Addressing the link between paraoxonase-1 gene variants and the incidence of early onset myocardial infarction

Mohamed F. Abdel Rahman, Ingy M. Hashad, Khaled Abou-Aisha, Sahar M. Abdel-Maksoud, Mohamed Z. Gad

Clinical Biochemistry Unit, Faculty of Pharmacy and Biotechnology, German University, Cairo, Egypt

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Corresponding author:
Prof. Dr. Mohamed Z. Gad
Biochemistry Department
Faculty of Pharmacy
and Biotechnology
German University in Cairo
New Cairo City – 11835
Al Tagamoa Al Khames
Cairo, Egypt
Phone: +202-27590717
Mobile: +20100-1429689
Fax: +202-27581041
E-mail:
mohamed.gad@guc.edu.eg

Abstract

Introduction: The enzyme paraoxonase-1 (PON1) represents an endogenous defense mechanism against vascular oxidative stress, thereby contributing to the prevention of atherosclerosis. Several polymorphisms have been reported in the PON1 gene, including Q192R. PON1 phenotype is commonly expressed as the paraoxonase/arylesterase ratio (PON/ARE). The major aim of this study was to investigate the association between PON1 Q192R polymorphism, PON1 phenotypes and the incidence of early-onset acute myocardial infarction (AMI) in Egyptians.

Material and methods: The study subjects consisted of 102 AMI patients and 72 age-matched healthy controls. Genotyping and enzyme activities were determined using PCR-RFLP and kinetic spectrophotometric assays, respectively.

Results: The genotype distribution for the PON1 gene was significantly different between AMI patients (QQ = 38.24%, QR = 49.02%, RR = 12.75%) and controls (QQ = 66.67%, QR = 25%, RR = 8.33%). Allele frequencies were also significantly different between patients (Q = 62.75%, R = 37.25%) and controls (Q = 79.17%, R = 20.83%). The genotypes QR and RR showed higher risk for AMI compared to the homozygous QQ (odds ratio (OR) = 3.231, $p < 0.001$). The average PON/ARE ratio in MI patients (1.187 ±0.1) did not differ significantly from controls (1.118 ±0.26). However, it showed a significant difference among different genotypes in both AMI patients (QQ = 0.91 ±0.11, QR = 1.09 ±0.11 and RR = 2.65 ±0.4) ($p = 0.0002$) and controls (QQ = 0.68 ±0.1, QR = 1.07 ±0.11 and RR = 4.89 ±2.84) ($p < 0.0001$).

Conclusions: PON1 192R allele represents an independent risk factor for early-onset AMI in Egyptians, and PON1 Q192R polymorphism modulates the paraoxonase phenotype.

Key words: paraoxonase, arylesterase, PON polymorphism, acute myocardial infarction, Egyptians.

Introduction

Myocardial infarction (MI) is a complex multifactorial polygenic disorder that is thought to result from an interaction between a person's genetic makeup and various environmental factors [1]. In general, there are several conventional risk factors for occurrence of MI including hypertension, diabetes mellitus, and hypercholesterolemia. Although each

risk factor is partly under genetic control, a family history of MI is also an independent predictor, suggesting the existence of additional susceptibility genes for this condition [2]. Furthermore, some patients who have had a myocardial infarction do not have any conventional risk factors, suggesting the contribution of an uncharacterized genetic component [3].

Human serum paraoxonase (PON) was first identified as a protective barrier against organophosphorus poisoning [4]. The PON gene family in mammals includes at least 3 members: PON1, PON2, and PON3 [5, 6]. The three PON genes share about 65% similarity at the amino acid level and are located adjacent to each other on chromosome 7 (7q21.3) in humans [7]. Both PON2 and PON3 possess antioxidant properties and lactonase activity, but unlike PON1, they lack paraoxon or phenyl acetate-hydrolyzing activity [8].

The PON 1 (EC.3.1.8.1, aryldialkylphosphatase) is synthesized in the liver and is closely associated with high-density lipoprotein (HDL). This most likely explains its ability to metabolize lipid peroxides and to protect against their accumulation on low-density lipoprotein (LDL) [9]. The serum concentration of HDL has long been known to show an inverse correlation with the development of atherosclerosis. Some studies have demonstrated decreased serum PON1 activity in survivors of myocardial infarction [10, 11].

The PON1 gene has 2 common polymorphisms in the coding region, which lead to a glutamine-to-arginine substitution at position 575A>G (PON1 Q192R) and a leucine to-methionine substitution at position 163T>A (PON1 L55M). Both independently influence PON1 activity and have been referred to as the molecular basis for this inter-individual variability [12]. Genetic polymorphisms that affect PON1 could be predisposing risk factors both in environmental toxicology and in cardiovascular diseases. The PON1 polymorphisms are important in determining the capacity of HDL to protect LDL against oxidative modification in vitro, which may explain the relationship between the PON1 alleles and coronary heart disease in case-control studies [13]. However, it was suggested that PON1 phenotype (enzyme activity) is a better predictor of vascular disease than PON1 genotype [14].

The PON1 192 activity polymorphism is substrate dependent. It was reported by some studies that the PON1 R192 isoform has higher activity for the hydrolysis of paraoxon [15], while both isoforms hydrolyze phenylacetate at approximately the same rate [16]. The PON1 phenotype is expressed as the paraoxonase/arylesterase ratio (PON/ARE) as the arylesterase activity is generally accepted to be a measure of PON1 serum enzyme level [17].

The aim of the current study was to investigate the association between the PON1 Q192R polymorphism and early onset acute myocardial infarction (AMI) and to correlate PON/ARE with AMI in the male Egyptian population.

Material and methods

Study population

One hundred and two random unrelated AMI patients, divided into 36 women (age range 34 to 55 years) and 66 men (age range 35 to 55 years) were recruited from the intensive care unit of the National Heart Institute, Imbaba, Giza. Seventy-two random unrelated healthy controls were recruited for the study from the volunteers attending the blood bank at 57357 Hospital in Cairo, Egypt. Out of the controls, 13 were women, aged between 26 and 42 years, and 59 were men, 25 to 55 years of age.

Patients were included if they had a diagnosis of an acute single or multi-vessel coronary artery disease (CAD) verified by clinical presentation, ECG changes, and/or elevation of cardiac markers. Written informed consent was obtained from each participant in the study that abided by the Helsinki declaration. Information on personal and family medical history and health-relevant behaviors, including exercise and diet, was obtained by a routine questionnaire filled in by blood donors at the time of venesection. Exclusion criteria for both patients and controls included any concomitant acute or chronic severe diseases such as renal failure, hepatic insufficiency, cardiovascular disease other than MI, and diabetes mellitus.

Specimen collection

Fasting blood samples (4 ml) were collected into 2 sets of tubes; the first set consisted of EDTA-coated vacuum tubes stored at 4°C for DNA extraction. The second set consisted of non-EDTA-coated vacuum tubes, which were centrifuged at 1000 rpm for 10 min, and the resulting serum was separated and stored at –20°C in 0.25 ml aliquots. The aliquots were used for determination of PON and ARE activities.

Determination of the PON1 Q192R polymorphism

Genomic DNA was prepared from blood leukocytes by the QIAamp DNA Mini Kit (QIAGEN). Determination of the PON1 Q192R polymorphism was carried out by PCR followed by restriction digestion (PCR-RFLP, restriction fragment length polymorphism). The primers used were: forward: 5'-TGTTCCATTATAGCTAGCACGA-3' and reverse: 5'-TTTCACCCCCTGAAAAATTA-3'. The resulting PCR

product size was 496 bp. The nucleotide substitution corresponding to position 192 (Q/R) creates an AlwI (New England Biolabs) restriction site. Digestion resulted in 287- and 209-bp fragments for the R allele (Figure 1) [18].

Assay of serum PON activity

The PON activity was determined using paraoxon as a substrate and measured by increases in the absorbance at 412 nm due to the formation of 4-nitrophenol. The activity was measured at 25°C by adding 50 µl of serum to 1 ml of Tris-HCl buffer (100 mM at pH 8.0) containing 2 mM $CaCl_2$ and 5 mM paraoxon. The rate of generation of 4-nitrophenol was determined at 412 nm. Enzymatic activity was calculated by using the molar extinction coefficient 17 100 M^{-1} cm^{-1} [19]. One unit (U) of paraoxonase activity produced 1 µmol of *p*-nitrophenol per min [20].

Assay of serum ARE activity

ARE activity was measured using phenylacetate as a substrate. Serum was diluted 400-fold in 100 mM Tris-HCl buffer, pH = 8.0. The reaction mixture contained 2.0 mM phenylacetate and 2.0 mM $CaCl_2$ in 100 mM Tris-HCl buffer, pH = 8.0. Initial rates of hydrolysis were determined by following the increase of phenol concentration at 270 nm at 25°C [19]. One unit (U) of arylesterase activity is equal to 1 µmol of phenyl acetate per minute [21].

Assay of lipid parameters

Serum oxidized LDL (ox-LDL) was determined by quantitative enzyme-linked immunosorbent assay (ELISA) using a commercial kit provided by Immundiagnostik, Germany [22]. Serum triacylglycerols (TG) and total cholesterol (TC) were determined using an enzymatic colorimetric assay according to the methods of Wahlefeld and Richmond, respectively, using diagnostic kits provided by Stanbio Laboratory, USA [23, 24].

Data processing and statistical analysis

All statistical analysis was performed using the statistical programs SPSS and GraphPad Prism. Data are represented as mean ± SEM. To compare differences between groups, odds ratio, Student *t*-test and one-way analysis of variance (ANOVA) were used. A two-tailed p value ≤ 0.05 was considered statistically significant.

Results

Genotyping of PON1 Q192R polymorphism

The genotype distribution for the PON1 gene was significantly different between AMI patients (QQ = 38.24%, QR = 49.02% and RR = 12.75%) and controls (QQ = 66.67%, QR = 25%, RR = 8.33%) (Mann-Whitney test, $p = 0.0006$) (Figure 2). The corresponding allele frequencies were significant as well; patients were Q = 62.75% and R = 37.25% and controls Q = 79.17% and R = 20.83% (Mann-Whitney test, $p = 0.0011$) (Figure 3). The odd ratio between QQ genotype and QR + RR genotypes was 3.231 ($p < 0.001$), while the odd ratio between the Q allele and the R allele was 2.256 ($p = 0.001$).

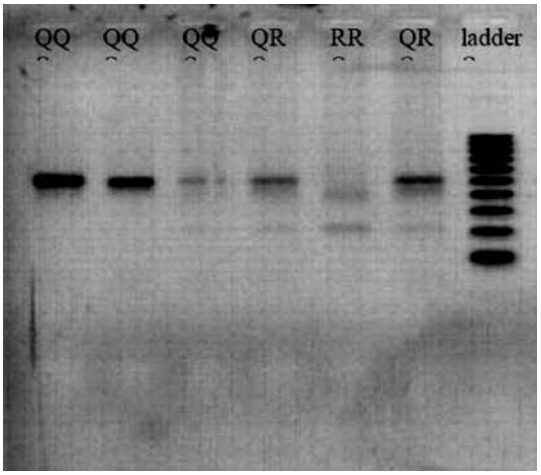

Figure 1. Possible Q192R polymorphism outcomes

PON phenotyping

No significant difference was observed (Student *t*-test; $p = 0.7413$) between the PON activities in AMI patients (82.71 ±7.15 U/ml) (mean ± SEM) and controls (86.44 ±8.88 U/ml). PON/ARE ratio in MI patients (1.187 ±0.1) also did not differ significantly (Student *t*-test; $p = 0.7851$) from controls (1.118 ±0.26).

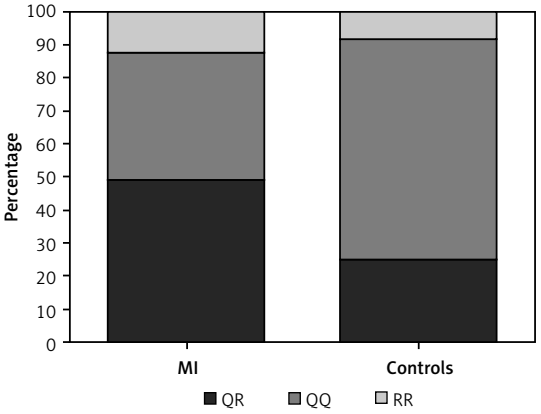

Figure 2. The PON1 genotype distribution in MI and controls. The genotype distribution for the PON1 gene was significantly different between AMI patients (QQ = 38.24%, QR = 49.02% and RR = 12.75%) and controls (QQ = 66.67%, QR = 25%, RR = 8.33%) (Mann-Whitney test, $p = 0.0006$)

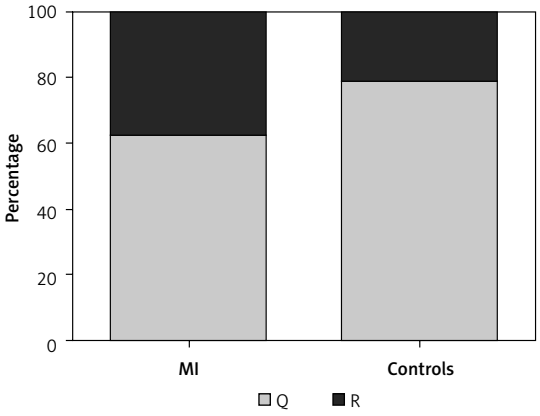

Figure 3. The PON1 allele frequencies in MI and controls. The allele frequencies were significantly different between AMI patients (Q = 62.75% and R = 37.25%) and controls (Q = 79.17% and R = 20.83%) (Mann-Whitney test, $p = 0.0011$)

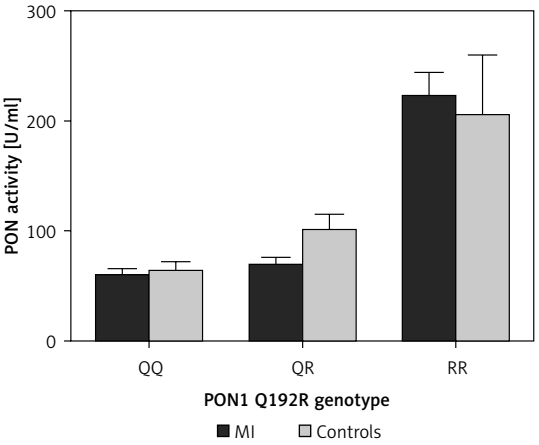

Figure 4. The PON activities among different genotypes in MI patients and controls. There was a significant difference in the PON activities among the different genotypes in both AMI patients (QQ = 60.88 ±6.36, QR = 69.66 ±6.56 and RR = 222.7 ±20.75 U/ml) (Kruskal-Wallis; $p < 0.0001$) and control subjects (QQ = 65.58 ±7.9, QR = 101.4 ±14.15 and RR = 206.6 ±53.65 U/ml) (Kruskal-Wallis; $p = 0.0025$)

Correlation of PON1 genotypes with PON/ARE ratio in control and AMI subjects

There was a significant difference in the PON activities among the different genotypes in both AMI patients (QQ = 60.88 ±6.36, QR = 69.66 ±6.56 and RR = 222.7 ±20.75 U/ml) (Kruskal-Wallis; $p < 0.0001$) and control subjects (QQ = 65.58 ±7.9, QR = 101.4 ±14.15 and RR = 206.6 ±53.65 U/ml) (Kruskal-Wallis; $p = 0.0025$) (Figure 4). No significant difference was observed in the ARE activities among the different genotypes in both AMI patients (QQ = 79.56 ±6.62, QR = 69.08 ±4.73 and RR = 95.2 ±11.38 U/ml) (Kruskal-Wallis; $p = 0.1170$) and control subjects (QQ = 106.9 ±6.86, QR = 101.5 ±10.54 and RR = 86.28 ±31.62 U/ml) (Kruskal-Wallis; $p = 0.8092$) (Figure 5). On the other hand, PON/ARE ratios showed a significant difference among different genotypes in both AMI patients (QQ = 0.91 ±0.11, QR = 1.09 ±0.11 and RR = 2.65 ±0.4) (Kruskal-Wallis; $p = 0.0002$) and control subjects (QQ = 0.68 ±0.1, QR = 1.07 ±0.11 and RR = 4.89 ±2.84) (Kruskal-Wallis; $p < 0.0001$) (Figure 6).

Results of lipid profile

A summary of lipid profile data in AMI and controls is shown in Table I. A highly significant dif-

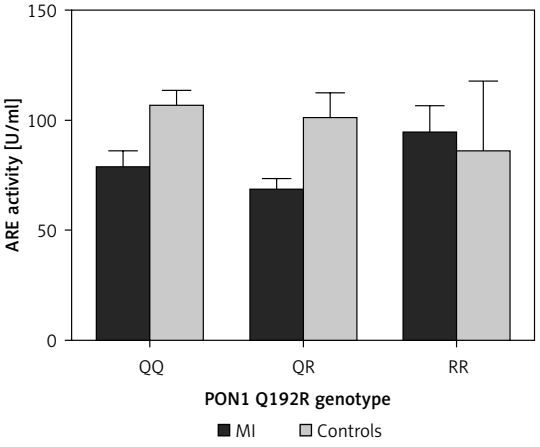

Figure 5. The ARE activities among different genotypes in MI patients and controls. No significant difference was observed in the ARE activities among the different genotypes in both AMI patients (QQ = 79.56 ±6.62, QR = 69.08 ±4.73 and RR = 95.2 ±11.38 U/ml) (Kruskal-Wallis; $p = 0.1170$) and control subjects (QQ = 106.9 ±6.86, QR = 101.5 ±10.54 and RR = 86.28 ±31.62 U/ml) (Kruskal-Wallis; $p = 0.8092$)

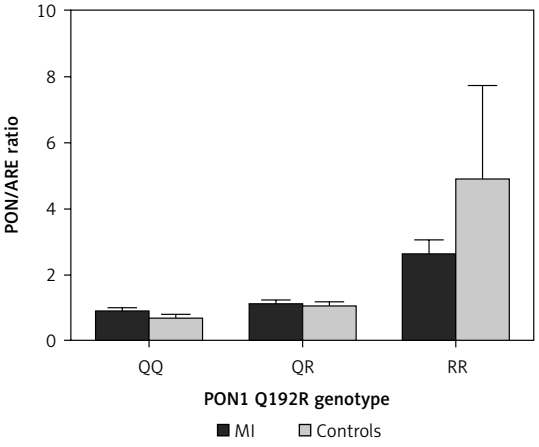

Figure 6. PON/ARE ratios among different genotypes in MI patients and controls. PON/ARE ratios showed a significant difference among different genotypes in both AMI patients (QQ = 0.91 ±0.11, QR = 1.09 ±0.11 and RR = 2.65 ±0.4) (Kruskal-Wallis; $p = 0.0002$) and control subjects (QQ = 0.68 ±0.1, QR = 1.07 ±0.11 and RR = 4.89 ±2.84) (Kruskal-Wallis; $p < 0.0001$)

ference was observed in the levels of ox-LDL and TG in AMI relative to those of controls. However, TC was not significantly affected, as most patients were on hypocholesterolemic therapy. The significant difference in ox-LDL and TG in AMI patients was not reflected in noticeable differences among the different PON genotypes (Table II).

Table I. Summary of lipid profile data in study groups

Parameter	AMI	Controls
Total cholesterol [mg/dl]	208.6 ±6.20	219.7 ±9.07
Total triglycerides [mg/dl]	162.1 ±7.89*	110.8 ±7.57
Ox-LDL [ng/ml]	203.4 ±21.33*	113.5 ±4.9

**Significant difference was observed; p < 0.0001.*

Discussion

Genotyping of PON1 Q192R polymorphism

Many reports have provided evidence that PON1 has a protective role from cardiovascular disease due to its ability to inhibit LDL oxidation *in vitro*. The role of PON1 in vascular protection was supported by knockout and transgenic mouse studies. While knockout mice lacking serum PON1 have an increased susceptibility to atherosclerosis [25], human PON1 transgenic mice with 2- to 4-fold increased PON1 levels have reduced susceptibility [26]. The SREBP-2 (an important cholesterol transcriptional regulator) present in the region from bp –104 to –95 in the proximal PON1 promoter suggests an important PON1-lipoprotein relationship, as SREBP-2 was found to increase PON1 promoter activity in a dose-dependent manner, indicating an additional lipid-related mechanism of PON1 activation [27].

The two most common PON1 coding region polymorphisms are 192 Gln(Q)/Arg(R) and 55 Leu(L)/Met(M). Recently, several studies aimed to explore the relationship between PON1 polymorphisms and the risk of coronary artery disease.

This study investigated the clinical significance of PON1 Q192R polymorphism in CAD in an Egyptian population. The study results showed that subjects having genotypes QR + RR have a significantly higher risk for development of AMI than QQ subjects, suggesting that the PON1 R allozyme is less efficient at retarding the oxidation of LDL than is the Q allozyme. These results are supported by Mackness *et al.*, who observed decreased hydrolysis of lipid peroxides by the R allozyme [28]. The R allele frequency among our studied AMI patients was also significantly different from control subjects.

These results are consistent with a meta-analysis carried out in 2001 which demonstrated an increase in the frequency of the PON1-192R allele in coronary heart disease (CHD) patients [13]. The results were also consistent with several studies carried out in Euro- and Afro-Brazilian [29], Indian [30, 31], Turkish [32, 33], and Polish populations [34] and also with another study performed on the Egyptian population [35]. All these studies showed that genotype distributions and allele frequencies of the PON1 Q192R polymorphism were significantly different between control subjects and AMI patients.

However, our study results were in contrast with others done on Japanese [36], British [13], German [37], Portuguese [38], American [39], Brazilian [40], Turkish [41–43] and Finnish [44] populations which showed that genotype distributions and allele frequencies of the PON1 Q192R polymorphism were not significantly different between control subjects and AMI patients. The inconsistent association of PON1 with CAD may be attributed to the variable factors among studies including ethnicity, environmental factors, sampling scheme and trial size.

PON phenotyping

The results showed that the PON activities among AMI patients were slightly lower than PON activities in control subjects; however, the difference was not significant. The difference in PON/ARE ratios was not significant as well. So far there have been only a few studies that have assessed PON1 activity in CAD. These studies showed that the PON activities [11, 45, 46] and ARE activities [11, 46] are significantly lower in AMI patients than control subjects. Our results are in harmony with these studies in ARE results; however, the insignificant difference in the PON activities in the current study might be explained by the relatively young age of our study subjects compared to oth-

Table II. Summary for the total cholesterol, total triglycerides and oxidized LDL (ox-LDL) in different variants of PON1 Q192R polymorphism in AMI patients

AMI	QQ	QR	RR	Value of *p*
Total cholesterol [mg/dl]	211.8 ±11.74	205.3 ±7.64	211.0 ±15.57	0.7968
Total triglycerides [mg/dl]	148.6 ±11.65	174.3 ±11.73	159.3 ±24.58	0.3420
Ox-LDL [ng/ml]	189.9 ±34.02	196.9 ±27.68	208.7 ±86.57	0.8515

er studies. Animal studies also provide evidence on the inverse relationship between age and PON1 activity [47]. We do not exclude the possibility of having a significant difference in PON activities if a larger number of study subjects were included.

Correlation of PON1 genotypes with PON/ARE ratio in control and AMI subjects

In the current study both patients and controls with RR genotype had the highest serum paraoxonase activities, followed by QR then QQ. The difference in PON activities was significant in the AMI patients as well as the control subjects; however, the arylesterase activities of paraoxonase enzyme did not change significantly among different PON1 Q192R variants either in the AMI patients or in the control subjects. This was reflected in the PON/ARE ratios, which varied significantly with the PON1 Q192R polymorphism in both study groups. These results indicated that PON1 Q192R polymorphism modulates the PON activity without affecting the ARE activity of the enzyme. The results are consistent with previous studies that investigated the relationship between the PON1 Q192R polymorphism and the phenotype of the enzyme [14–17]. Mackness *et al.* [48] suggested that the polymorphism does not appear to affect the synthesis and secretion or clearance, but might have an effect on the protein conformation or binding to HDL altering its substrate specificity.

Demonstration of 192RR as a risk factor for AMI seems contradictory to its high paraoxonase activity. Despite the familiarity of the use of paraoxon as a substrate to assay for PON activity, the direct relationship between the hydrolytic activity of PON1 against organophosphate substrates such as paraoxon and the protection against LDL oxidation is still not fully understood. While the organophosphate hydrolytic activity of PON1 is highly calcium dependent, it was found that the sulfhydryl group at amino acid position 283, not calcium, is essential to prevent the accumulation of lipid peroxides [49]. This suggests either that conformational changes in the active site of PON1 take place to shift the enzyme from hydrolytic to antioxidant activity, or that the enzyme possesses two active sites: the antioxidant site, dependent on Cys283, and the hydrolytic site, dependent on calcium. In either case, it cannot be assumed that the capacity to hydrolyze substrates such as paraoxon or phenylacetate necessarily reflects the presence or absence of significant PON1 antioxidant capacity [9].

In conclusion, although it was suggested that the PON phenotype is more important than genotype [14], our results suggest that carrying the PON1 192R allele represents an independent risk factor for early onset AMI in the Egyptian population. Among the limitations of this study is the narrow selection of MI patients with regard to number, sex and age. Further, wider studies are still required to unravel the exact physiological role of PON and the nature of its physiological substrate.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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