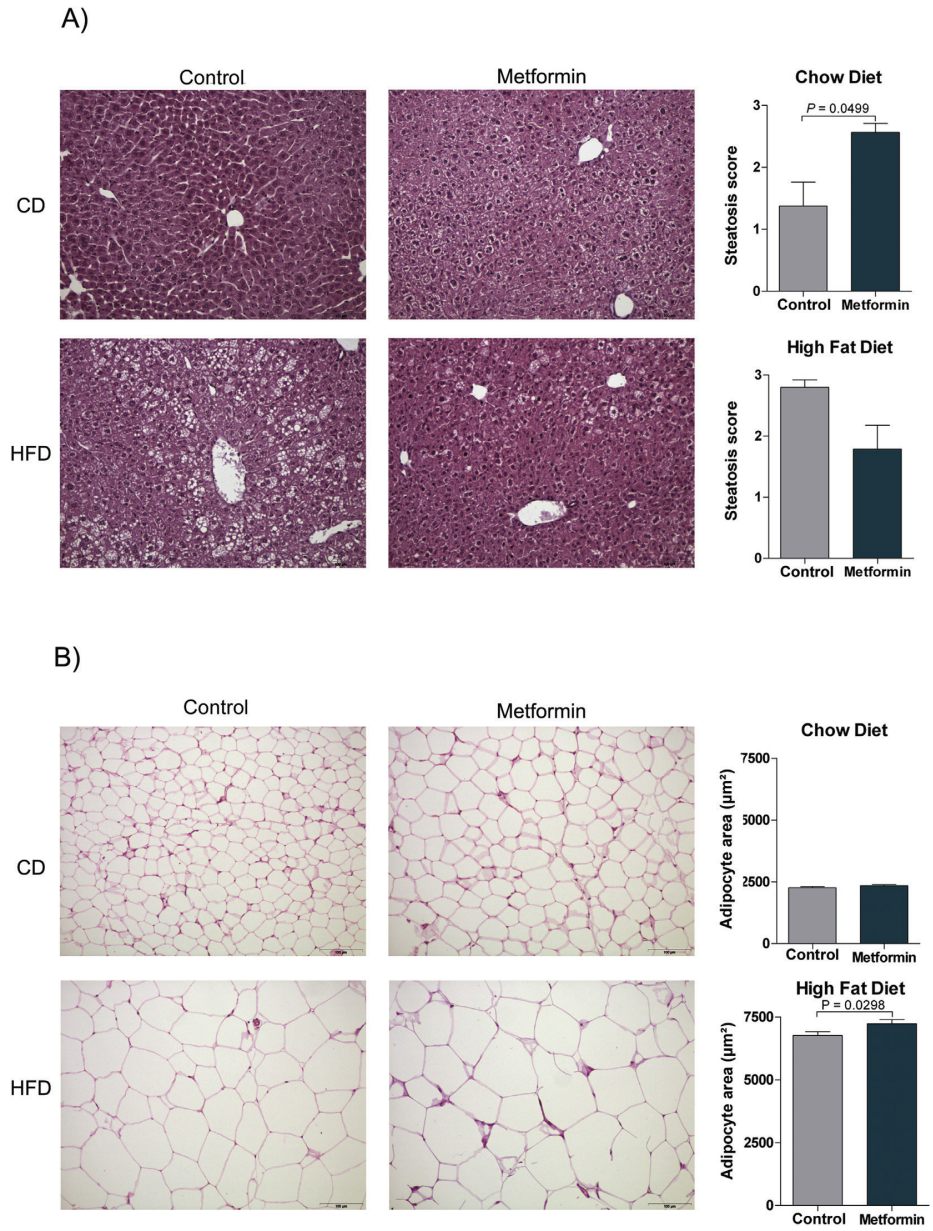

Fig. 2. Hematoxylin-eosin staining of the liver and eWAT of PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). The arrows show ballooning hepatocytes. Magnification ×10.

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

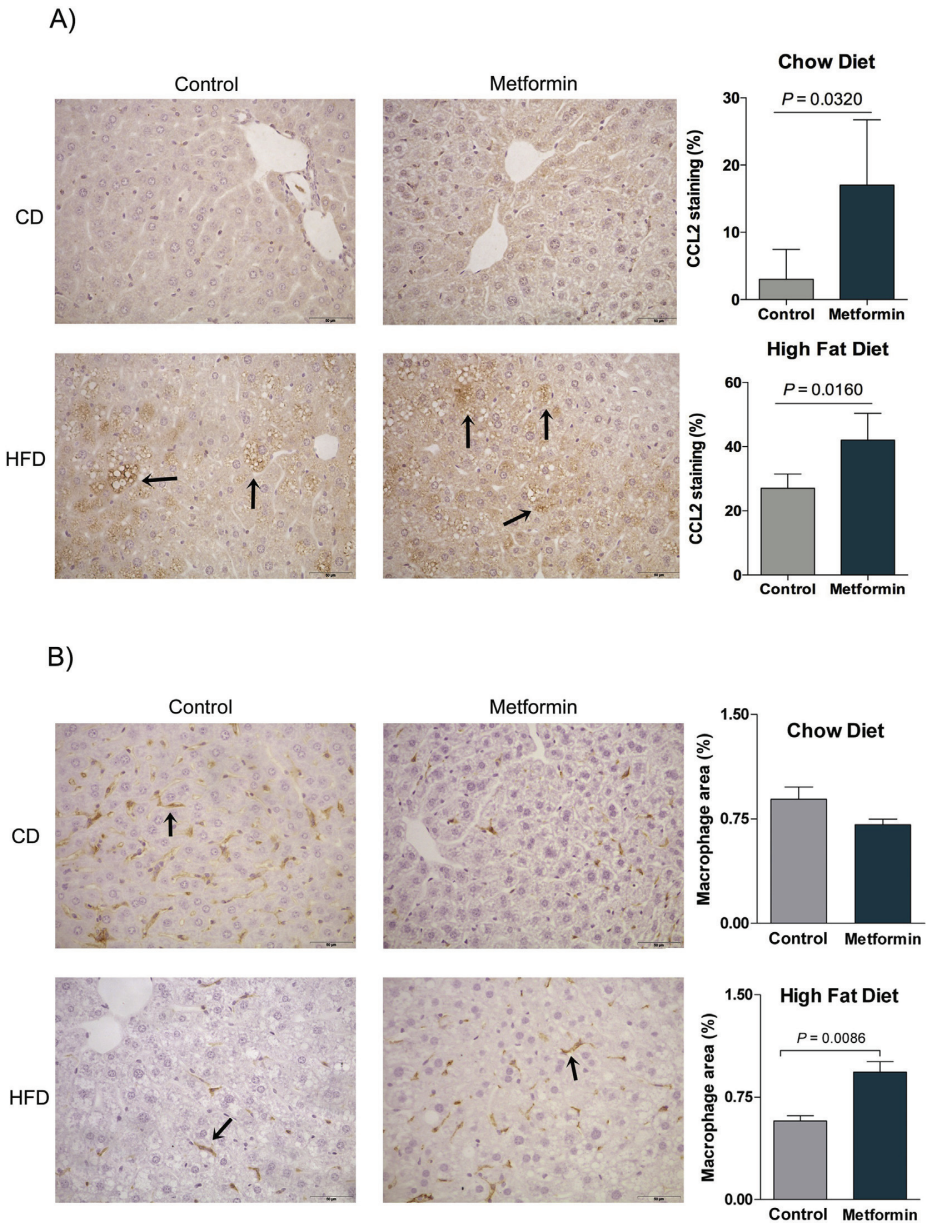

Fig. 3. Immunohistochemical analyses of liver tissues of PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). A) Immunochemical staining for CCL2. The arrows show positively-stained areas. B) Immunochemical staining for F4/80 and macrophage area quantification. The arrows show positive staining for F4/80. Magnification ×20.

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

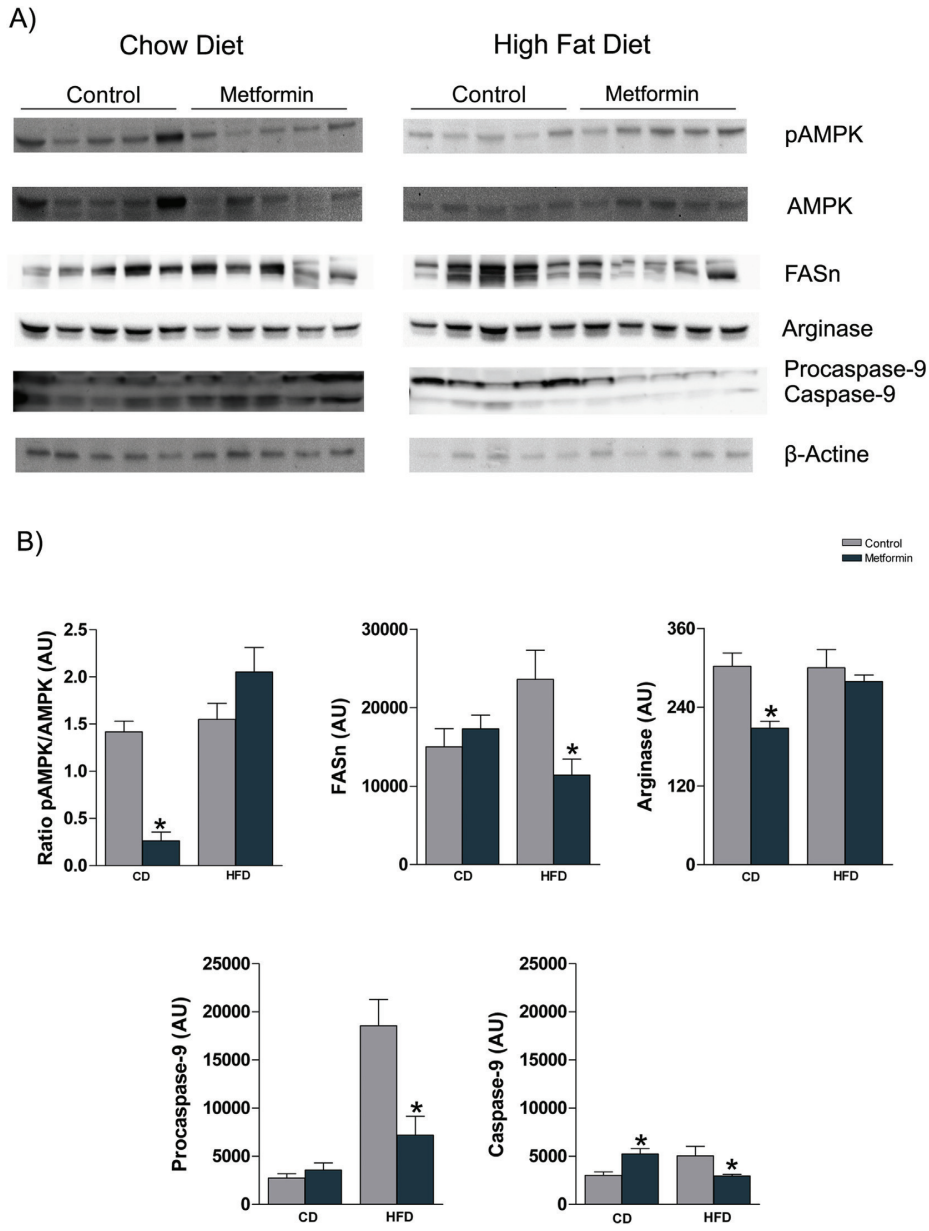

Fig. 4. Western blot analyses of liver in PON1-deficient mice fed chow diet (CD) and high fat diet (HFD). A) Immunoblots for pAMPK, AMPK, FASn, arginase, procaspase-9 and caspase-9. B) Quantification of these immunoblots. Results are shown as arbitrary units (AU). [a] $P < 0.05$ with respect to the control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

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.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cbi.2016.03.004.

Transparency document

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Supplementary information corresponding to the manuscript:

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

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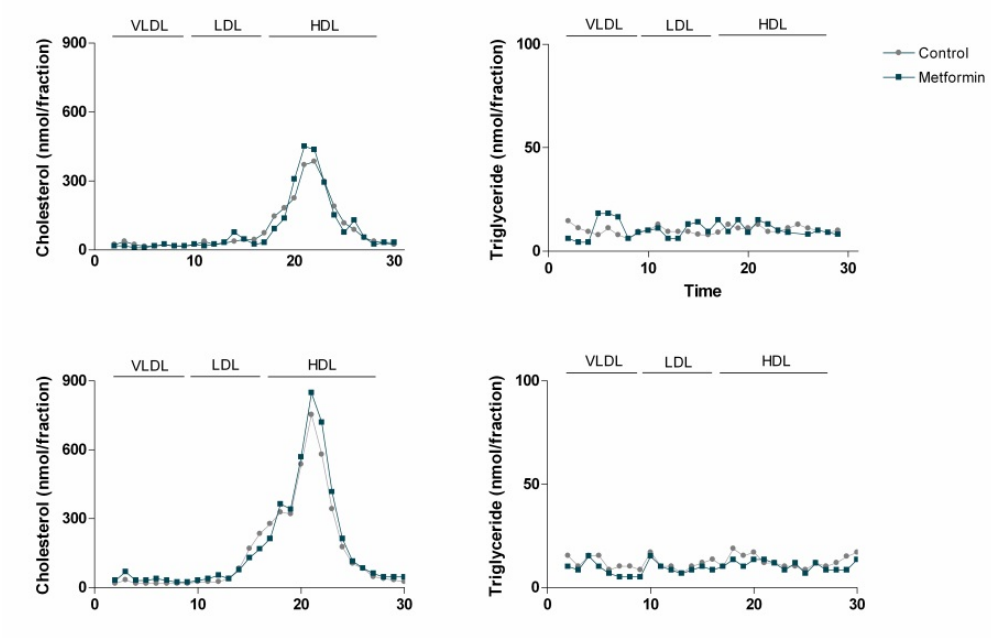
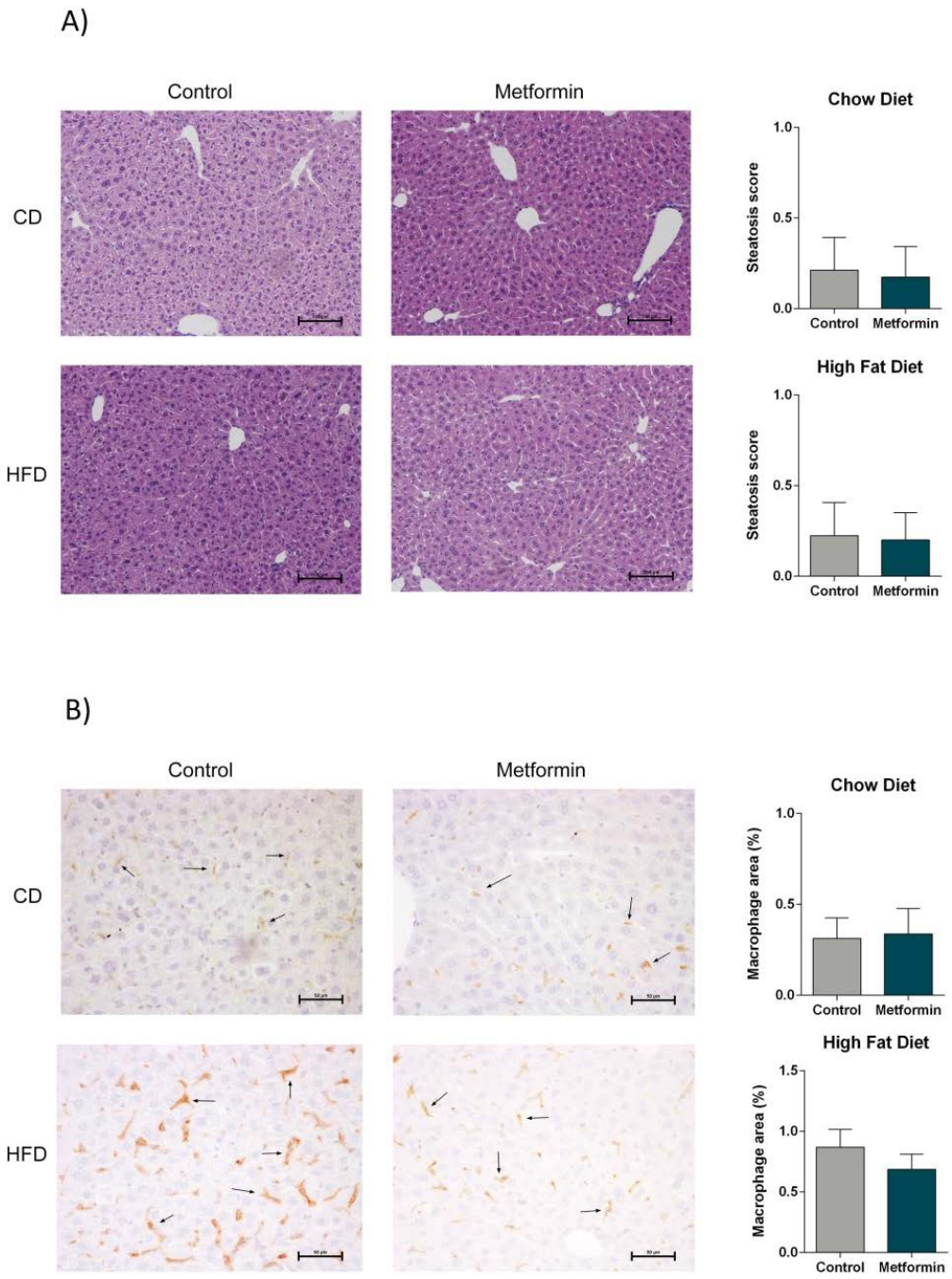


Supplementary Fig. 1. Serum lipoprotein distribution in PON1-deficient mice. **A)** Mice fed chow diet. **B)** Mice fed high fat diet. HDL: high-density lipoproteins; LDL: low-density lipoproteins; VLDL: very low-density lipoproteins



Supplementary Fig. 2. A) Hematoxylin-eosin staining of the liver of wild-type mice fed chow diet (CD) and high fat diet (HFD). Magnification x10. **B)** Immunochemical staining for F4/80 and macrophage area quantification. The arrows show positive staining for F4/80. Magnification x20.