Paraoxonase 1 Response to a High-Fat Diet: Gender Differences in the Factors Involved

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Diets consumed in industrialized countries are rich in fat and increase the incidence of atherosclerosis, a process reported to be influenced by gender. Considering the anti-atherogenic role attributed to serum Paraoxonase 1 (PON1) activity, and given the pro-atherogenic effects described for saturated fatty acids (SFA), as opposed to the beneficial ones conferred to monounsaturated fatty acids (MUFA), the aim of this study was to investigate the response of male and female rat serum PON1 activity and its related factors to a high-fat (HF), hypercaloric diet (fat representing 55.2% of the energy) containing similar amounts of SFA and MUFA. The HF diet feeding did not alter total body weight, but increased adiposity. Nevertheless, and in spite of the increased adiposity, the HF diet did not entail a more pro-inflammatory serum adipokine or lipid profile or increased lipid peroxidation. Paraoxonase activity was reduced in both male and female HF fed rats, due to a reduction of PON1 mRNA levels in males and to a reduced stability and/or number of HDL particles responsible for PON1 transport in females. Both the maintenance of body weight and the MUFA content in the diet would be among the factors responsible for the attenuation of the negative effects usually related to excessive fat intake and for the reduction in PON activity, whose antioxidant activity would be less necessary in this situation.

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INTRODUCTION

Dietary fat represents an important regulatory factor of pro-inflammatory events. In fact, eating fat in excess is usually associated with increased adiposity, which has been reported to be related to higher levels of pro-inflammatory adipokines, such as plasminogen activator inhibitor 1 (PAI-1) and leptin, and to reduced levels of adiponectin, an adipokine related to a higher insulin sensitivity and to a more cardioprotective status (1,2).

Concerning dietary fat composition, it is widely believed that diets rich in saturated fatty acids (SFA) are usually associated with a less favorable inflammatory profile than those rich in unsaturated fatty acids (3,4). Moreover, diets rich in monounsaturated fatty acids (MUFA) are associated with a lower LDL susceptibility to oxidation than those rich in polyunsaturated fatty acids (PUFA), which, due to their intrinsic nature and despite being related to beneficial effects on pro-inflammatory factors and insulin resistance (4,5), are more prone to oxidation (4,6).

Paraoxonase 1 (PON1) is an esterase closely associated to HDL containing both apolipoprotein A-I and apolipoprotein J (apoA-I and apoJ) and is believed to confer antioxidant properties to HDL (7). In this sense, PON1 has been shown to effectively hydrolyze the oxidized phospholipids present in LDL, thus retarding the oxidation of these lipoproteins and attenuating their pro-inflammatory effects (8-10). LDL oxidation is known to be influenced by diet, altering both LDL susceptibility to oxidation and serum PON1 activity (6,11). Thus, and as far as PON1 response to dietary fat is concerned, the consumption of high-fat (HF), as well as hypercholesterolemic diets, has been associated with a reduction of PON1 activity in both mice and rabbits (9,12). However, and in agreement with the improvement of the antioxidant and anti-inflammatory protection reported to be exerted by oleic acid (13), supplementation and in vitro studies have reported a higher MUFA-related PON1 stability and activity in comparison with PUFA (14-16).

Diets usually consumed in industrialized countries are rich in animal fat and have been associated with an increased incidence of cardiovascular diseases, which seems to be influenced by gender. Given the pro-atherogenic effects described for SFA, as opposed to the beneficial ones conferred to MUFA, together with the protective role of PON1 against atherosclerosis development and its modulation by dietary factors, the aim of this study was to investigate the response of serum PON1 activity and dif-

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ferent inflammatory markers to the consumption of a high-fat, hypercaloric diet containing similar amounts of SFA and MUFA on male and female rats. We also analyzed serum apoA-I and apoJ levels, as factors involved in PON1 function and stability in the serum, and liver PON1 mRNA levels, as the main source of serum PON1, to further characterize the PON1 response to the diet.

MATERIALS AND METHODS

Materials

Oligonucleotide primer sequences, Lightcycler-FastStart DNA Master SYBR Green I for real-time PCR and Tripure isolation reagent were purchased from Roche Diagnostics (Basel, Switzerland). RT-PCR chemicals were from Applied Biosystems (Foster City, CA, USA). Rabbit polyclonal antibody to human PON1 was provided by Dr. M. Mackness and Dr. B. Mackness (17). Rabbit polyclonal antibodies to human apoA-I (Cat. Num. 178422; San Diego, CA, USA) and to rat SR-BI (NB 400-104G2, Littleton, CO, USA) were supplied by Calbiochem and Novus Biological, respectively. Goat polyclonal antibodies to rat apoJ and rat PAI-1 were purchased from Santa Cruz Biotechnology (Cat. Num. sc-13747 and sc-6644 respectively; Santa Cruz, CA, USA). Chemiluminescence kit (ECL) for immunoblot development was purchased from Amersham (Little Chalfont, UK).

Kits for measurement of serum lipid profile—HDL-cholesterol direct (Cat. Num. 1133505), LDL-cholesterol (Cat. Num. 1142005) and Total cholesterol MR (Cat. Num. 1118005)—were purchased from Linear Chemicals SL (Barcelona, Spain). Accutrend triglyceride and glucose test strips (Cat. Num. 1538144 and 11443054, respectively) and the Accutrend GCT-meter were purchased from Roche Diagnostics (Basel, Switzerland). Non esterified fatty acid assay (Cat. Num. 999-75406) was purchased from Wako Chemicals (Osaka, Japan). Enzyme immunoassays for measurement of rat adiponectin and ghrelin (Cat. Num. EK-ADI-02 and EK-031-31, respectively) were purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Enzyme immunoassays for the measurement of leptin (Cat. Num. MOB00) and insulin (Cat. Num.10-1124-01) were from R&D Systems (Minneapolis, MN, USA) and Mercodia (Uppsala, Sweden), respectively.

Substrates for the measurements of PON1 activities (diethyl p-nitrophenyl phosphate and phenylacetate) were from Sigma-Aldrich (St. Louis, MO, USA). Routine chemicals used were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain) and Sigma-Aldrich (St. Louis, MO, USA).

Animals, Diets, and Sample Collection

Animal experiments were performed in accordance with the general guidelines approved by our institutional ethics committee and EU (86/609/EEC) regulations. Eight-week old Wistar rats (Charles River, Barcelona, Spain), 14 males and 14 females, were housed in groups of two-three rats per cage and kept at 22°C on a 12 h light-dark cycle. For each gender, rats having similar mean body weight were divided into two dietary groups: a control group—fed a pelleted standard diet with protein, carbohydrate, and fat representing 18.7%, 73.3%, and 8% of the total energy content of the diet (14104 kJ/kg diet), respectively—and a high fat fed group (HF group)—fed a high-fat diet with 13.5% of energy from protein, 31.3% from carbohydrate, and 55.2% from fat (21602 kJ/kg diet). Nutrient composition of diets (Panlab, Barcelona) is given in detail in Table 1. For 14 weeks, rats had free access to the standard diet (control group) or to the HF diet (HF group). After a period of 12 h of fasting, 22-week old rats were killed by decapitation. Liver and gonadal, lumbar and inguinal white adipose tissue (WAT) depots were rapidly dissected, weighed, frozen in N_2 liquid and stored at –70°C until processed. Serum samples were also stored at –70°C until analyzed. Small pieces of liver and WAT depots were homogenized with a teflon/glass homogenizer in Tris-HCl/sucrose buffer (5mM Tris-HCl, 0.25mM sucrose, 2mM EGTA, pH = 7.4)

Measurement of Serum Lipid Profile and Serum Glucose, Hormone, and Adipokine Levels

Total, HDL, and LDL cholesterol, as well as non-esterified free fatty acids lev-

Table 1. Diet composition.

	Standard diet	HF diet
Fat	29	300
SFA	6	130
MUFA	7	140
PUFA	15	30
C14:0	—	5.3
C15:0	—	0.6
C16:0	4.7	77
C18:0	1.3	53
C14:1	—	0.03
C16:1	0.4	11
C18:1	6.5	133
C18:2	14	36
C18:3	1.2	—
Protein	155	166
Carbohydrate	605	206
Fiber	39	36
Mineral mix	53	35
Vitamin mix	10	8
Energy content	14104	21062

Nutrient composition and energy content of the diets are given in g/kg and kJ/kg of diet, respectively.
Mineral composition of the diets (mg/kg): standard diet: Ca, 8800; P, 5900; Na, 2500; Cl, 3100; K, 6250; Hg, 1400. high-fat diet: Ca, 6347; P, 4598; Na, 231; Cl, 624; K, 309; Hg, 85.5; S, 9.2; Fe, 86; Cu, 7.4; Mn, 463; MnO, 463; Zn, 259; ZnO, 259; I, 3.6; Co, 0.6; salt, 572.
Vitamin composition of the diets: standard diet: A, 15000 IU/kg; D3, 1500 IU/kg; E, 20 mg/kg; menacine, 2 mg/kg; B1, 1 mg/kg; B2, 4 mg/kg; B3, 20 mg/kg; B5, 15 mg/kg; B6, 4 mg/kg; B8, 0.1 mg/kg; B9, 1 mg/kg; B12, 20 mg/kg; coline, 500 mg/kg. high-fat diet: A, 0.017 IU/mg; D3, 0.002 IU/mg; E, 1000 mg/kg; K3, 15 mg/kg; B1, 17 mg/kg; B2, 13 mg/kg; PP, 86 mg/kg; B5, 5.9 mg/kg; B6, 8.6 mg/kg; B9, 4.3 ppm; H, 0.268 ppm, B12, 0.043 ppm; C, 0.7 mg/kg; coline, 1179 mg/kg; inositol, 129 mg/kg.

els, were measured by using spectrophotometric assay kits. Triglyceride and glucose levels were measured by using the Accutrend system. Adiponectin, leptin, insulin, and ghrelin serum levels were measured by using enzyme immunoassay kits.

Measurement of Serum Thiobarbituric Acid-Reactive Substances (TBARS) and PON1

Levels of TBARS, as an index of lipid peroxides, were measured in serum and liver homogenate samples as previously described (18). PON1 activities were assayed by measuring the rate of hydrolysis of phenylacetate (arylesterase activity) and paraoxon (paraoxonase activity) as previously described (19).

Western Blot Analysis of Serum PON1, apoA-1, apoJ, and PAI-1, and Liver SR-BI Protein Levels

Equal amounts of serum and liver protein (30 μg) were fractionated on 15% SDS-PAGE gels and electrotransferred onto a nitrocellulose filter. Ponceau S staining was performed systematically to check the correct loading and electrophoretic transfer. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands in films were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). The apparent molecular weights of PON1 (43 kDa), apoA-I (28 kDa), apoJ (50 kDa), PAI-1 (50 kDa), and SR-BI (80 kDa) were estimated using protein molecular-mass standards.

Analysis of PON1 mRNA Levels by Real-Time RT-PCR

Total cellular RNA was isolated from liver samples by using Tripure isolation reagent according to the manufacturer's protocol. One μg of the total RNA was reverse transcribed to cDNA and subsequently diluted 1/10 and frozen at –70°C until the PCR reactions were carried out. Real-time PCR was performed using SYBR Green® detection technology in a Lightcycler Rapid Thermal Cycler (Roche Diagnostics, Basel, Switzerland) as previously described (20).

Real-time PCR efficiencies were calculated as previously described (20). PON1 and 18S real-time PCR efficiencies were 1.87 and 1.89 respectively.

Statistical Analysis

All data were expressed as mean values ± SEM of 7 animals per group. Statistical analyses were performed by using a statistical software package (SPSS 13.0 for Windows, Inc., Chicago, IL, USA). Statistical differences between experimental groups were analyzed by two-way analysis of variance (ANOVA). Student t-test, as post-hoc comparison, was performed when an interactive effect of gender and diet was shown. A *P*-value of less than 0.05 was considered statistically significant.

The statistical PCR data analysis was performed by using the Relative Expression Software tool (REST 2005 β V1.9.9) (21). Differences in mRNA levels between groups were analyzed by Pair Wise Fixed Reallocation Randomization Test® (22), a proper model to avoid the normal distribution of data.

RESULTS

Food and Energy Intake and Biometrical Parameters

HF fed rats reduced their food consumption (22% and 18% for males and females, respectively), maintaining both their energy intake and body weight, compared with control rats (Table 2). Consumption of the HF diet increased the weight of different WAT depots studied, especially in male rats, whereas no changes were found in liver weight.

Effect of HF Diet Feeding on Serum Lipid Profile, Glucose, Hormone, and Adipokine Levels

Consumption of the HF diet decreased serum triglycerides in both genders and free fatty acid levels only in male rats (Table 3). No differences were found between control and HF fed groups as regards total, HDL, and LDL cholesterol

Table 2. Food and energy intake and biometrical parameters.

	Male		Female		
	Control	HF fed	Control	HF fed	ANOVA
Food intake, g/kg body weight$^{0.75}$ × day	49.4 ± 2.7	38.4 ± 2.6	46.9 ± 1.1	38.3 ± 1.2	D
Energy intake, kJ/kg body weight$^{0.75}$ × day	959 ± 32	1022 ± 43	906 ± 72	929 ± 86	NS
Body weight, g	452 ± 9	470 ± 13	257 ± 8	263 ± 3	G
Relative tissue weight, g/100g body weight					
Liver	2.45 ± 0.08	2.37 ± 0.04	2.50 ± 0.17	2.36 ± 0.07	NS
Lumbar WAT	1.37 ± 0.22	2.56 ± 0.23	1.12 ± 0.18	1.53 ± 0.22	G,D
Inguinal WAT	1.71 ± 0.17	2.50 ± 0.25	1.60 ± 0.17	1.71 ± 0.20	G,D
Gonadal WAT	1.68 ± 0.20	2.75 ± 0.28	1.87 ± 0.28	2.26 ± 0.41	D

Values are expressed as the mean ± S.E.M. of seven animals per group.
ANOVA ($P < 0.05$).
G, gender effect.
D, diet effect.
NS, non significant.

Table 3. Effect of HF diet feeding on serum lipid profile and ghrelin and adipokine serum levels.

	Male		Female		
	Control	HF fed	Control	HF fed	ANOVA
Triacylglycerols (mM)	2.48 ± 0.16	2.08 ± 0.15	2.90 ± 0.41	2.05 ± 0.27	D
Free fatty acids (mM)	0.91 ± 0.04	0.67 ± 0.04[a]	0.93 ± 0.08	0.95 ± 0.05[b]	G,G*D
Total cholesterol (mM)	1.74 ± 0.12	1.63 ± 0.15	1.50 ± 0.10	1.26 ± 0.14	G
HDL-cholesterol (mM)	0.21 ± 0.01	0.23 ± 0.01	0.25 ± 0.03	0.20 ± 0.02	NS
LDL-cholesterol (mM)	0.63 ± 0.23	0.59 ± 0.08	0.71 ± 0.07	0.53 ± 0.06	NS
LDL/HDL ratio	2.90 ± 0.93	2.51 ± 0.24	2.63 ± 0.26	2.72 ± 0.55	NS
Glucose (mM)	8.20 ± 0.27	8.10 ± 0.60	6.62 ± 0.28	7.00 ± 0.32	G
Insulin (µg/L)	0.28 ± 0.03	0.42 ± 0.06	0.23 ± 0.02	0.37 ± 0.10	D
HOMA-IR	2.92 ± 0.27	4.06 ± 0.45	1.98 ± 0.23	3.40 ± 1.02	D
Ghrelin (ng/mL)	0.76 ± 0.24	0.30 ± 0.10	0.48 ± 0.15	0.29 ± 0.07	D
Adiponectin (µg/mL)	8.17 ± 0.82	12.6 ± 1.51[a]	9.23 ± 1.02	8.32 ± 0.87[b]	G*D
Leptin (ng/mL)	0.98 ± 0.22	1.25 ± 0.19	0.77 ± 0.17	0.52 ± 0.11	G
(TBL)PAI-1 (AU)	100 ± 14	106 ± 9	135 ± 12	120 ± 9	NS

[a]HF vs. control.
[b]female vs. male.
Values are expressed as the mean ± S.E.M. of seven animals per group.
HOMA-IR was calculated as (blood fasting glucose (mmol/L) × blood fasting insulin (µU/mL))/22.5.
PAI-1 levels were measured by immunoblot.
ANOVA ($P < 0.05$).
G, gender effect.
D, diet effect.
G*D, interactive effect.
NS, non significant.
t-Test ($P < 0.05$).

levels. Total cholesterol levels were lower in female rats compared with males.

In both genders, serum insulin levels increased with the HF diet feeding, whereas those of glucose were not modified. Thus, the HOMA-IR ratio was significantly higher in rats fed the HF diet than in control rats. Ghrelin levels were lower in the HF fed than in the control fed rats. Serum adiponectin levels were significantly higher in male rats fed the HF diet compared with those fed with the standard diet and did not change in female rats. Leptin levels, which were higher in male rats compared with females, were not affected by the dietary treatment. No changes were observed in serum PAI-1 levels.

Effect of HF Diet Feeding on Lipid Peroxide levels (TBARS), Serum PON1 Activities, Serum PON1, apoA-I and apoJ Levels, and Liver SR-BI Levels.

There were no differences between groups in serum lipid peroxide levels (Table 4). Serum paraoxonase activity was significantly decreased by HF diet consumption (24%-26%), being higher in female rats compared with males. No changes were observed in serum arylesterase activity. Serum PON1 levels were higher in female rats compared with males, and showed a tendency to decrease with HF diet feeding in both genders, although they did not reach statistical significance. ApoA-I levels were also higher in female control rats compared with males, and decreased in females fed with the HF diet. Similarly, apoJ levels were reduced only in HF fed female rats. SR-BI levels were higher in female rats, but were not modified by HF diet feeding.

Effect of HF Diet Feeding on Liver PON1 mRNA Levels

PON1 mRNA levels decreased in HF fed male rats compared with controls (Table 5). In female rats, a tendency to decrease was also shown, although it did not reach statistical significance ($P = 0.081$).

DISCUSSION

In this study we found that consumption of a diet highly rich in fat (55.2% of the energy content of the diet) and with similar amounts of SFA and MUFA resulted in an enhanced weight of WAT and a reduction of serum paraoxonase activity, but with no evidence of an enhanced inflammatory state.

Consumption of the HF diet induced a satiating effect, which is reflected in the reduced levels of the appetite-stimulating peptide ghrelin (23). In consistence with this fact, rats reduced their food intake, thus maintaining their energy intake and their body weight closer to those of the control rats. Nevertheless, the HF fed rats showed a marked increment of adipose depots. This enhanced adiposity was greater in male rats com-

Table 4. Effect of HF diet feeding on serum lipid peroxide levels (TBARS), PON1 activities, PON1, apoA-I and apoJ levels, and liver SR-BI levels.

	Male		Female		
	Control	HF fed	Control	HF fed	ANOVA
Serum					
TBARS (µmol/L)	1.40 ± 0.03	1.73 ± 0.19	1.53 ± 0.22	1.44 ± 0.09	NS
Paraoxonase activity (nKat/mL)	1.16 ± 0.09	0.96 ± 0.03	1.38 ± 0.06	1.01 ± 0.06	G,D
Arylesterase activity (µKat/mL)	2.34 ± 0.28	2.25 ± 0.17	2.70 ± 0.34	2.43 ± 0.28	NS
PON1(%)	100 ± 18	70 ± 20	218 ± 36	157 ± 17	G
ApoA-I (%)	100 ± 8	89 ± 18	152 ± 21[b]	60 ± 14[a]	D, G*D
ApoJ (%)	100 ± 13	111 ± 16	137 ± 16	87 ± 11[a]	G*D
Liver					
SR-BI (%)	100 ± 5	73 ± 39	184 ± 34	159 ± 17	G

[a]HF vs. control
[b]female vs. male.
Values are expressed as the mean ± S.E.M. of seven animals per group.
Serum levels of PON1, apoA-I and apoJ, and liver levels of SR-BI were detected by immunoblot.
Levels of control rats were set as 100%.
ANOVA ($P < 0.05$).
G, gender effect.
D, diet effect.
G*D, interactive effect.
t-Test ($P < 0.05$).

pared with females, which, together with the lower serum free fatty acid levels observed in this gender, could point to a lesser lipid mobilization.

The HF diet, despite causing an increased adiposity, did not entail either a more pro-inflammatory and pro-atherogenic serum adipokine profile, because neither leptin nor PAI-1 levels were increased. Moreover, adiponectin levels were not decreased, but rather increased in male rats. This observed attenuation of the pro-inflammatory consequences usually resulting from excessive fat consumption (24,25) would be related in part to the reduction of energy intake, as well as to the high MUFA content in the HF diet. This idea would be in accordance with previous studies reporting a reduction of PAI-1 activity and levels after MUFA consumption (26) and describing the modulation of adipokine expression by PUFA (2,27), the content of which is also increased in the HF diet compared with the control diet. Along the same lines, the HF diet did not alter serum lipid profiles, thus again suggesting that the HF diet, probably due to its high MUFA content, brings about the attenuation of the deleterious effects of SFA (28), thus predominating the beneficial effects of MUFA (3,29) over the negative ones described for SFA. Neverthe-

Table 5. Effect of HF diet feeding on liver PON1 mRNA levels.

	Male		Female		
	Control	HF fed	Control	HF fed	REST
PON1					(α=0.013)
Expression	1	0.452[α]	1.313	0.504	
REST error		0.274-0.814	0.657-2.666	0.362-0.782	
18S					NS
Expression	1	0.942	0.623	0.827	
REST error		0.529-1.89	0.357-1	0.653-0.922	

[α]HF fed males vs. control males.
mRNA levels of control male rats were set as 1.
18S mRNA expression was used as a housekeeper.
Statistical differences between experimental groups were assessed by Pair Wise Fixed Reallocation Randomization Test® ($P < 0.05$).
Statistical comparisons were performed between HL vs. control rats and female vs. male rats.

less, the hyperinsulinemia and enhanced insulin resistance that the HF fed rat HOMA-IR values indicate would suggest that a prolonged consumption of the diet would lead to a more pro-inflammatory status.

The reduction of serum paraoxonase activity in HF fed rats, which is in accordance with previous studies in mice and rabbits fed with diets rich in fat (9,12), as well as the lack of differences in serum lipid peroxide levels, suggest that the antioxidant protection by PON1 would be less necessary in these animals, and could be attributed to the high content of MUFA and vitamin E in the diet. These results contrast with previous studies reporting a MUFA-associated increase of PON1 activity (14-16), discrepancy that could be explained by the longer duration of the dietary treatment used in our study (3 months), which would lead to an adaptation of PON1 response to a situation entailing a lower oxidative risk. In fact, LDL particles obtained after an MUFA-diet consumption or after vitamin E supplementation have been shown to be less susceptible to oxidation (6,13).

The observed reduction of paraoxonase activity with the HF diet would correspond with a reduction of serum PON1 levels, such as the positive correlation found between both parameters would indicate ($R = 0.442$, $P = 0.03$). These decreased PON1 levels cannot be attributed to alterations in the SR-BI mediated PON1 release from the liver (30). We have found gender differences in the main factors responsible for serum PON1 activity, i.e. its expression in the liver (31) and the serum levels of apoA-I and apoJ -apolipoproteins present in the HDL subpopulation to which PON1 associates and which contribute to the stability of the PON1-HDL association and to PON1 function (32,33). In male rats, the observed paraoxonase activity decrease would be justified by a reduction of the PON1 mRNA levels in the liver, thus suggesting either a reduction of the hepatic transcriptional activity of the PON1 gene or a lower stability of the PON1 mRNA molecules. In female rats, although liver PON1 mRNA levels tended to decrease, a reduced stability of the PON1-HDL association and/or of the number of HDL particles responsible for PON1 transport in the blood would rather be the factors responsible for the reduced serum paraoxonase activity and PON1 levels, as the lower apoA-I and apoJ would indicate.

In summary, the HF diet used in this study did not entail either a more pro-inflammatory serum adipokine or lipid profile or increased lipid peroxidation, but caused a reduction in serum paraoxonase activity. These effects could probably be a result of both the isocaloric intake induced by the diet and its high MUFA content, such that, the less oxidative situation the diet involved would make serum paraoxonase activity less necessary. The reduction of serum paraoxonase activity entailed gender differences as far as factors involved in PON1 production, stability, and function are concerned, reflecting different PON1 adaptive mechanisms of male and female rats in response to the consumption of a HF diet.

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