Lactonase activity and status of paraoxonase 1 in Chinese women with polycystic ovarian syndrome

Yujin Zhang¹, Hongwei Liu¹, Jin He², Kelei Xu², Huai Bai³, Ying Wang¹, Feng Zhang¹, Jinxia Zhang³, Li Cheng² and Ping Fan³

¹Department of Obstetrics and Gynecology, West China Second University Hospital, ²West China School of Pharmacy and ³Laboratory of Genetic Disease and Perinatal Medicine and Key Laboratory of Birth Defects and Related Diseases of Women and Children of Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu, Sichuan 610041, People's Republic of China

Correspondence should be addressed to P Fan
Email
fanping15@scu.edu.cn

Abstract

Objective: To study the relationship between the lactonase activities and status of paraoxonase 1 (PON1) and its association with the *PON1* genetic polymorphisms in women with polycystic ovarian syndrome (PCOS).

Design: A case–control study.

Methods: A total of 455 PCOS patients and 441 control women were included in this study. The lactonase activities and concentrations of PON1 were assayed using 5-thiobutyl butyrolactone (TBBL) and 7-O-diethylphosphoryl-3-cyano-4-methyl-7-hydroxycoumarin (DEPCyMC) respectively. A normalized lactonase activity (NLA) was estimated based on the ratio of TBBLase:DEPCyMCase activity. The *PON1* genotypes, serum malondialdehyde (MDA) levels and total antioxidant capacity were analyzed.

Results: The lactonase activities and levels of PON1 were higher in PCOS patients than in the control women. However, the NLA did not significantly differ between groups. The $-108C \rightarrow T$ variation of the *PON1* gene showed decreased lactonase activities and levels of PON1 in a genotype-dependent manner (CC > CT > TT); the $192Q \rightarrow R$ variation of the *PON1* gene showed increased PON1 lactonase activities and NLA; and the $55L \rightarrow M$ variation of the *PON1* gene showed decreased lactonase activities and levels of PON1 but an increased NLA. A multivariable regression analysis showed that the $-108C/T$, $192Q/R$, and $55L/M$ variations of the *PON1* gene, serum apolipoprotein A1, and MDA levels were significant predictors of PON1 lactonase activity, PON1 level, and NLA.

Conclusions: The serum lactonase activities and concentrations of PON1 are increased in PCOS patients. The increased oxidative stress and the $-108C/T$, $192Q/R$, and $55L/M$ genetic polymorphisms of *PON1* may be associated with these changes.

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Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine metabolic disorder characterized by oligo- or anovulation (OA), biochemical or clinical hyperandrogenism (HA), and the presence of polycystic ovaries (PCO) (1). PCOS affects 6–15% of reproductive-age women (2, 3). PCOS is often associated with obesity (4), increased oxidative stress (5), systemic chronic inflammation (6), elevated prevalences of metabolic syndrome and type 2 diabetes (7), and potentially increased risks of

atherosclerosis and cardiovascular disease (8, 9). The etiology of PCOS remains unclear, but studies have suggested that PCOS appears to have a complex, multifactorial etiology in which a variety of predisposing genes interact with environmental factors to produce the disease (10, 11).

Serum paraoxonase 1 (PON1) is a calcium-dependent multifunctional enzyme that is mainly expressed by the liver and circulates within HDL particles (12, 13).

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PON1 has paraoxonase (POase), arylesterase (AREase), lactonase, and Hcy-thiolactonase (HTase) activities (13, 14). Thus, it can not only detoxify several organophosphorus insecticides and nerve agents to protect against xenobiotic toxicity, but it can also inhibit LDL oxidation and increase macrophage-associated cholesterol efflux. It also possesses antioxidant, anti-inflammatory, and anti-atherogenic properties (13, 15, 16, 17). Recent studies have shown that the physiological roles of PON1 are related to its lactonase and HTase activities. PON1 can hydrolyze a wide range of lactones that are structurally similar to many oxidized mediators of polyunsaturated fatty acids (13, 18, 19) and homocysteine thiolactone, which can increase the homocysteinylation of proteins and lead to protein inactivation and cell damage. Thus, PON1 plays beneficial roles in the prevention of atherosclerosis (12, 14, 20).

Recently, new serum PON1 activity tests have been developed using 5-thiobutyl butyrolactone (TBBL) or 7-O-diethylphosphoryl 3-cyano 4-methyl 7-hydroxycoumarin (DEPCyMC) as substrates (21, 22). TBBL is a synthetic chromogenic lactone that resembles the most natural PON1 lactone substrates, thus allowing a specific evaluation of PON1 lactonase activity (18, 23). DEPCyMC is a chromogenic phosphotriester substrate that allows the total PON1 concentration to be estimated (21). The ratio of TBBL:DEPCyMC activity has been suggested to provide the so-called 'normalized lactonase activity (NLA)', which may reflect the level of PON1 lactonase catalytic stimulation by HDL (21, 24).

The POase and/or AREase activities are reportedly lower (25, 26, 27, 28) or similar (29, 30) and the HTase activity is decreased (31) in PCOS patients compared with control women. However, the level, lactonase activity, and NLA of PON1 have not yet been assessed in PCOS patients. The aim of this study was to investigate these new PON1 activities and PON1 levels, and their association with PON1 genetic polymorphisms in PCOS patients.

Subjects and methods

Subjects

This is a case-control study. Women with or without PCOS between 17 and 40 years of age were randomly recruited from the Outpatient Clinic of Reproductive Endocrinology, West China Second University Hospital, Sichuan University from 2006 to 2013 as described before (32, 33). All participants gave their informed consent and the study was approved by the Institutional Review Board of the West China Second University Hospital.

PCOS was defined by the presence of two or more of the following features based on the revised 2003 Rotterdam diagnostic criteria (1): OA was assessed as oligomenorrhoea, i.e., less than eight cycles per year. HA was assessed as total testosterone (TT) levels above the 95th percentile of the levels (2.60 nmol/l) detected in a group of normal menstruating women with normal cycles and/or clinically by the presence of obvious acne (33) and/or hirsutism with a F-G score of more than 6 (33, 34, 35). PCO was confirmed if 12 or more follicles in each ovary measured 2–9 mm in diameter and/or the ovarian volume was increased (>10 ml) by ultrasonic examination with exclusion of other etiologies, such as congenital adrenal hyperplasia, androgen-secreting carcinomas, and Cushing's syndrome. All control women had regular menstrual cycles (between 21 and 35 days), exhibited normal circulating androgen levels, the absence of obvious acne or hirsutism on physical examination, and normal ovarian morphology as determined by ultrasound.

The subjects were excluded if they met one of the following criteria: i) clinically evident chronic or acute diseases, such as infection, tumors, thyroid dysfunction, cardiovascular diseases, endometriosis, hyperprolactinemia, hypogonadotropic hypogonadism, or premature ovarian failure; ii) pregnant or in the luteal phase based on progesterone measurement (>9.54 nmol/l); iii) taking medication known to affect the metabolism of carbohydrates, lipids, or hormones within 3 months before the study; iv) age <20 years; and v) smokers.

Clinical and anthropometrical variables, including the waist circumference (WC), hip circumference, waist-to-hip ratio (WHR), BMI (kg/m²), systolic and diastolic blood pressure (SBP and DBP), and the degree of hirsutism and acne, were measured or assessed in all subjects. The ultrasound ovarian volume was also assessed using the formula for the volume of an ellipsoid (36): 0.523 × length × width × thickness.

Blood samples were obtained in the morning after overnight fasting, placed on ice immediately, and centrifuged at 1500 g for 15 min at 4 °C within 2 h. The serum and plasma samples were stored in 200–300 µl aliquots at –80 °C for later analysis, and blood cells in tubes containing EDTA were stored at 4 °C.

PON1 activity assays

The substrates TBBL and DEPCyMC were synthesized as shown in the [Supplemental data](#), see section on [supplementary data](#) given at the end of this article, according to previous reports (21, 22) with some modifications.

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The PON1 lactonase activity was determined as previously described (22, 24). A 100 mM stock of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was prepared in DMSO. The serum was diluted 400-fold in a sample buffer (50 mM Tris pH 8.0, 1 mM CaCl₂, and 1 mM DTNB). TBBL was diluted 500-fold from a 500 mM stock (in acetonitrile) using substrate buffer (50 mM Tris pH 8.0, 1 mM CaCl₂, and 2% acetonitrile). The reaction was initiated by adding 100 µl of TBBL (1 mM) to 100 µl diluted serum, and the kinetics were immediately measured using the Varioskan Flash Multimode Microplate Spectrophotometer (Thermo Scientific, Vantaa, Finland) by monitoring the absorbance at 412 nm for 5 min at 25 °C (ϵ =7000 OD/M). The rate of spontaneous hydrolysis of TBBL in the buffer was subtracted from all measurements. The activities are expressed as U/ml (1 unit=1 µmol of TBBL hydrolysed/min per ml of undiluted serum).

The serum PON1 levels were assessed by measuring the activity using DEPCyMC as a substrate (21). A 100 mM stock of DEPCyMC was prepared in DMSO. The kinetics were measured using 20 µl of serum and 180 µl of assay buffer (50 mM bis-trispropane pH 9.0, 1 mM CaCl₂, and 1 mM DEPCyMC) by monitoring the absorbance at 400 nm for 5 min at 25 °C (ϵ =22 240 OD/M). The rate of spontaneous hydrolysis of DEPCyMC in buffer was subtracted from all measurements. The activities are expressed as mU/ml (1 milli unit=1 nmol of DEPCyMC hydrolysed/min per ml of undiluted serum).

The samples were plated in duplicate, and a pooled serum sample from healthy volunteers was measured on each plate as a quality control. The intra- and inter-assay coefficients of variation (CV) for all measurements were <5%.

Genotype analysis

The genomic DNA was isolated from the peripheral blood leukocytes of subjects and the *PON1* –108C/T, 192Q/R, and 55L/M genotypes were assessed by PCR amplification and restriction analysis as described previously (34, 37).

Analysis of serum total antioxidant capacity and malondialdehyde levels as well as hormonal and metabolic profiles

The levels of serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), TT, estradiol, and progesterone as well as the concentrations of fasting plasma insulin (Ins) and glucose (Glu), total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglyceride (TG), apolipoprotein A1 (apoA1) and apoB,

homeostasis model insulin resistance index (HOMA-IR), and atherogenic index (AI) were measured or assessed as described before (33). The serum total antioxidant capacity (T-AOC) and malondialdehyde (MDA) levels were determined by spectrophotometry using T-AOC kits and micro-MDA kits (NanJing Jiancheng Bioengineering Institute, Nan Jing, China) respectively. The intra- and inter-assay CV for all measurements were <5 and 10% respectively.

Statistical analyses

The data are presented as the mean±s.d. Differences in variables between the PCOS and control groups were evaluated with an independent sample *t*-test, a Mann–Whitney *U* test or an analysis of covariance (ANCOVA). A χ^2 analysis was used to test deviations of the genotype distribution from the Hardy–Weinberg equilibrium and to determine allele or genotype frequencies between patients and controls. The effects of PCOS phenotypes or *PON1* genotypes on the clinical and metabolic parameters were estimated with an ANOVA or ANCOVA. A Pearson's correlation was performed to define correlations between the PON1 lactonase activities and the PON1 levels. Multivariate stepwise regression analyses were used to assess the effect of other parameters, including the –108C/T, 192Q/R, and 55M/L genotypes of *PON1*; PCOS; age; BMI; SBP; DBP; WC; F–G score; average ovarian volume; TT; FSH; LH; the fasting insulin and glucose levels; and the TG, LDL-C, apoA1, apoB, MDA, and T-AOC levels on the PON1 lactonase activity, PON1 level, or NLA in all subjects. We excluded some parameters, such as the WHR, LH:FSH ratio, HOMA-IR, TC, HDL-C, AI, and TG:HDL-C ratio, from the regression models due to the presence of a co-linearity between these parameters and some of the independent variables. A value of $P<0.05$ was considered to be statistically significant. All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS) 13.0 for Windows.

Results

Selection of subjects

Women with or without PCOS ($n=1778$) were randomly recruited in the same manner and during the same period. Of these subjects, 755 women met the revised 2003 Rotterdam diagnostic criteria for PCOS and 702 women met the control women's inclusion criteria. After excluding

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subjects meeting one or more of the exclusion criteria and those missing data for PON1 activities or the *PON1* genetic polymorphisms, 455 PCOS patients and 441 control women were finally included in this study.

The controls consisted of: i) infertile women (59.9%) due to Fallopian obstruction (28.8%) or male factor infertility; ii) women presenting for preconception counseling (35.8%); and iii) healthy volunteers, doctors, and nurses (4.3%).

Clinical and biochemical characteristics of the study population

As given in Table 1, the BMI, WC, WHR, F-G score, DBP, and average ovarian volume were significantly higher and the age was significantly lower in the PCOS group than in the control group.

The TT and LH levels, LH:FSH ratio, fasting insulin, HOMA-IR, TC, TG, LDL-C and apoB levels, AI, TG:HDL-C

Table 1 Clinical and biochemical characteristics of the subjects. Values are presented as the mean $\pm$ s.d.

	Controls (n = 441)	PCOS (n = 455)	P	P ^a
Clinical characteristics				
Age (years)	28.11 ± 4.08	25.11 ± 3.75	< 0.001	
BMI (kg/m ²)	21.01 ± 2.74	23.14 ± 4.32	< 0.001	
Waist circumference (cm)	73.11 ± 7.95	79.70 ± 11.59	< 0.001	
Waist-to-hip ratio (WHR)	0.81 ± 0.06	0.85 ± 0.07	< 0.001	
Ferriman–Gallwey score	0.21 ± 0.67	1.78 ± 2.05	< 0.001	
SBP (mmHg)	113.21 ± 11.70	114.67 ± 11.07	0.058	
DBP (mmHg)	73.52 ± 8.71	75.29 ± 8.72	0.003	
Ovarian volume (ml)	7.81 ± 2.71	10.22 ± 4.20	< 0.001	
Hormonal levels				
TT (nmol/l)	1.57 ± 1.05	2.42 ± 0.76	< 0.001	< 0.001
LH (IU/l)	8.50 ± 10.98	14.07 ± 11.78	< 0.001	< 0.001
FSH (IU/l)	6.57 ± 2.96	5.97 ± 2.36	0.001	0.066
LH/FSH	1.31 ± 1.35	2.39 ± 1.28	< 0.001	< 0.001
Metabolic profiles				
Fasting Ins (pmol/l)	65.06 ± 35.14	105.12 ± 75.56	< 0.001	< 0.001
Fasting Glu (mmol/l)	5.29 ± 0.46	5.44 ± 0.74	< 0.001	0.398
HOMA-IR	2.25 ± 1.36	3.82 ± 3.31	< 0.001	0.001
TG (mmol/l)	1.01 ± 0.54	1.42 ± 1.15	< 0.001	< 0.001
TC (mmol/l)	4.24 ± 0.70	4.40 ± 0.82	0.001	< 0.001
HDL-C (mmol/l)	1.51 ± 0.32	1.38 ± 0.35	< 0.001	0.046
LDL-C (mmol/l)	2.33 ± 0.61	2.53 ± 0.77	< 0.001	< 0.001
AI	1.90 ± 0.67	2.37 ± 1.01	< 0.001	< 0.001
TG/HDL-C	0.74 ± 0.63	1.21 ± 1.43	< 0.001	< 0.001
ApoA1 (g/l)	1.43 ± 0.19	1.38 ± 0.20	< 0.001	0.451
ApoB (g/l)	0.75 ± 0.16	0.82 ± 0.21	< 0.001	< 0.001
MDA (nmol/ml)	3.60 ± 1.20	4.25 ± 1.58	< 0.001	< 0.001
T-AOC (U/ml per min)	14.97 ± 2.80	16.28 ± 3.18	< 0.001	0.003
PON1 lactonase activity (U/ml)	9.43 ± 1.62	9.73 ± 1.70	0.007	0.006
PON1 level (mU/ml)	44.23 ± 6.43	45.41 ± 6.70	0.007	0.004
NLA	213.01 ± 16.79	214.00 ± 17.14	0.384	0.563
PON1 genetic polymorphisms				
–108C/T, % (n)				
CC	30.4 (134)	29.0 (132)	0.331 ^b	
CT	52.8 (233)	50.3 (229)		
TT	16.8 (74)	20.7 (94)		
192Q/R, % (n)				
QQ	14.5 (64)	11.0 (50)	0.017 ^b	
QR	50.3 (222)	44.8 (204)		
RR	35.1 (155)	44.2 (201)		
55L/M, % (n)				
LL	92.5 (408)	94.1 (428)	0.354 ^b	
LM	7.5 (33)	5.9 (27)		

AI, atherosclerosis index; apoA1, apolipoprotein A1; apoB, apolipoprotein B; DBP, diastolic blood pressure; FSH, follicle-stimulating hormone; Glu, glucose; HDL-C, HDL-cholesterol; HOMA-IR, homeostasis model insulin resistance index; Ins, insulin; LDL-C, LDL-cholesterol; LH, luteinizing hormone; MDA, malondialdehyde; NLA, normalized lactonase activity; PCOS, polycystic ovarian syndrome; SBP, systolic blood pressure; T-AOC, total antioxidant capacity; TC, total cholesterol; TG, triglyceride; TT, total testosterone.

^aAll comparisons of parameters were corrected for differences in age and BMI between the two groups.

^b χ^2 tests.

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ratio, MDA concentrations, T-AOC, PON1 levels, and PON1 lactonase activities were significantly higher and the HDL-C levels were significantly lower in the PCOS patients than in the controls (Table 1).

The genotypic distributions of the *PON1* –108C/T, 192Q/R, and 55L/M gene sites were in Hardy–Weinberg equilibrium in the PCOS and control groups. No significant differences were found in the frequencies of the *PON1* –108C/T and 55L/M genotypes between the PCOS and control groups. However, *PON1* 192RR genotype was significantly more prevalent in the PCOS patients compared with the control women (44.2% vs 35.1%, $P=0.017$).

Lactonase activity and status of PON1 and oxidative stress parameters in different PCOS phenotypes

We further analyzed the age- and BMI-adjusted level, lactonase activity and NLA of PON1, *PON1* genetic polymorphisms, and oxidative stress parameters in control women and in patients with different key clinical phenotypes of PCOS: i) HA+OA+PCO, ii) HA+OA, iii) HA+PCO, and iv) OA+PCO (Table 2). Compared with the control subjects, each of the four PCOS subgroups had higher MDA levels; the HA+OA+PCO, HA+OA, and OA+PCO subgroups had a higher T-AOC; the HA+OA+PCO and HA+PCO subgroups had higher PON1 levels and

PON1 lactonase activities and increased or tended to increase the *PON1* 192RR genotypic frequencies (for HA+OA+PCO subgroup: 35.1% vs 45.0%, $P=0.015$ and for HA+PCO subgroup: 35.1% vs 50.0%, $P=0.065$); the HA+OA+PCO subgroup had a higher NLA. Patients with OA+PCO also had lower MDA levels compared with the other three PCOS subgroups, lower T-AOC levels, lower PON1 levels and PON1 lactonase activities, and a lower NLA compared with the HA+OA+PCO subgroup; the levels and lactonase activities of PON1 were lower or tend to be decreased compared with the HA+PCO subgroup ($P<0.062$).

Effects of *PON1* –108C/T, 192Q/R, and 55L/M genetic variants on the lactonase activity and status of PON1 and oxidative stress parameters

As given in Table 3, the *PON1* –108C→T variants decreased PON1 levels and PON1 lactonase activities in a genotype-dependent manner ($CC>CT>TT$) but did not significantly affect the NLA in patients with PCOS or the control women. The T-AOC was higher in patients with *TT* genotype than in patients with *CC* genotype.

The PON1 lactonase activities and NLA were significantly increased in patients with *RR* genotype compared with patients with the *QQ* or *QR* genotype. In the control women, *PON1* 192Q→R variants increased the PON1

Table 2 Lactonase activity, level, NLA, and genotypic frequencies of PON1 and markers of oxidative stress in different PCOS phenotypes and control women. Values are as means±s.d. Comparisons of the lactonase activity, level, and NLA of PON1 and markers of oxidative stress were corrected for differences in age and BMI between the two groups; the genotypic distributions of PON1 were compared by χ^2 tests.

	Controls (n=441)	PCOS			
		HA+OA+PCO (n=191)	HA+OA (n=93)	HA+PCO (n=26)	OA+PCO (n=145)
Age (years)	28.11±4.08	24.85±3.55*	24.71±3.83*	26.03±4.45*	25.61±3.80*
BMI (kg/m ²)	21.01±2.74	22.70±3.86*	23.01±4.70*	22.50±4.39	23.84±4.58*
MDA (nmol/ml)	3.60±1.20	4.30±1.64*	4.55±1.76*	4.59±1.55*	3.96±1.41 ^{*,†,‡,§}
T-AOC (U/ml per min)	14.97±2.80	16.73±3.16*	16.09±3.21*	15.40±2.84	16.01±3.22 ^{*,†}
PON1 lactonase activity (U/ml)	9.43±1.62	9.93±1.65*	9.59±1.60 [†]	10.27±2.27*	9.47±1.68 [†]
PON1 level (mU/ml)	44.23±6.43	46.01±6.51*	44.89±6.47	48.38±9.24 ^{*,‡}	44.42±6.38 ^{†,§}
NLA	213.01±16.79	215.57±16.37*	213.70±18.61	211.14±19.45	212.61±16.69 [†]
<i>PON1</i> genotypes, % (n)					
–108CC	30.4 (134)	29.8 (57)	28.0 (26)	42.3 (11)	26.2 (38)
CT	52.8 (233)	47.6 (91)	59.1 (55)	38.5 (10)	50.3 (73)
TT	16.8 (74)	22.5 (43)	12.9 (12)	19.2 (5)	23.4 (34)
192QQ	14.5 (64)	7.9 (15)*	11.8 (11)	23.1 (6)	12.4 (18)
QR	50.3 (222)	47.1 (90)	47.3 (44)	26.9 (7)	43.4 (63)
RR	35.1 (155)	45.0 (86)	40.9 (38)	50.0 (13)	44.1 (64)
55LL	92.5 (408)	93.7 (179)	94.6 (88)	100 (26)	93.1 (135)
LM	7.5 (33)	6.3 (12)	5.4 (5)	0 (0)	6.9 (10)

HA, hyperandrogenism; MDA, malondialdehyde; NLA, normalized lactonase activity; OA, oligo- and/or anovulation; PCO, polycystic ovaries; PON1, paraoxonase 1; PCOS, polycystic ovarian syndrome; T-AOC, total antioxidant capacity. * $P<0.05$ compared with the controls. [†] $P<0.05$ compared with the HA+OA+PCO subgroup. [‡] $P<0.05$ compared with the HA+OA subgroup. [§] $P<0.05$ compared with the HA+PCO subgroup.

Table 3 Lactonase activity, level, and NLA of PON1 and markers of oxidative stress according to *PON1* genetic polymorphisms in PCOS patients and control women. Values are as means $\pm$ s.d.

	<i>PON1</i> – 108CT				<i>PON1</i> 192Q/R				<i>PON1</i> 55L/M			
	CC	CT	TT		QQ	QR	RR		LL		LM	
Controls												
<i>n</i>	134	233	74		64	222	155		408		33	
Age (years)	28.51 $\pm$ 4.00	27.96 $\pm$ 4.05	27.82 $\pm$ 4.32		27.64 $\pm$ 3.96	28.04 $\pm$ 4.20	28.40 $\pm$ 3.96		28.11 $\pm$ 4.07		28.09 $\pm$ 4.24	
BMI (kg/m ²)	21.14 $\pm$ 2.46	20.90 $\pm$ 2.87	21.12 $\pm$ 2.85		21.14 $\pm$ 2.90	20.81 $\pm$ 2.52	21.24 $\pm$ 2.97		21.04 $\pm$ 2.77		20.64 $\pm$ 2.44	
MDA (nmol/ml)	3.46 $\pm$ 1.14	3.69 $\pm$ 1.26*	3.52 $\pm$ 1.11		3.42 $\pm$ 1.16	3.57 $\pm$ 1.23	3.70 $\pm$ 1.18		3.60 $\pm$ 1.21		3.54 $\pm$ 1.12	
T-AOC (U/ml per min)	14.78 $\pm$ 2.54	14.90 $\pm$ 3.13	15.46 $\pm$ 2.15		14.70 $\pm$ 2.69	14.90 $\pm$ 2.76	15.20 $\pm$ 2.93		14.83 $\pm$ 2.74		16.64 $\pm$ 3.08	
PON1 lactonase activity (U/ml)	10.03 $\pm$ 1.64	9.32 $\pm$ 1.58*	8.69 $\pm$ 1.27* [†]		8.70 $\pm$ 1.24	9.24 $\pm$ 1.48 [†]	10.01 $\pm$ 1.76 ^{†,§}		9.50 $\pm$ 1.63		8.66 $\pm$ 1.16	
PON1 level (mU/ml)	47.19 $\pm$ 6.55	43.65 $\pm$ 6.06*	40.72 $\pm$ 5.00* [†]		42.98 $\pm$ 5.48	43.59 $\pm$ 6.20	45.68 $\pm$ 6.88 ^{†,§}		44.66 $\pm$ 6.35		38.95 $\pm$ 5.04	
NLA	212.50 $\pm$ 16.75	213.25 $\pm$ 17.17	213.17 $\pm$ 15.84		202.57 $\pm$ 15.42	211.93 $\pm$ 16.06 [†]	218.87 $\pm$ 16.02 ^{†,§}		212.21 $\pm$ 16.51		222.83 $\pm$ 17.40	
PCOS												
<i>n</i>	132	229	94		50	204	201		428		27	
Age (years)	24.78 $\pm$ 3.70	25.43 $\pm$ 3.95	24.81 $\pm$ 3.24		24.69 $\pm$ 3.97	25.26 $\pm$ 3.69	25.06 $\pm$ 3.76		25.12 $\pm$ 3.76		24.89 $\pm$ 3.50	
BMI (kg/m ²)	22.87 $\pm$ 4.43	23.20 $\pm$ 4.31	23.36 $\pm$ 4.22		22.14 $\pm$ 4.74	23.16 $\pm$ 4.20	23.35 $\pm$ 4.32		23.20 $\pm$ 4.34		22.05 $\pm$ 3.87	
MDA (nmol/ml)	4.22 $\pm$ 1.43	4.17 $\pm$ 1.73	4.48 $\pm$ 1.73		4.29 $\pm$ 1.49	4.18 $\pm$ 1.54	4.31 $\pm$ 1.64		4.25 $\pm$ 1.55		4.18 $\pm$ 2.00	
T-AOC (U/ml per min)	15.90 $\pm$ 2.41	16.20 $\pm$ 3.50	16.96 $\pm$ 3.29*		15.83 $\pm$ 2.13	16.28 $\pm$ 2.86	16.39 $\pm$ 3.67		16.30 $\pm$ 3.19		15.87 $\pm$ 3.12	
PON1 lactonase activity (U/ml)	10.58 $\pm$ 1.81	9.59 $\pm$ 1.52*	8.88 $\pm$ 1.42* [†]		9.33 $\pm$ 1.48	9.49 $\pm$ 1.48	10.08 $\pm$ 1.89 ^{†,§}		9.79 $\pm$ 1.70		8.80 $\pm$ 1.40	
PON1 level (mU/ml)	49.22 $\pm$ 7.33	44.69 $\pm$ 5.82*	41.76 $\pm$ 4.94* [†]		45.25 $\pm$ 6.49	44.75 $\pm$ 6.08	46.13 $\pm$ 7.28		45.79 $\pm$ 6.57		39.47 $\pm$ 5.98	
NLA	215.00 $\pm$ 17.25	214.22 $\pm$ 17.17	212.03 $\pm$ 16.90		206.50 $\pm$ 18.41	212.00 $\pm$ 14.88	217.87 $\pm$ 18.03 ^{†,§}		213.39 $\pm$ 16.77		223.54 $\pm$ 20.25	

MDA, malondialdehyde; NLA, normalized lactonase activity; PCOS, polycystic ovarian syndrome; PON1, paraoxonase 1; T-AOC, total antioxidant capacity. * $P < 0.01$ compared with the *PON1* gene – 108CC genotype subgroup. [†] $P < 0.001$ compared with the *PON1* gene – 108CT genotype subgroup. [‡] $P < 0.05$ compared with the *PON1* gene 192QQ genotype subgroup. [§] $P < 0.05$ compared with the *PON1* gene 192QR genotype subgroup. ^{||} $P < 0.05$ compared with the *PON1* gene 55LL genotype subgroup.

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lactonase activities and NLA in a genotype-dependent manner ($RR > QR > QQ$); the PON1 levels were also increased in the RR genotype compared with the controls with the QQ or QR genotype.

The $PON1$ $55L \rightarrow M$ variants significantly decreased PON1 levels and PON1 lactonase activities and increased the NLA in the PCOS patients and the control women.

Relationship of the lactonase activity and status of PON1 with PON1 genetic variants, clinical, hormonal, metabolic, and oxidative stress parameters in subjects

A bivariate analysis showed that the PON1 lactonase activities positively correlated with the PON1 levels ($r=0.898$, $P<0.001$).

The multivariate stepwise regression analysis showed that the $PON1$ $-108C/T$, $192Q/R$, and $55L/M$ genetic variants and the apoA1, MDA, and LDL-C levels were

significant predictors of the PON1 lactonase activities; the $PON1$ $-108C/T$, $192Q/R$, and $55L/M$ genetic variants; the apoA1 and MDA levels; and PCOS status were significant predictors of the PON1 levels; the $PON1$ $-108C/T$, $192Q/R$, and $55L/M$ genetic variants; SBP; and the apoA1, LDL-C, TG, and MDA levels were significant predictors of the NLA (Table 4).

Discussion

In this study, we found that the lactonase activities and concentrations of PON1 as well as the $PON1$ $192RR$ genotypic frequencies were increased in PCOS patients with HA+OA+PCO and HA+PCO, and the NLA was increased in patients with HA+OA+PCO among the four PCOS phenotypes (based on the Rotterdam criteria), suggesting that a compensatory stimulation of PON1 lactonase activities and some genetic components may

Table 4 Association of important independent variables with lactonase activity, level, and NLA of PON1 by multivariate regression analysis in subjects.

Independents	Unstandardized coefficients		Standardized coefficients	t	P
	B	S.E.M.	β		
Model I: PON1 lactonase activity as dependent variable					
Constant	-3.052	0.974		-3.133	0.002
PON1 - 108C/T ^a	1.444	0.116	0.595	12.413	<0.001
PON1 192Q/R ^b	1.083	0.125	0.421	8.688	<0.001
ApoA1 (g/l)	2.660	0.415	0.283	6.403	<0.001
MDA (nmol/ml)	0.215	0.048	0.198	4.437	<0.001
LDL-C (mmol/l)	0.374	0.106	0.154	3.534	<0.001
PON1 55M/L ^c	0.974	0.326	0.132	2.991	0.003
Model II: PON1 level as dependent variable					
Constant	-3.564	3.784		-0.942	0.347
PON1 - 108C/T	5.507	0.459	0.584	12.007	<0.001
PON1 192Q/R	2.791	0.494	0.279	5.646	<0.001
ApoA1 (g/l)	10.202	1.643	0.279	6.210	<0.001
PON1 55M/L	6.816	1.283	0.237	5.313	<0.001
MDA (nmol/ml)	0.716	0.193	0.170	3.713	<0.001
PCOS ^d	1.808	0.772	0.105	2.343	0.020
Model III: NLA as dependent variable					
Constant	133.904	16.375		8.177	<0.001
PON1 192Q/R	9.046	1.523	0.350	5.938	<0.001
PON1 - 108C/T	5.587	1.420	0.229	3.935	<0.001
ApoA1 (g/l)	16.826	5.153	0.178	3.266	0.001
LDL-C (mmol/l)	3.514	1.313	0.145	2.677	0.008
PON1 55M/L	-10.341	4.018	-0.139	-2.574	0.011
SBP (mmHg)	0.239	0.093	0.138	2.561	0.011
TG (mmol/l)	2.025	0.967	0.115	2.094	0.037
MDA (nmol/ml)	1.219	0.595	0.112	2.048	0.041

ApoA1, apolipoprotein A1; LDL-C, LDL-cholesterol; MDA, malondialdehyde; NLA, normalized lactonase activity; PCOS, polycystic ovarian syndrome; PON1, paraoxonase 1; SBP, systolic blood pressure; TG, triglycerides.

^a $PON1$ $-108C/T$ (TT=1, CT=2, and CC=3).

^b $PON1$ $192Q/R$ (QQ=1, QR=2, and RR=3).

^c $PON1$ $55M/L$ (ML=1 and LL=2).

^dPCOS (control=0 and PCOS=1).

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be present in these patients. We also showed that the *PON1* –108C/T, 192Q/R, and 55L/M genetic polymorphisms, oxidative stress, serum apoA1 and lipid levels, and PCOS status might influence the lactonase activities and status of PON1.

This study showed that the MDA levels, PON1 lactonase activities, and PON1 levels were higher in patients with PCOS than in control women, and the MDA level was one of the significant predictors of the PON1 lactonase activities and PON1 levels in the multivariate regression models, suggesting that oxidative stress and the lactonase activities or concentrations of PON1 might be closely linked in these patients. Studies have shown that PON1 inhibits LDL oxidation (38) and stimulates cholesterol efflux from macrophages (39). Impairing the lactonase activity of PON1 via mutations of the catalytic histidine dyad (H115Q and H134Q) reduces the ability of PON1 to prevent LDL oxidation and stimulate macrophage cholesterol efflux, indicating that the anti-atherogenic functions of PON1 may be mediated by its lactonase activity (23, 40). PON1 evidently plays an antioxidant role via hydrolyzing lipid peroxides by means of its lactonase activity (13, 18, 19). PCOS is associated with increased oxidative stress (5) and systemic inflammation (6). Our previous study showed that the increased MDA levels were correlated with elevated TT levels in PCOS patients (41). Gonzalez *et al.* (42) reported that hyperandrogenemia activated and sensitized leukocytes to glucose and increased leukocytic NADPH oxidase P47(phox) subunit gene expression and reactive oxygen species generation in normal-weight PCOS women. The increased oxidative stress was reportedly associated with insulin resistance (43), systemic inflammation, endothelial dysfunction (44, 45), stimulated proliferation, testosterone production of theca cells (45, 46), and increased hepatic PON1 protein expression (47, 48). These findings support our results that the elevated lactonase activities and levels of PON1 might be a compensation for increased oxidative stress and inflammation in PCOS patients. Conversely, PON1 is sensitive to the oxidative stress status which can lead to enzyme inactivation and consumption (49), which may explain the decreased serum PON1 activity and/or levels that are associated with increased PON1 protein expression in some disease statuses (47, 48). In brief, PON1 plays an important role in preventing oxidative stress and controlling inflammation, and the absolute or relative lack of PON1 lactonase activity may contribute to PCOS pathology.

This study also showed that apoA1 levels were significant predictors of PON1 lactonase activities, PON1 levels, and the NLA; the PON1 lactonase activities positively correlated with PON1 levels ($r=0.898$), suggesting that the PON1 protein concentrations and apoA1 levels are important factors that influence the PON1 lactonase activities. The PON1 DEPCyMCase activities have been shown to be a good marker of PON1 protein levels because they are not influenced by the degree of catalytic stimulation by HDL and can provide information similar to direct PON1 quantification by ELISA (21). The PON1 AREase activity is also considered as the classical surrogate marker of PON1 concentration, but it undergoes an approximately twofold higher stimulation upon HDL binding; thus, phenylacetate may not be a suitable substrate for assessing PON1 concentration (22). ApoA1–HDL or apoE–HDL binding to PON1 can significantly increase the stability of PON1 and stimulate the lactonase activities of PON1 (50, 51), while the dissociation of PON1 from HDL to the lipoprotein-deficient serum fraction is accompanied by a loss of PON1 lactonase activities and anti-atherogenic properties (52). In addition, we found that a high stimulation of PON1 lactonase activities due to increased NLA is associated with the HA+OA+PCO phenotype, and the significant predictors of NLA were TG, LDL-C, and SBP. Consistent with our findings, NLA was also increased in patients with coronary artery disease (CAD) (24). Recent studies have shown that HDLs from CAD patients enriched in PON1 (53) and proatherogenic small and density LDLs tend to be associated with increased PON1 activity (54). Based on these findings, we speculate that PCOS-related changes in the HDL composition and presence of proatherogenic lipidemia could, at least partly, be associated with a compensatory stimulation of PON1 lactonase activity.

Our study showed that the *PON1* –108C/T, 192Q/R, and 55L/M genetic variants were important predictors of PON1 lactonase activities, PON1 levels, and the NLA in the multivariate regression analysis; the *PON1* –108C→T variants genotype-dependently decrease the serum PON1 concentrations and thus decrease the PON1 lactonase activities, but these variants did not significantly affect the degree of catalytic stimulation by HDL in the patients and the controls; the *PON1* 192Q→R variants genotype-dependently increase the PON1 lactonase activities and the degree of catalytic stimulation by HDL in the control women; the serum lactonase activities and concentrations of PON1 are higher in subjects with *PON1* 55LL genotype than in subjects with the 55LM genotype. Consistent with our results, the *PON1* genetic polymorphisms have been

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shown to regulate the PON1 status on HDL in circulation and account for more than 60% of the interindividual variation in enzyme concentration and activity (12, 13, 34). Studies have found that the promoter region $-108C \rightarrow T$ variants decrease the PON1 expression and protein level, the *PON1* Q192R polymorphism itself does not affect PON1 protein concentrations but greatly influences the PON1 activities, and the *PON1* 55L/M polymorphism influences both the PON1 concentrations and activities (12, 55). The PON1 mRNA and protein levels are significantly higher in 55L allele carriers (12) and the POase and lactonase activities were higher in the 55LL homozygotes (56). In addition, unlike most other studies, we find that the PON1 concentrations were higher in the controls harboring the *PON1* 192RR genotype than in controls harboring the QQ or QR genotype. The increases may be due to the *PON1* $-108C$ allele, which increases PON1 expression and may partly compensate for the PON1 concentrations afforded by the R isoform because the *PON1* 192R allele has been reported to be in linkage disequilibrium with the *PON1* $-108C$ allele (34, 56). Similarly, the effects of *PON1* 55L/M polymorphism on the PON1 concentrations and activities might be largely due to the linkage disequilibrium among the 55L, 192R, and $-108C$ alleles and between 55M and 192Q (34, 56).

In addition, we showed that the lactonase activities and levels of *PON1* and the 192RR genotypic frequencies were significantly higher or tended to be increased in PCOS patients with HA+OA+PCO or HA+PCO than in the controls, suggesting that the increased 192RR genotypic frequencies may contribute to the increased lactonase activities and levels of PON1 in these patients. On the other hand, we also found that unlike the control 192QR or RR genotypic subgroups, the increase in PON1 lactonase activities and NLA is of limited in PCOS patients with 192QR or RR genotype (Table 3). Our previous study showed that the PCOS patients with QR or RR genotype had significantly higher or tend to increase WC, WHR, fasting insulin and TG levels, and HOMA-IR compared with patients with QQ genotype, while such relationships were not detected in the control women (34), suggesting that abdominal obesity and insulin resistance, which is often associated with the decreased apoA1 levels and the increased plasma glucose levels, may impair PON1 lactonase activities by the glycation of HDL and the reduced stimulation of apoA1 on PON1 (13). In addition, we found that the PON1 concentrations were lowest and the degree of catalytic stimulation by HDL was highest in the subjects harboring the 55LM genotype. However, our previous study showed that the degree of obesity and

insulin resistance was similar between the PCOS patients harboring the 55LM genotype and the patients harboring the LM genotype (34). Thus, the association of this high stimulation by HDL with the amino acid change in the PON1 55 site remains to be determined.

Notably, the allele frequencies for *PON1* polymorphisms differ among ethnic groups. The frequency of the 192R allele among Chinese subjects in the current study (60%) is higher compared with other populations, such as Greek (9.5%), Caucasians (24–31%), Asian Indian (31%), and Hispanic Americans (42–46%), and similar to Korean (60%) and Japanese (59–66%) (57, 58). The frequency of the 55M allele was 4% in this study carried out in the Chinese population. It was shown to be higher in Caucasians (26–38%), and similar to Korean (3%), but slightly higher in Japanese (9–10%) (58). The frequency of the $-108T$ allele among Chinese subjects in this study (43%) was lower compared with Caucasians (50–54%) (59), and similar to Greek (43.5%) (57), but higher compared with African Americans (15.5%) and Hispanics (35%) (58). Interestingly, the *PON1* 192RR genotype, but not $-108TT$ genotype, was associated with PCOS in our previous studies (34, 37). However, the *PON1* $-108TT$ and 192RR genotypes are more frequently encountered in Greek PCOS than in non-PCOS women (57). This inconsistency may be in part attributed to different genetic background between the two populations. In addition, environmental factors such as smoking, a high-fat diet, and consumption of antioxidants may also affect PON1 status on HDL in circulation or PON1 expression (60). Therefore, it is possible that the relationships between *PON1* genetic polymorphism and PON1 lactonase activity, PON1 level, or disease may be different among different populations.

This study has several limitations. First, we only measured the TT levels, which may be a less sensitive marker for HA than the free testosterone levels. Second, the presence of tubal factor may have biased the control group in this study. Finally, the sample size of the HA+PCO phenotype was relatively small, and a larger number of subjects is needed to properly evaluate their characteristics.

In summary, the increased serum lactonase activities and concentrations of PON1 and MDA levels in the PCOS patients with HA+OA+PCO and HA+PCO, along with the observed positive correlation between these parameters, suggests that increased oxidative stress may partly be related to a compensatory stimulation of PON1 lactonase activities in these patients with PCOS. In addition, the *PON1* $-108C/T$, 192Q/R, and 55L/M genetic

polymorphisms also significantly affect the lactonase activities and status of PON1.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-14-0863>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

P Fan designed, performed the study, analyzed the data, and wrote the paper. Y Zhang collected samples, did experiments, and analyzed the data. J He, K Xu, and L Cheng synthesized TBBL and DEPCyMC. J Zhang, Y Wang, and F Zhang collected samples and helped with the experiments. H Liu was responsible for patients screening. H Bai revised the paper. All authors discussed the results and commented on the manuscript.

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