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Original Article

Paraoxonase gene polymorphisms: Understanding the biochemical and genetic basis of coronary artery disease

Fathima J. Nasreen, MD [a] and Gayathri Balasubramaniam, MD [b,*]

[a] *Department of Biochemistry, Bioline Laboratories, Coimbatore, India*
[b] *Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore, India*

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الملخص

أهداف البحث: إن مصل باراوكسوناز هو بروتين سكري، مع أنشطة مضادة للتأكسد ومضادة لتصلب الشرايين. يعتبر تعدد الأشكال الجيني غلوتامين192أرجينين وتعدد الأشكال الجيني ليوسين55 مثيونين لمصل باراوكسوناز أحد عوامل الخطر لمرض الشريان التاجي، وهو أقل استكشافا في الهنود الجنوبيين. كنا نهدف إلى تحديد تعدد الأشكال الجيني غلوتامين192أرجينين و تعدد الأشكال الجيني ليوسين55مثيونين لمصل باراوكسوناز في مرضى مرض الشريان التاجي وتقييم النمط الجيني وترددات الأليل لتعدد الأشكال الجيني في مصل باراوكسوناز 1.

طرق البحث: تضمنت هذه الدراسة الاستباقية وضبط الحالات 20 شخصا في كل مجموعة مع مرضى مرض الشريان التاجي مثبتين في تصوير الأوعية الدموية في مجموعة الحالة. تم قياس نشاط مصل باراوكسوناز 1 وتم تحديد تعدد الأشكال الجيني لمصل باراوكسوناز. تم تحليل مصل مصل باراوكسوناز كميا باستخدام مختصر مقايسة الممتز المناعي المرتبط بالإنزيم لمصل باراوكسوناز التابع لشركة راي بايو. تم استخدام اختبار مربع كاي لمعرفة ارتباط الأنماط الجينية بالجنس، وأي أمراض مصاحبة في الحالات والضوابط.

النتائج: كان النوع المتحور من تعدد الأشكال ليوسين55مثيونين شائعا في 50٪ من المرضى الذين أظهروه بينما في غلوتامين192أرجينين أظهر 42.5٪ من المشاركين النمط السوي. لم تظهر قيم مصل باراوكسوناز المتوسطة بين المجموعتين فرقا إحصائيا بينما أظهرت أنشطة مصل باراوكسوناز فرقا إحصائيا بين المجموعتين بقيم أقل في مجموعة الحالة. لم يكن فرق النوع المتحور من تعدد الأشكال ليوسين55مثيونين ذو دلالة إحصائية بين الحالة ومجموعات التحكم بينما كان النوع المتحور من تعدد الأشكال غلوتامين192أرجينين ذو دلالة إحصائية في الحالات مقارنة بالمجموعة الضابطة.

الاستنتاجات: لوحظ انخفاض مستويات مصل باراوكسوناز 1 والبروتين الشحمي المرتفع الكثافة في البلازما، وارتفاع البروتين الشحمي المنخفض الكثافة، وإجمالي مستويات الكوليسترول والدهون الثلاثية في مرضى مرض الشريان التاجي. لوحظ أن النوع المتحور من تعدد الأشكال غلوتامين192أرجينين أعلى في مرضى مرض الشريان التاجي مقارنة بالأفراد الأصحاء.

الكلمات المفتاحية: باراوكسوناز 1؛ مضادات الأكسدة؛ تصلب الشرايين؛ مضاعفات القلب والأوعية الدموية؛ داء السكري من؛ النمط 2

Abstract

Objectives: Serum paraoxonase (PON) is a glycoprotein with antioxidant and anti-atherosclerotic activities. The Q192R and L55M gene polymorphisms of PON have been implicated as risk factors for coronary artery disease (CAD) but have not been extensively explored in South Indians. We aimed to assess the Q192R and L55M genetic polymorphisms of the PON1 gene in participants with CAD, including genotypes and allele frequencies for PON1 gene polymorphism.

Methods: This prospective case-control study involved 20 participants in each group. Patients with angiographically demonstrated CAD were included in the case group. PON1 activity was measured, and PON gene polymorphism was determined. Serum PON was quantitatively analyzed with a RayBio® Human PON1 ELISA kit. Chi square tests were used to assess the association of the genotypes with sex and any comorbidities in cases and controls. A p value ≤ 0.05 was considered significant.

Results: Mutant type L55M polymorphism was observed in 50% of patients, whereas wild type Q192R polymorphism was observed in 42.5% of the participants. The mean PON values between groups did not significantly differ, whereas PON U/L was significantly ($p = 0.001$) lower in the case

* Corresponding address: Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore, India.
E-mail: drgayathrikeerthivasan@gmail.com (G. Balasubramaniam)

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group. The L55M polymorphism did not significantly differ between the case and the control groups ($p = 0.213$), whereas the Q192R polymorphism was statistically significant in cases compared with controls ($p \leq 0.001$)

Conclusion: Low plasma PON1 and HDL levels, and higher LDL, total cholesterol and triglyceride levels were observed in patients with CAD. More patients with CAD than healthy individuals had Q192R polymorphism.

Keywords: Antioxidants; Atherosclerosis; Cardiovascular complications; Paraoxonase 1; Type 2 diabetes

Introduction

Cardiovascular diseases are disorders that involve the heart and the vascular system and include primarily coronary artery disease (CAD).[1] Atherosclerosis is a major cause of CAD development.[1] The World Health Organization has estimated 7 million deaths associated with CAD, more so in developing countries.[2–4]

A key factor in the development of CAD is a low concentration of plasma high density lipoprotein (HDL).[3] HDL has been found to decrease oxidation of low density lipoprotein (LDL). Furthermore, HDL has an additional role in preventing CAD through reverse cholesterol transport, stabilization of atherosclerotic plaques and exertion of anti-inflammatory effects.[5]

Serum paraoxonase (PON) is a glycoprotein with antioxidant and anti-atherosclerotic activities.[6,7] This HDL bound enzyme that rescinds the dynamic lipoperoxide is responsible for the conversion of LDL to atherosclerotic particles as well as oxidation of HDL itself.[6,8]

The PON family comprises three genes—PON1, PON2 and PON3—each encoding a different protein.[9] The PON1 gene initiates cholesterol flow facilitated by HDL. All three PON enzymes inhibit lipid oxidation.[7] PON1 has two forms: the L55M and Q192R polymorphisms.[8] The single nucleotide polymorphisms (SNPs) of PON1 genes are 192QQ and 55LL, which are wild type genotypes, whereas 192QR, 192RR, 55LM and 55MM are mutants.[10] Nearly 200 PON1 SNPs have been identified to date.[11,12]

Some studies have focused on the link between PON1 and CAD, because PON, particularly the Q192R polymorph, has been implicated in vascular pathology.[6,8] Serum concentrations of PON1 are significantly diminished in people with low HDL concentrations, and low serum PON1 is considered an independent risk factor for CAD.[1,5] PON1 is a biomarker under development for CAD whose catalytic efficiency may be regulated by its polymorphs, such as Q192R.[6]

Previous literature has reported ethnic variations in PON1 activity, and several studies have evaluated PON1 activity in northwestern and North Indian populations; however, studies on South Indians are scarce.[12–15]

Hence, we sought to study the PON1 gene polymorphism in the participants with CAD in South India. Our objectives were to assess the Q192R and L55M genetic polymorphisms of the PON1 gene in participants with CAD visiting a tertiary care hospital, and to examine the genotype and allele frequencies of PON1 gene polymorphism (PON1 gene; Q192R and L55M).

Materials and Methods

This was a prospective case-control study conducted in our Department of Biochemistry. All participants provided written consent to participate in this research. Participants above the age of 30 years with confirmed CAD composed the case group, which consisted of 20 participants; 20 participants attending health check-ups formed the control group. Participants with malignancy, known genetic malformation, acute illness, pregnancy, valvular dysfunctions or endocrine abnormalities were excluded. Blood samples of the participants were collected, processed and stored for analysis. Additional data, including height, weight and age, were retrieved from medical records with consent. Histories of both cases and controls, including diseases such as diabetes, hypertension and family history of CAD, were also recorded.

Collection of blood samples

The blood samples of cases and controls were collected in sodium fluoride vacutainers for estimation of fasting and postprandial plasma glucose, or in EDTA vacutainers for estimation of HbA1c and DNA extraction. Red topped serum tubes were used for lipid profiling and estimation of urea and creatinine. The samples for DNA extraction were transferred to sterile cryovials, which were labeled and stored at −80 °C for further use.

The parameters analyzed were plasma glucose, glycosylated hemoglobin, serum cholesterol, triglycerides, HDL, LDL, total bilirubin, direct bilirubin, alkaline phosphatase, serum alanine amino transferase, gamma glutamyl transferase, total serum proteins, serum albumin, creatinine and urea.

PON 1 gene polymorphism analysis

DNA extraction

The blood samples were thawed, and DNA extraction was performed with a Macherey Nagel- Genomic DNA purification NucleoSpin Blood kit. Lysis was achieved by incubation of whole blood in a solution containing large amounts of chaotropic ions in the presence of proteinase K. Appropriate conditions for binding of DNA to the silica membrane of the corresponding NucleoSpin Blood Columns were achieved by addition of ethanol to the lysate. Pure genomic DNA was finally eluted under low ionic strength conditions in a slightly alkaline elution buffer. Polymerase chain reaction (PCR) was performed to amplify the 172 bp L55 M SNP, through a standard PCR procedure with Readymix (R2523 or P4600), forward and reverse primers, DNA extract and nuclease-free water. Sequences of forward and reverse primers used for amplification of polymorphic regions are provided in Table 1. Subsequently, restriction fragment length polymorphism (RFLP) was assessed after digestion with the restriction enzymes AlwIII for the SNP Q192R and NlaIII for the SNP

L55M. In a 50 µL total reaction volume, 1 µg of substrate was digested with enzyme not exceeding 10% of total volume. PCR products of SNP Q192R were digested with AlwIII by incubation for 2 h at 37 °C, and PCR products of SNP L55M were digested with NlaIII by incubation for 1 h and 30 min at 37 °C. The Q192R digestion did not require inactivation, but the L55M digestion reaction was inactivated at 65 °C for 20 min.

Identification of genotypes

Three genotypes were identified for each SNP.

Q192R:

- Restriction did not occur when the A allele was present, thus yielding a fragment of 238 bp.
- Restriction occurred when the G allele was present, thus yielding fragments of 175 bp and 63 bp.
- Restriction occurred when both the A allele and G allele were present, thus yielding fragments of 238 bp, 175 bp and 63 bp.

L55M

- Restriction did not occur when the T allele was present, thus yielding a fragment of 172 bp.
- Restriction occurred when the A allele was present, thus yielding fragments of 106 bp and 66 bp.
- Restriction occurred when both the T allele and A allele were present, thus yielding fragments of 172 bp, 106 bp and 66 bp.

Serum paraoxonase level

A RayBio® Human PON1 ELISA kit was used to perform in vitro enzyme-linked immunosorbent assays for the quantitative measurement of human PON1 in serum. This assay used antibodies specific to human PON1 coated on a 96-well plate. Standards and samples were pipetted into the wells, and PON1 present in samples was bound to the wells by the immobilized antibodies. The wells were washed, and biotinylated antihuman PON1 antibodies were added. After unbound biotinylated antibodies were washed away, HRP conjugated streptavidin was pipetted into the wells. The wells were again washed, a TMB substrate solution was added to the wells, and color developed in proportion to the amount of PON1 bound. Stop solution changed the color from blue to yellow, and the color intensity was measured at 450 nm.

Statistical analysis

Data were analyzed in the statistical software R 4.0.3 and Excel. Continuous variables are represented as median and IQR, and categorical variables are shown in a frequency table. The normality of the data was assessed with the Kolmogorov–Smirnov test. Chi square tests were used to assess associations of genotypes, sex and any comorbidities between cases and controls. Two-sample t test/Welch's t test and Mann Whitney U test were used to compare means of blood parameters between cases and controls. A p-value ≤ 0.05 indicated statistical significance.

Results

The study included 27 male and 13 female participants between 35 and 73 years of age, with a mean age of 55.40 ± 10.04 years. Blood parameters such as creatinine,

Table 1: Forward and reverse primer sequences for amplification of polymorphic regions.

SNP	Forward primer	Reverse primer
L55M	5′-TTTCTGTTCTCTTTTCTGGCAGAAA-3′	5′-GAAAACACTCACAGAGCTAATGAAAAGC-3′
Q192R	5′-GGACCTGAGCACTTTTATGGCA-3′	5′-GACAACATACGACCACGCTAAACC-3′

Table 2: Normality of the variables tested in cases and controls.

Variables	Case Mean (SD)	*p*-value	PON control Mean (SD)	*p*-value
Age (years)	58.95 (9.61)	0.200	51.85 (9.38)	0.200
BMI	27.59 (2.2)	0.200	30.84 (3.86)	0.200
Total cholesterol (mg/dL)	173.25 (49.38)	0.200	216.25 (46.61)	0.200
HDL (mg/dL)	43.3 (8.6)	0.200	41.75 (9.65)	0.200
TGL (mg/dL)	185.3 (59.63)	0.024#	200.15 (43.43)	0.078
LDL (mg/dL)	133.45 (42.58)	0.200	126.15 (23.42)	0.200
Creatinine (mg/dL)	0.96 (0.19)	0.139	1.07 (0.09)	0.003#
Urea (mg/dL)	40.9 (11.85)	0.090	44.25 (6.69)	0.200
Total bilirubin (mg/dL)	1.05 (0.42)	0.001#	1 (0.27)	0.110
SGPT (U/L)	40.2 (13.13)	0.200	58.5 (25.29)	0.023#
SGOT (U/L)	40.2 (7.48)	0.020#	59.45 (22.87)	0.003#
ALP (U/L)	65.15 (10.38)	0.200	80.4 (12.22)	0.200
GGT (U/L)	44.2 (25.52)	0.000#	61.35 (24.85)	0.091
Total protein (g/dL)	7.12 (0.4)	0.200	7.22 (0.24)	0.001#
PON (ng/ml)	65.99 (31.38)	0.009#	87.46 (3.33)	0.144

Table 3: Distribution of diabetes, hypertension and family history of coronary artery disease.

Variables	Frequency	Percentage
Diabetes		
No diabetes	11	27.50
Diabetes	29	72.50
Hypertension		
No	21	52.50
Present	19	47.50
Family history of CAD		
Present	5	12.50
Absent	35	87.50

Table 4: Distribution of L55M and Q192R genotypes.

	Frequency	Percentage
L55M		
Wild type	2	5.00
Heterozygous mutant	18	45.00
Mutant type	20	50.00
Q192R		
Wild type	17	42.50
Heterozygous mutant	12	30.00
Mutant type	11	27.50

SGPT, SGOT and total protein in the control group followed non-normal distributions, and TGL, SGOT, GGT and PON followed non-normal distributions in the case group (Table 2). The mean PON was 3.83 ng/ml, whereas the mean PON U/L was 76.73. The distribution of diabetes, hypertension and family history of coronary artery disease among participants is shown in Table 3. The frequency of occurrence of the L55M and Q192R polymorphisms is summarized in Table 4. Accordingly, the mutant type of L55M polymorphism was most common and was found in 50% of participants, whereas for the Q192R polymorphism, 42.5% of the participants were wild type.

The participants in the case group were significantly older than those in the control group ($p = 0.023$). Most participants were men in both groups. The mean plasma glucose was significantly higher, and the serum cholesterol was significantly lower, in the case group than the control group ($p = 0.007$).

No statistically significant differences were observed in triglycerides, HDL, LDL, urea and total bilirubin values between groups. Table 5 provides a comparison of these parameters between groups.

The mean PON values between groups did not show statistical differences, whereas PON U/L was significantly ($p = 0.001$) lower in the case than the control group.

Table 5: Comparison of parameters between groups.

Variables	Case Median (IQR)	Control Median (IQR)	*p*-value
Age (years)	59.5 (51.75, 67)	51.50 (47, 56)	0.023*^{W}
BMI	27.56 (26.08, 28.99)	30.12 (27.82, 34.54)	0.002*^{T}
Total cholesterol (mg/dL)	174.50 (136.25, 200.50)	206 (179.50, 255.25)	0.007*^{W}
HDL (mg/dL)	43 (38, 49.75)	42 (35, 48.75)	0.595^{W}
TGL (mg/dL)	162 (147.50, 206.25)	192 (173.75, 221.50)	0.105^{MU}
LDL (mg/dL)	1299 (96.50, 160.50)	124.5 (108.75, 138.25)	0.507^{T}
Creatinine (mg/dL)	0.9 (0.87, 1.08)	1.1 (0.98, 1.14)	0.018*^{MU}
Urea (mg/dL)	39 (34.50, 43.50)	44.5 (41.5, 48)	0.054^{MU}
Total bilirubin (mg/dL)	1.1 (0.8, 1.20)	0.99 (0.89, 1.12)	0.902^{MU}
SGPT (U/L)	41 (35.75, 48)	50 (42.5, 65.25)	0.013*^{MU}
SGOT (U/L)	40 (35, 45)	50 (45, 74)	<0.001*^{MU}
ALP (U/L)	62.5 (48, 71.25)	82.5 (73.5, 90.25)	<0.001*^{W}
GGT (U/L)	38 (29.75, 42.50)	58 (40.5, 86.25)	0.016*^{MU}
Total protein (g/dL)	7.10 (6.9, 7.4)	7.35 (7.08, 7.4)	0.411^{MU}
PON (ng/ml)	3.4 (3.13, 4.15)	3.15 (2.5, 3.73)	0.244^{MU}
PON (U/L)	61 (49.5, 72.50)	88.45 (85.5, 90.03)	<0.001*^{MU}

Abbreviations: T: two sample t test, W: Welch's t test, MU: Mann Whitney U test.
* indicates statistical significance.

Table 6: Gene polymorphism in cases and controls.

Variables		Case	Control	*p*-value
L55M	Wild type	2 (10)	0	0.213
	Heterozygous mutant	7 (35)	11 (55)	
	Mutant type	11 (55)	9 (45)	
Q192R	Wild type	2 (10)	15 (75)	<0.001*
	Heterozygous mutant	7 (35)	5 (25)	
	Mutant type	11 (55)	0	

* Statistically significant.

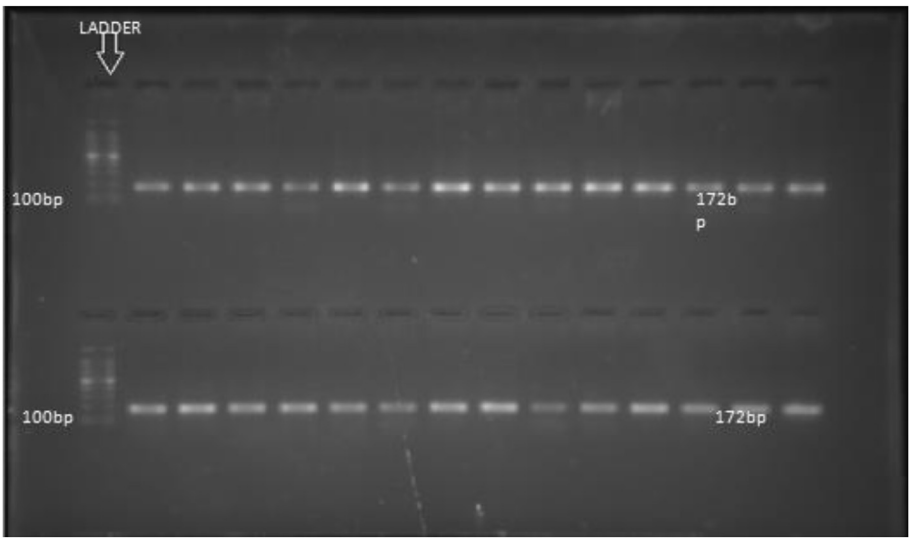

Figure 1: Photograph showing 172 bp PCR products of SNP L55M.

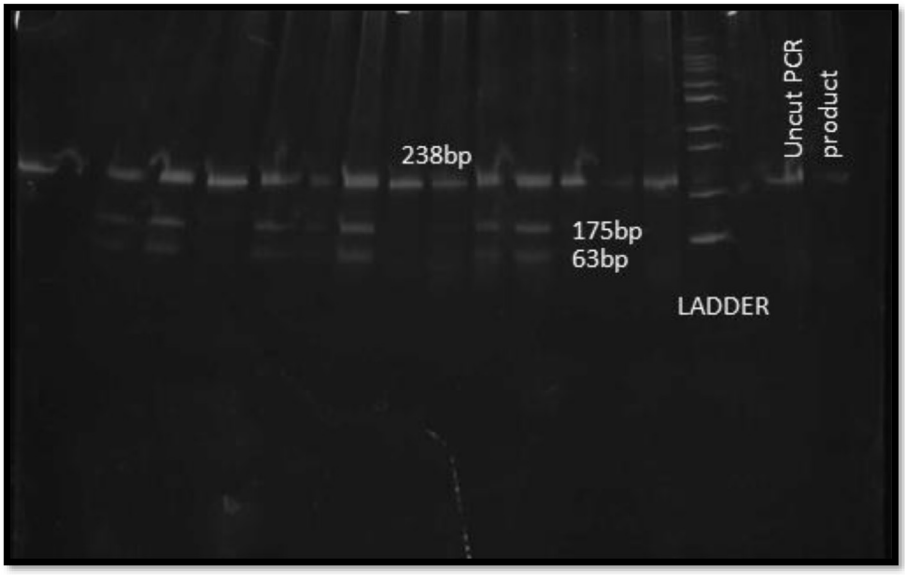

Figure 2: Photograph showing RFLP products of SNP Q192R.

The L55M polymorphism difference was not statistically significant between the case and the control groups ($p = 0.213$), whereas the Q192R polymorphism was statistically significant: more participants in the case than the control group exhibited heterozygous mutants and wild type ($p < 0.001$). The details are summarized in Table 6. Figures 1, 2 and 3 show the genetic analysis results.

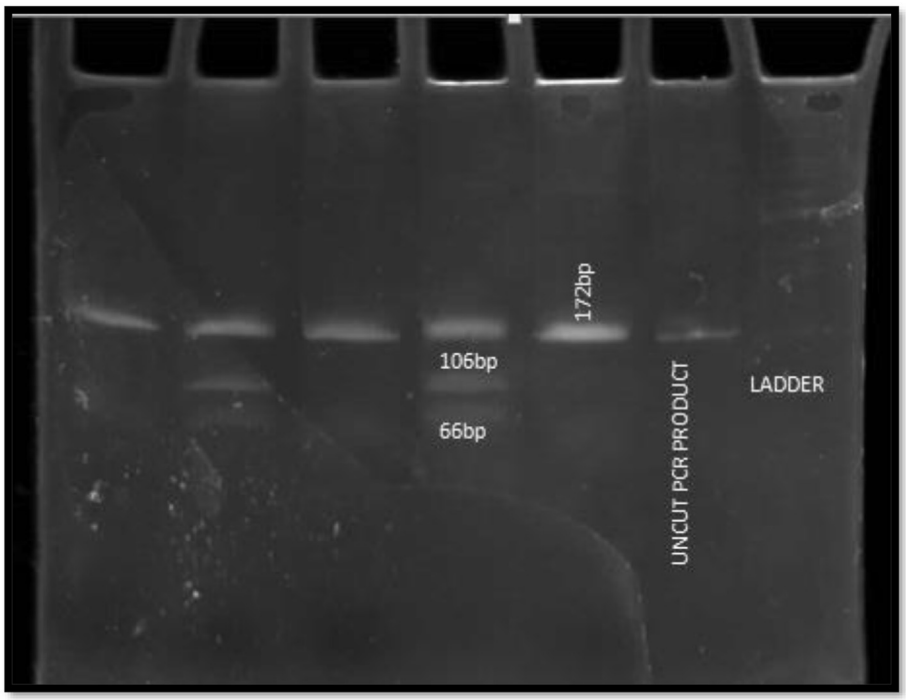

Figure 3: Photograph showing RFLP products of SNP L55M.

Discussion

Previous research has indicated that Asian-Indians are particularly predisposed to the development of CAD, and have higher susceptibility than people of European, Chinese or Japanese descent.[13]

In India, CAD is considered a prominent health disorder, particularly because of genetic predisposition as well as lifestyle modification trends.[6] Additionally, oxidative stress and weakened antioxidant action may result in initiation and advancement of atherosclerosis.[4]

Similarly to the results of the present study, Singh et al. have reported lower plasma PON1 and HDL levels, and higher LDL, total cholesterol and triglyceride levels in individuals with CAD than controls.[15] Likewise, increased serum glucose, HbA1C, triglycerides, LDL, total cholesterol and lower serum PON1 and HDL levels in CAD have been observed by El-Lebedy et al.[4]

Gupta et al. have reported that the Q192R polymorphism is significantly associated with CAD, and the allele frequency is much higher in people with than without CAD.[13] In accordance with these findings, in the present research, all alleles of Q192R were significantly associated with CAD.

Similarly to the results of our study, Godbole et al. have found low PON1 activity (U/L) in participants with CAD.[10] The authors also observed the wild type of Q192R predominantly in the control group (51.01%) and the mutant type predominantly in the CAD group. In the present study, mutant type L55M was more common in the CAD group, and heterozygous mutants were common in the control group. In contrast, in a study by Gupta et al., wild type L55M was predominant in the CAD group, whereas the mutant was predominant in the control group. People with CAD are predominantly men (77.3%) and have higher BMI (24.52%).[11,13] Similar results have been found in a study conducted by Hasan et al.[15]

A study by Shahsavari et al. has found that the LM and LL genotypes of the L55M polymorph are positively associated with atherosclerosis development, but no such association has been observed with Q192R.[16] In contrast, a Columbian study has found a positive correlation of CAD with Q192R.[17]

Reports of PON1 activity and CAD have been controversial. Some reports have suggested low PON1 activity in cardiovascular disorders, whereas others have reported contrasting findings. Measuring PON1 activity alone may not be suitable for risk assessment, and one study has suggested that genotypic and phenotypic assessment should also be performed.[11] Further support has been provided by Grubisa et al., who have assessed 60 participants with angiographically confirmed atherosclerosis for the allelic and genotypic frequencies of Q192R and found equal frequencies in both case and control groups. Logistic regression analysis showed that alleles or genotypes were not risk factors for atherosclerosis.[18] In a Chinese study, the PON1 Q192R polymorphism has been found to have a major effect on the risk of CAD development, and logistic regression analysis has indicated that the 192R allele is associated with a higher risk of CAD than other alleles. Significantly lower serum PON1 activity and concentration have also been observed in patients with CAD than controls.[19]

Previous research has indicated that measuring PON1 activity is insufficient to assess the risk of CAD development, because this activity is influenced by the Q192R polymorphism. Hence, both enzyme activity and genetic polymorphism must be measured to ascertain the risk.[12]

Diminished PON1 activity has been observed in obesity, whereas diets rich in olive oil, vegetables and fruit have been

associated with elevated PON1 activity.[14,20] Certain oral antidiabetics may increase the activity of PON1, whereas statins are known to decrease its activity.[21]

This study has several limitations. First, diet plays an important role in PON1 activity, but in the present research, the diets of the individuals, such as their consumption of olive oil, vegetables or red wine, were not considered. In addition, oral antidiabetics have been shown to increase the activity of PON1, and statins have been found to decrease the activity of PON1. The present research included individuals with diabetes as well as those taking statins, thus potentially confounding the results. In addition, further studies with larger sample sizes and populations could help identify PON gene polymorphism in patients with CAD.

Conclusion

In the current study, a higher proportion of the Q192R polymorphism, but not the L55M polymorphism, was observed in participants with CAD in a South Indian population. Low plasma PON1 and HDL levels, and higher LDL, total cholesterol and triglyceride levels were found in individuals with CAD. Further research should investigate larger sample sizes and categorize participants on the basis of confounding variables to avoid biased results.

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This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Conflict of interest

The authors declare that there are no competing interests.

Ethical approval

Ethical clearance for conducting the study was obtained from the Institutional Human Ethics Committee (ref No. 17/371 dated December 29, 2017), before initiation of the research.

Consent

All participants provided written consent to participate in this research.

Authors contributions

JFN was involved in screening of patients, selection and recruitment of study participants, informed consent, selection and recruitment of patients, laboratory investigations, laboratory report interpretation, data collection and monitoring of data, interpretation of data, statistical analysis and interpretation, maintaining master files of the project and drafting the final report. BG was involved in the concept, design, laboratory report interpretation, interpretation of data, statistical analysis and interpretation, and drafting the final report. Both authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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