Paraoxonase 1 phenotype distribution in a cohort of healthy Sri Lankan population

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Abstract: Human serum paraoxonase 1(PON1) is an enzyme synthesised mainly by the liver. Paraoxonase 1 Q192R polymorphism involves amino acid substitution of glutamine (Q isoform) to arginine (R isoform) at position 192. Paraoxonase 1 activity and polymorphism is associated with many disease conditions. In the Sri Lankan context, data is lacking on the distribution of Paraoxonase 1 phenotypes. The aim of this study was to assess the phenotypic distribution of Paraoxonase 1 in a cohort of healthy Sri Lankan individuals, using the dual substrate method. Serum samples of 77 apparently healthy individuals were used for the study. Paraoxonase activity was measured by a kinetic method using paraoxon as the substrate at 412 nm and 25° C. Salt stimulated paraoxonase 1 activity was measured in the presence of 1M NaCl. Arylesterase activity was measured by a kinetic method using phenylacetate as the substrate at 270 nm and 25° C. The ratio of salt stimulated paraoxonase activity to arylesterase activity was used to assign phenotypes. Basal and salt stimulated paraoxonase activities were bimodally distributed. Arylesterase activity was unimodally distributed. Salt stimulated paraoxonase activity/ arylesterase activity ratio was trimodally distributed. The three modes corresponded to QQ (lower activity), QR (intermediate activity) and RR (higher activity) phenotypes. The percentage distribution of QQ, QR and RR phenotypes were 36 %, 51 % and 13 %, respectively. This study has set the baseline data on phenotypic distribution of paraoxonase1 in a cohort of healthy Sri Lankan individuals.

Keywords: Arylesterase, distribution, paraoxonase, phenotypes, polymorphism.

INTRODUCTION

Human serum paraoxonase 1(PON1; EC3.1.8.1) is a glycoprotein with a molecular mass of 43kDa. It is synthesised mainly in the liver and contains 354 amino acids. In blood, it is associated with Apo-A component of high density lipoprotein (HDL) (Gaidukov & Thawfik, 2005). PON1 is a calcium-dependent esterase which shows both paraoxonase and arylesterase activity (Mackness et al., 2001). It has the ability to hydrolyse organophosphates (Mackness et al., 1998) and biologically active lipoperoxides (Hashemi et al., 2011), rendering a protective effect against organophosphate toxicity and formation of atherosclerotic plaques. Recent studies have revealed its ability to hydrolyse homocystein thiolactone, a risk factor for cardiovascular diseases (Zafiropoulos et al., 2010). Cardio protective characteristic of PON1 is supported by several studies conducted in vitro (Mackness et al., 1991, González et al., 2019; Grzegorzewska et al., 2021) and in vivo (Shih et al., 1998).

Serum PON 1 activity significantly differs among individuals with a 40-fold variation (Mackness &
Mackness, 2015). These differences are modulated by hereditary and acquired factors (Draganov & La Du, 2004). The PON1 activity is influenced by diet, drugs, alcohol consumption, smoking and environmental toxins. Serum PON1 activity was reported to be decreased by intake of high fat diet (Sutherland et al., 1999). The intake of antioxidant polyphenols such as quercetin and glabridin was associated with increased PON1 activity (Aviram et al., 1999). Few studies reported the ability of statins and fibrates to increase PON1 activity (Balogh et al., 2001; Paragh et al., 2003). However, contrasting results were observed in some studies, emphasising the inability of statins and fibrates to influence PON1 activity (Durrington et al., 1998). Consumption of 40 g/day of alcohol was associated with increased PON1 activity and mass in mg/L (van der Gaag et al., 1999). A study has revealed the inhibitory effect of smoking on PON1 activity (Nishio & Watanabe, 1997). In another study, serum PON 1 concentration and activity were decreased due to smoking (James et al., 2000). The PON1 activity was inhibited by organophosphates (Sözmen et al., 2002).

The PON1 gene is located on the long arm of chromosome 7q21-22. The 192 R/Q polymorphism of human serum PON1 has two isoforms, namely, PON1 Q and PON1 R (Mackness & Mackness, 2015). The PON1 Q contains a glutamine at position 192 and it shows low activity towards paraoxon hydrolysis. PON1 R contains an arginine at position 192 (Deakin & James, 2004). It shows a six-fold higher activity towards paraoxon hydrolysis compared to Q isoform. But the arylesterase activity is similar in both isozymes (Gaidukov et al., 2006). The R allozyme shows a greater degree of stimulation of its paraoxon-hydrolyzing activity in 1 M NaCl than the Q allozyme (Eckerson et al., 1983a). This qualitative property can be used to identify two different allozymes based on their response to salts. Eckerson et al. (1983b) proposed that heterozygous PON1 phenotype (QR) can be obviously differentiated from both homozygous phenotypes (QQ and RR) on the basis of its ratio of salt stimulated paraoxonase activity to arylesterase activity. The ratio of salt-stimulated PON1 activity to arylesterase activity (P/A ratio) was trimodally distributed (Eckerson et al., 1983b). The trimodal distribution corresponds to QQ, QR and RR phenotypes. The least frequent value between two modes is called the antimode. The population was divided at the two antimodes to segregate the three phenotypes (QQ, QR and RR).

PON1 activity is found to be reduced in many disease conditions such as cardiovascular diseases (González et al., 2019; Murillo-González et al., 2020; Grzegorzewska et al., 2021), thyroid disorders (Azizi et al., 2003), cancers (El-Lebedy et al., 2014), diabetes mellitus (Savu et al., 2014), chronic renal failure (Prakash et al., 2010), chronic liver damage (Ferre et al., 2002) and psychiatric disorders (Moreira et al., 2019). The comparison of PON1 activity and polymorphism between healthy individuals and patients require baseline data of healthy individuals. In the Sri Lankan context, data of PON1 activity and distribution of PON1 phenotypes among healthy individuals are lacking. The aim of this study was to evaluate the PON1 activity and phenotype distribution in a cohort of healthy Sri Lankan individuals using the dual substrate method.

MATERIALS AND METHODS

The study protocol and procedures were approved by the Ethics Review Committee, Postgraduate Institute of Science, University of Peradeniya on 10th November 2013. Informed consent was obtained from all participants before commencing the study. The study was conducted in the Department of Biochemistry, Faculty of Medicine, University of Peradeniya.

Study subjects

Seventy seven apparently healthy individuals without any history of hypertension, smoking, cardiovascular diseases, diabetes, renal or hepatic diseases and cancer were enrolled for the study. The study group comprised 43 females and 34 males with a mean age of 57.82 ± 16.38 years. The number of subjects recruited was based on previous studies conducted as described below. Organophosphate intoxication on human serum paraoxonase was studied in 28 organophosphate poisoning patients with 66 control subjects by Sözmen et al. (2002). The study of Paraoxonase -1 gene polymorphism in a healthy population of Khorramabad, Iran involved 64 healthy volunteers by Bełtowski et al. (2002). The study of Paraoxonase -1 gene polymorphism in a healthy population of Peradeniya was studied in 28 organophosphate poisoning patients with 66 control subjects by Sözmen et al. (2002). Species and substrate specific stimulation of human plasma paraoxonase 1 by high chloride concentration was conducted in 15 male healthy volunteers by Beltowski et al. (2002). The study of Paraoxonase -1 gene polymorphism in a healthy population of Peradeniya was studied in 28 organophosphate poisoning patients with 66 control subjects by Sözmen et al. (2002).

Sample collection

Blood was collected by venipuncture with minimal stasis from individuals who have been resting for at least 20 minutes prior to collection of blood. Precautions were taken to avoid haemolysis of blood. Blood was collected with only moderate suction. The needle was always removed from the syringe before emptying it slowly into
the collecting tube. The samples were not collected in EDTA containing vacutainers as paraoxonase activity is irreversibly inactivated by the chelation of Ca. Blood was allowed to clot, and the serum was separated by centrifuging at 4000 rpm for 10 min. The serum samples were stored frozen at -20 °C and assayed within two weeks of collection. The samples were completely thawed before the assay.

Paraoxonase activity assay

Serum paraoxonase activity was estimated according to the protocol described by Eckerson et al. (1983a). The rate of hydrolysis of paraoxon (diethyl-p-nitrophenyl phosphate) was measured by monitoring the increase in absorbance at 412 nm at 25°C for 75 s in 15 s intervals. The basal assay mixture included 1.0 mM paraoxon and 1.0 mM CaCl₂ in 0.05 M glycine buffer at a pH of 10.5. One unit of paraoxonase activity was defined as 1 µmol of p-nitrophenol formed per minute and activity was expressed as U/L of serum. The liberated amount of p-nitrophenol was calculated using molar extinction coefficient of p-nitrophenol (18290 M⁻¹cm⁻¹). Salt stimulated PON1 activity was measured in the presence of 1 M NaCl. A blank without serum was used to correct any spontaneous hydrolysis of the substance.

Arylesterase activity assay

Arylesterase activity was measured using phenylacetate as the substrate (Josse et al., 1999). The change in absorbance was measured at 270 nm for 75 s in 15 s intervals. The amount of phenol formed was calculated using molar extinction coefficient (1310 M⁻¹cm⁻¹) for phenol. One unit of arylesterase activity was defined as 1 µmol of phenol generated/min at a pH of 8.0 and at 25°C. The activity is expressed as kU/L. Paraoxon, phenylacetate, calcium chloride, and sodium chloride were purchased through local suppliers from Sigma-Aldrich (St. Louis, Missouri, United States).

PON1-Q192R phenotyping

PON1-Q192R phenotype distributions were determined using both paraoxonase and arylesterase activity according to the method of Eckerson et al. (1983b). Paraoxon is identified as a distinguishing substrate with a polymorphic distribution of activity, and phenylacetate is a non-distinguishing substrate for two allozymes. The distribution normality of variables was assessed using Kolmogorov-Smirnov test. Univariate density estimation was performed using R statistical software (R 3.4.2). Each variable was fitted a mixture of normal distributions with model-based clustering.

Statistical analysis

Statistical analysis was performed using MINITAB16 software (MINITAB inc., State College, PA, USA). A p value < 0.05 was considered as statistically significant. All values were presented as mean ± SD. The range of each variable was calculated by dividing the lowest observed value from the highest observed value. The significance of differences between groups was assessed by using independent Students’ t-test, Mann Whitney test and ANOVA. The significance of association between variables was evaluated by using chi-square test.

RESULTS AND DISCUSSION

The cohort comprised 55.8 % females and 44.2 % males. Mean age of the participants was 57.82 ± 16.38 years. There was no significant difference in age between males and females. The mean basal paraoxonase activity was 205.27 ± 115.00 U/L, ranging from 34.54 U/L to 628.30 U/L (Figure 1). The mean salt stimulated PON1 activity was 320.4 ± 218.9 U/L, ranging from 50.3 U/L to 1233.6 U/L (Figure 2). The mean arylesterase activity was 159.53 ± 37.11 kU/L, ranging from 56.2 kU/L to 253.9 kU/L (Figure 4). The range for basal PON1 activity, salt stimulated activity, and arylesterase activity among the population was 593.8, 118.3, and 179.7 respectively. The basal paraoxonase (Figure 1) and salt stimulated paraoxonase (Figure 2) activities were bimodally distributed, whereas arylesterase activity (Figure 4) was unimodally distributed. Degree of stimulation of PON1 activity by 1 M NaCl was normally distributed (Figure 3).

Figure 1: Estimated density curve of basal paraoxonase activity in healthy individuals
The addition of NaCl caused approximately $1.52 \pm 0.44$ fold increment in PON1 activity in the range of 0.55 to 2.72. However, the addition of NaCl also caused an inhibition of PON1 activity in twenty-three individuals, ranging from 0.63% to 45.4%.

Eckerson et al. (1983a) also reported that the degree of salt stimulation separated individuals into two very discrete classes; one stimulated -23% to 35% (the non-salt responsive type), and the other stimulated 60% and greater (the salt-responsive type).

Frequency distribution histograms for percent stimulation of paraoxonase activity by 1.0 M NaCl was obtained by Eckerson et al. (1983a), and the present study included both non-salt responsive and salt-responsive types, which implies that both attributes to salt response should be considered for phenotype categorisation.

We observed the relationship between age and salt responsiveness of an individual. The number of individuals whose PON1 activity was inhibited by the addition of NaCl was higher in the younger age group (18–54 years) than in the older age group (54–90 years). The number of individuals whose PON1 activity was stimulated by the addition of NaCl was higher in the older age group (54–90 years) than in the younger age group (18–54 years) ($p = 0.005$) (Table 1).

| Age group (years) | Number of individuals whose PON1 activity was inhibited by NaCl | Number of individuals whose PON1 activity was stimulated by NaCl |
|-------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| 18–54             | 17                                                            | 48                                                            |
| 54–90             | 6                                                             | 66                                                            |

Table 1: Number of individuals whose PON1 activity was inhibited / stimulated by addition of 1M NaCl according to age

There were no significant differences in basal and salt stimulated PON1 activity and P/A ratio between males and females. Arylesterase activity was significantly higher in females than in males (Table 2).

| Parameters                          | Female (n=87) | Male (n=50) | p value |
|-------------------------------------|---------------|-------------|---------|
| Basal paraoxonase activity (U/L)   | $212.3 \pm 116.7$ | $193.1 \pm 112.1$ | 0.344   |
| Salt stimulated PON1 activity (U/L)| $331.2 \pm 226.7$ | $301.6 \pm 205.4$ | 0.436   |
| Arylesterase activity (kU/L)*       | $168.2 \pm 39.9$ | $148.5 \pm 30.3$ | 0.029   |
| P/A ratio                           | $2.07 \pm 1.18$ | $1.79 \pm 1.23$ | 0.059   |

p value < 0.05. * Significantly different between males and females
The ratio of salt stimulated paraoxonase activity to arylesterase activity disclosed a trimodal distribution in the studied cohort (Figure 5). The three modes corresponded to QQ, QR and RR phenotypes. Subjects with salt stimulated PON1 activity to arylesterase activity ratio < 1.5 were classified as the QQ group (homozygous low activity) (n = 28), those with ratios between 1.5 and 3.5 as the QR group (heterozygous moderate activity) (n = 39), and those with ratios > 3.5 (n = 10) as the RR group (homozygous high activity). The percentage distribution of QQ, QR and RR phenotypes were 36 %, 51 % and 13 %, respectively.

Means of basal PON1 activity (p < 0.001), salt stimulated PON1 activity (p < 0.001), and P/A ratio (p < 0.001) were significantly different among the 3 phenotypes (Table 3). The above three parameters were in the order of QQ < QR < RR phenotypes. Mean arylesterase activity was significantly higher in QQ phenotype compared to QR phenotype (p = 0.034). There were no significant differences in mean arylesterase activity between QR and RR phenotypes and QQ and RR phenotypes. Mean basal PON1 activity of RR phenotype was 3-fold higher than the mean basal PON1 activity of QQ and mean salt stimulated PON1 activity of RR phenotype was 5 fold higher than the mean salt stimulated PON1 activity of QQ.

Table 4 depicts the differences in mean PON1 and arylesterase activities between males and females, according to PON1 phenotypes. Arylesterase activity was significantly higher in females than in males in QQ phenotype. P/A ratio was significantly higher in males compared to females in QQ phenotype. Basal PON 1 activity, salt stimulated PON1 activity and arylesterase activity was significantly higher in females compared to males in QR phenotype. Significant differences were not observed in all four parameters between males and females in RR phenotype.

**Table 3:** Paraoxonase and arylesterase activities in healthy individuals according to phenotype

| Parameter                  | Phenotype | QQ (n=28)   | QR (n=39)   | RR (n=10)   |
|----------------------------|-----------|-------------|-------------|-------------|
| Basal PON1 activity (U/L)  |           | ± 115.3     | ± 218.0     | ± 382.3     |
| Salt stimulated PON1 activity (U/L) | ± 76.2 | ± 78.0     | ± 113.3     |
| Arylesterase activity (kU/L) |           | ± 126.6     | ± 344.4     | ± 670.2     |
| P/A ratio                 |           | ± 173.7     | ± 150.1     | ± 156.8     |

1, 2, 3 Significantly different among phenotypes p < 0.001; a, b different superscript in the row for Arylesterase activity indicate significantly different, p = 0.034

PON1 is an antioxidant enzyme with paraoxonase, esterase and lactonase activity. PON1 is involved in metabolism of many drugs containing lactone or cyclic carbonate moieties. However, natural substrate of PON1 inside human body is still unclear (Draganov & La Du, 2004). Numerous studies have revealed associations between PON1 activity and various disease conditions (Aldonza et al., 2017; Ertürk et al., 2017; Wei et al., 2017; Passaro et al., 2018; Moreira et al., 2019; Matsumoto et al., 2020; Murillo-González et al., 2020). Determination of PON1 activity and phenotypic distribution in healthy individuals may facilitate the unveiling of any possible relationships between PON1 activity and/or phenotypic distribution and particular disease conditions. Identification of relationships between PON1 activity/polymorphism and disease conditions may improve the existing knowledge on pathophysiology of disease conditions. The ability of paraoxonase in destroying biologically active lipids in mildly oxidised LDL was first documented by Watson et al. (1955). The inhibitory effect of PON on HDL oxidation was established based on the reduced HDL peroxide and aldehyde formation (Aviram et al., 1998). Since then, numerous studies have been carried out on the cardio-protective role of PON. A recent review concluded that the true physiological substrates for PON are still not known (Taler-Verčič et al., 2020). As such, further studies on the enzymes’ molecular mechanism may help to identify possible natural substrates for PON1. Modulation of PON1 activity by pharmacological, environmental and behavioural interventions may exert a beneficial therapeutic approach to hinder the detrimental effects of certain diseases, which exhibit an association with serum levels of PON1 within the body.
Table 4: Paraoxonase and arylesterase activities according to phenotypes and gender

| Parameter                  | Phenotype | Male       | Female       | p value |
|----------------------------|-----------|------------|--------------|---------|
| Basal PON1 activity (U/L)  | QQ        | 104.9 ± 46.6 | 129.2 ± 104.4 | 0.926   |
|                            | QR        | 182.5 ± 42.5 | 235.8 ± 86.0  | 0.030   |
|                            | RR        | 347.2 ± 94.0 | 417.4 ± 130.2 | 0.531   |
| Salt stimulated PON1 activity (U/L) | QQ | 122.4 ± 51.4 | 132.1 ± 65.1 | 0.871   |
|                            | QR        | 296.0 ± 52.5 | 368.7 ± 117.0 | 0.031   |
|                            | RR        | 597.1 ± 139.6| 743 ± 297     | 0.676   |
| Arylesterase activity (kU/L) | QQ      | 161.44 ± 33.78| 190 ± 55     | 0.039   |
|                            | QR        | 134.02 ± 21.06| 158.12 ± 37.91| < 0.001 |
|                            | RR        | 144.9 ± 24.2  | 168.7 ± 48.3  | 0.403   |
| P/A ratio                  | QQ        | 0.72 ± 0.21   | 0.62 ± 0.16   | 0.048   |
|                            | QR        | 2.22 ± 0.3    | 2.31 ± 0.48   | 0.743   |
|                            | RR        | 4.10 ± 0.51   | 4.31 ± 0.48   | 0.531   |

Values are represented as mean ± SD. 1,2,3,4: Significantly different between males and females

In this study, salt stimulated PON1 and arylesterase activities were measured to determine the phenotypic distribution of PON1 in a cohort of healthy Sri Lankan individuals. Based on the salt-stimulated PON1 to arylesterase activity ratio, it was possible to distinguish the three paraoxonase phenotypes (QQ, QR and RR). The percentage distribution of QQ, QR and RR phenotypes in our cohort was 36 %, 51 % and 13 % respectively. The findings were compared with the results from a Croatian cohort, where 39 %, 48 % and 13 % individuals belonged to QQ, QR and RR phenotypes, respectively (Juretiæ et al., 2001). Close values were observed in another study conducted in Iran, in which frequencies of QQ, QR and RR phenotypes were reported to be 48.1 %, 41.3 % and 10.6 %, respectively (Sepahvand et al., 2007). The genotype frequencies for paraoxonase 1-Q192R were reported as 47 % (QQ), 41 % (QR) and 12 % (RR) in a healthy population of Khorramabad, Iran by Chehari et al. (2014), which was almost similar to that of reported by Sepahvand et al. (2007). Contrasting results were observed in a Thai population with 14.4 %, 51.9 % and 33.7 % of individuals belonging to QQ, QR and R, respectively (Porntadavity et al., 2009).

Mean PON1 activity of the studied Sri Lankan cohort was found to be 205.27 ± 115.00 U/L ranging from 34.54 U/L to 628.30 U/L. Basal PON1 activity exhibited close resemblance to values reported in a Croatian population 

(251 ± 143 U/L) [Juretiæ et al., 2001], with an interquartile range of 236 and a Thai populations 239.7 ± 83.9 U/L [Porntadavity et al., 2009]. Mean basal paraoxonase activity in the present study was considerably higher compared to those observed in many other populations including Iran [81.8 ± 57.0 U/L, ranging from 19.2 to 290 U/L] (Sepahvand et al., 2007) and 98.79 ± 68.79 U/L (Naderi et al., 2011), Czech Republic [125.2 ± 69.4 U/L (Novak et al., 2010)], Bulgaria [128.79 ± 15.1 U/L (Döneva-Basheva et al., 2013)], Hungary [188 ± 55 U/L (Paragh et al., 2002)] and Turkey [178 ± 79 U/L (Dirican et al., 2004)]. A study conducted by Elkiran et al. (2007) in Turkey revealed higher value (395.8 ± 116.6 U/L) than our finding for basal PON1 activity.

We observed significantly higher arylesterase activities in females than in males. However, opposite results were reported in a Thai study (Female: 137.0 ± 25.7 vs. Male: 160.0 ± 37.9) (Porntadavity et al., 2009), and no differences were reported in an Iranian study (Female: 79.8 ± 11.9 vs. Male: 81.6 ± 17) (Sepahvand et al., 2007). The significant differences among the three phenotypes in basal PON1 activity, salt stimulated PON1 activity and P/A ratio in our study was similar to the observation made on the three phenotypes in Thai study (Porntadavity et al., 2009). This should be reworded as ‘The range observed in basal PON1 activity and salt stimulated PON1 activity in our population was higher than that of
Thai population with basal PON1 activity of 384.5 U/L and salt stimulated PON1 activity of 1022.2 U/L (Porntadavity et al., 2009), whereas range in arylesterase activity was similar in both populations. Salt responsiveness of PON1 in Thai population (2.3 fold) (Porntadavity et al., 2009) was moderately higher than our population (1.52 ± 0.44). In our study, we found significantly higher arylesterase activity in females compared to males in the QQ phenotype. The P/A ratio was significantly higher in males compared to females in the QQ phenotype. Mean arylesterase activity was significantly higher in males (176.2 ± 41.3) than in females (146.4 ± 25.0) in the QQ phenotype in the Thai population. We observed significantly higher basal PON1 activity, salt stimulated PON1 activity and arylesterase activity in females compared to males in the QR phenotype. However, significantly higher mean salt stimulated PON1 activity (560.5 ± 157.5 Vs 469.4 ± 113.8) and mean arylesterase activity (171.8 ± 41.1Vs 136.4 ± 28.4) were observed in males than females in QR phenotype in the Thai population. Hence, there are contrasting differences in PON and arylesterase activities among the phenotypes in different populations.

The ability of PON1 to hydrolyse organophosphates varies depending on the PON1 phenotype. Previous studies have revealed that the QR and RR phenotypes are significantly more resistant to OP toxicity compared to the QQ phenotype (Sepahvand et al., 2007). Hence, we can suggest that 64 % of our population exhibits a resistance against OP toxicity. Sri Lanka is an agricultural country which uses considerable amounts of OP substances as pesticides and herbicides. Therefore, people may have genetically adapted to resist toxic effects of OP substances. Studies assessing the correlation between PON1 phenotype and atherosclerosis risk have revealed that QQ phenotype is more cardio protective compared to other two phenotypes (Sepahvand et al., 2007). According to the results of our study we can hypothesise that majority of our population doesn’t have this protective effect and may be susceptible to develop atherosclerosis. A study is in progress with acute coronary syndrome to verify our hypothesis.

CONCLUSION

In this study, basal and salt stimulated paraoxonase activities were bimodally distributed. Arylesterase activity was unimodally distributed. Salt stimulated paraoxonase activity/ arylesterase activity ratio was trimodally distributed. The three modes corresponded to QQ (lower activity), QR (intermediate activity) and RR (higher activity) phenotypes. The percentage distribution of QQ, QR and RR phenotypes were 36 %, 51 % and 13 %, respectively. This study has set the baseline data on phenotypic distribution of paraoxonase1 in a cohort of healthy Sri Lankan individuals. The findings may contribute in future studies involving assessment of PON1 activity and/or polymorphism in various disease conditions.

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