International Journal of Biomedical And Advance Research

ISSN: 2229-3809 (Online)
Journal DOI:10.7439/ijbar
CODEN:IJBABN

Research Article

Paraoxonase1 activity, its Q192R polymorphism and diabetic retinopathy in type 2 diabetes mellitus

Mahesh Harishchandra Hampe[*1] and Mukund Ramchandra Mogarekar[2]

[1]*Senior Manager, Preventine Lifecare, Turbhe (Navi Mumbai), India*
[2]*Professor and Head of the Department of Biochemistry, SRTRGMC, Ambajogai, Dist- Beed, India*

*** Correspondence Info:**
Mahesh Harishchandra Hampe,
Senior Manager,
Preventine Lifecare, Turbhe (Navi Mumbai), India
E-mail: mahesh.hampe27@gmail.com

Abstract

Background: The goal of this study was to investigate the role of paraoxonase1 (PON1) status i.e. activity and Q192R polymorphism, in the development of diabetic retinopathy (DR) in patients of type 2 diabetes mellitus.
Methods: This cross-sectional study included 55 normotensive type 2 diabetic patients (duration more than 5 years) admitted in the hospital divided into two groups (with and without DR) on the basis of fundus examination by direct ophthalmoscopy. Serum samples of all patients were subjected for PON1 activity with and without salt using paraoxon as substrate and arylesterase activity of PON1 using phenylacetate as substrate. PON1 phenotyping is carried out using ratio of salt stimulated PON1 activity to arylesterase activity.
Results: Arylesterase activity of PON1 was found significantly lower in diabetics with DR than diabetic patients without DR (P = 0.01). Multivariate testing also showed its independent protective association in the development of DR (OR 0.958, [95% CI (0.915-1.004)], P value = 0.023). Moreover, DR was associated significantly in diabetic patients with PON1 R allele carriers (71.05%) than diabetic patients with PON1 QQ homozygotes (29.41%). PON1 R carriers had higher Odds ratio (OR) for DR in univariate analysis (OR 5.891, [95% CI (1.676-20.705)], P value = 0.006) and in multivariate analysis (OR 14.12 [95% CI (0.926-215.2)], P = 0.057) after adjustment of conventional risk factors of diabetes. Also diabetics with homozygous PON1 RR were associated significantly with severe forms of DR.
Conclusions: Arylesterase activity of PON1 may be more important in the risk of retinopathy in diabetic individual than the paraoxon hydrolytic activity. Moreover PON1 R allele is associated with susceptibility to DR showing a graded risk relationship to the number of *R* alleles. Thus the determination of PON1 status may be useful in the risk assessment and management of DR.
Key words: paraoxonase1, polymorphism, diabetic retinopathy, diabetes mellitus.

1. Introduction

Diabetes mellitus (DM) is a major health problem world-wide and DR is the most common and devastating chronic ocular microvascular complication eventually leading to blindness.[1-3] The prevalence of DR in diabetic individuals is around 17% in India amongst which around 8% ends in vision threatening blindness.[4,5] The risk of retinopathy is directly related to the age at onset of hyperglycemic state, degree of glycemic control and duration of diabetes.[6-8] Several hypotheses have been proposed to explain the pathophysiological mechanism which contribute to the development of retinopathy in DM. These include polyol accumulation, formation of advanced glycation end products, oxidative stress and activation of protein kinase C which modulates the disease process through various cellular metabolic signaling. [9-11] Oxidative stress induced lipid peroxidation thought to play major role in the pathogenesis of DR.[10-14]

Poor diabetic control is strongly associated with an increase in lipid peroxides and consequent microangiopathies in DM.[3] High density lipoprotein (HDL) cholesterol has been shown to have antioxidant potential and its low levels are more prevalent with poor glycemic control.[15] PON1 is an aryldialkylphosphatase (E.C. 3.1.8.1) mainly found in the circulation anchored on HDL and is considered to be the major contributor of the protective effects displayed by HDL.[16] PON1 activity is found to be decreased in DM.[17] PON1 has the ability to hydrolyse specific oxidised phospholipids and hydroperoxides in oxidised low density lipoproteins (LDL) and destroying the proinflammatory molecules involved in the initiation and progression of microvascular complications in diabetes.[18] Serum PON1 activity, based on the ability of the enzyme to hydrolyse the substrate paraoxon, found to possess interindividual variability. [19] The molecular basis of these variations is the single nucleotide polymorphism in the PON1 gene (7q21-22).[20] An amino acid substitution at position 192 *(*Q192R polymorphism) give rise to two alleles PON Q and PON R and three phenotypes PON1 QQ, PON1 QR and PON1 RR . The amino acid arginine at position 192 of the protein specifies Q allele which is more abundant in the population and is responsible for protective effects of PON, while glutamine at that position denotes R allele which is proposed to be related with oxidative stress.[16] PON1 Q192R polymorphism thus offers differential protection towards oxidative stress induced microvascular damage in genetically susceptible individuals. This gave us the insight to investigate diabetic patients to determine the association of DR with PON1 phenotypes. The aim of this study was to investigate whether the PON1 status (activity and polymorphism) were associated with occurrence of retinopathy in type 2 diabetic patients besides other established risk factors.

2. Material and Methods

The cross-sectional study was conducted on 55 normotensive type 2 DM patients diagnosed as per the diagnostic criteria of American Diabetes Association 2007, selected randomly from the patients admitted in our hospital.[21] Diabetic patients with retinopathy formed the study group while diabetic patients without retinopathy served as the control group. Study protocol was approved by the institutional ethics committee of our medical College. Written informed consent was obtained from all patients. Patients with associated ischemic heart disease, rheumatoid arthritis, concomitant liver or kidney disease, history of exogenous hormone administration and DM of less than 5 years duration were excluded

from the study population. All cases included in this study were subjected to detailed history taking, a complete clinical examination and abdominal ultrasonography. Height and weight were measured in barefoot patients wearing only light clothes. Body mass index (BMI) was calculated by the Quetelet's index, Weight (kg) / height^2 (m).

2.1 Fundus examination

Fundus examination of both eyes was carried out by direct ophthalmoscopy 30 minutes after complete mydriasis with 1% tropicamide eye drops. Indirect ophthalmoscopy with + 20 D lens and examination of macula with + 90 D Volk's lens was carried out whenever indicated. Grading of DR was carried out according to Early Treatment Diabetic Retinopathy Study criteria.

2.2 Analysis of serum and plasma samples-

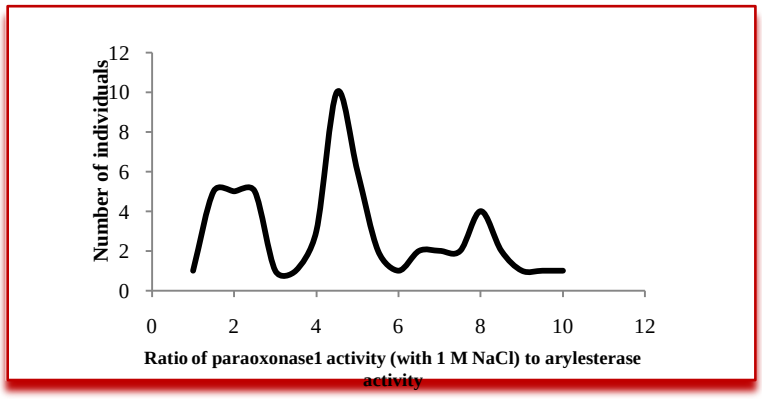
All fine chemicals of analytical grade were obtained from Sigma or Merck, India. Venous blood sample was collected aseptically from all patients in the morning after an overnight fast. Fasting blood sample collected in fluoride bulb was analyzed within a few hours for estimation of plasma glucose by glucose oxidase-peroxidase method.[22] Serum was collected from remaining blood sample by low speed centrifugation and was subjected to various biochemical analyses. Levels of total cholesterol, HDL cholesterol and triglycerides were measured using enzymatic techniques (Erba diagnostic kit). LDL cholesterol was calculated by the Friedewald formula.[23] Total oxidant status (TOS) was estimated by the method as described by Erel O and expressed as µmol H_2O_2 equivalents / L.[24]

2.2.1 PON1 activity measurements- The rate of formation of p-nitrophenol was measured on spectrophotometer using working reagent containing 2 mM/L paraoxon in 50 mM/L Tris-HCL buffer, p^H 8.0 with 0.9 mM/L $CaCl_2$ with or without 1 Mol /L NaCl. The reaction was initiated by 20 µl sample in 700 µl of working reagent.[19] The rate of p-nitrophenol formation was measured at 405 nm over 200 s with a 25 s lag time. Non-enzymatic hydrolysis was subtracted from the total rate of hydrolysis. The activity is expressed in U/L based on the molar absorption coefficient (18290 $M^{-1}cm^{-1}$) at 405 nm at p^H 8.0. Intra and interassay coefficients of variation (CVs) are 3.3% and 4.7% respectively.

2.2.2 Arylesterase activity measurements- Arylesterase activity was measured using phenylacetate as substrate. The initial rate of hydrolysis was measured spectrophotometrically using the assay mixture which contains 4.0 mM/L phenylacetate, 1 mM/L $CaCl_2$ in 20 mM /L Tris HCl buffer, p^H 8.0 at 25^{0}C. The rate of phenol formation was recorded at 270 nm following 20s lag time.[19] The activity is expressed in kU/L, based on the extinction coefficient of phenol of 1310 $M^{-1}cm^{-1}$ at 270 nm, p^H 8.0, and 25^{0}C. Blank samples containing water are used to correct for non-enzymatic hydrolysis. Intra and interassay CV were 2.4% and 3.7% respectively.

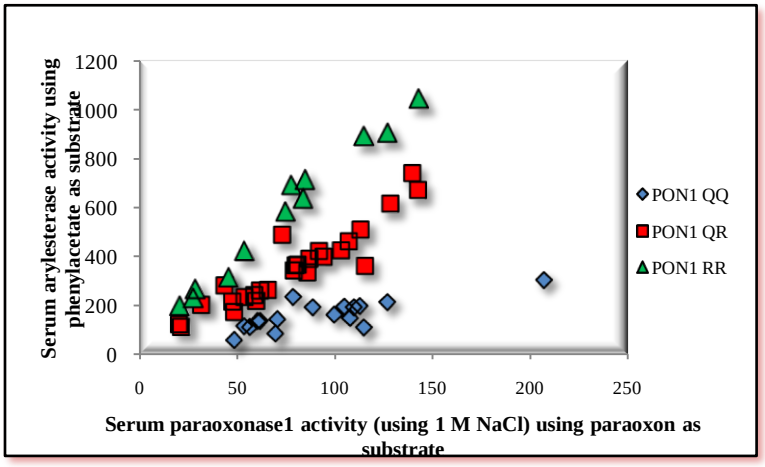
2.2.3 PON1 polymorphism- Individual human serum was phenotyped for the PON1 Q192R polymorphism by dividing the PON1 activity in the presence of 1M NaCl by arylesterase activity.

Figure 1: Population distribution of PON1 phenotypes



Cumulative frequency graph showing trimodal distribution of study population by the antimodes at 3.0 and 6.9.

Figure 2: Paraoxonase1 Q192R polymorphism



Two substrate hydrolysis assay resolving the study population into three PON1 phenotypes.

2.3 Statistical Analysis

Hardy-Weinberg equilibrium was evaluated by means of the χ^2 test. The continuous variables were tested for normality with Shapiro-Wilk test. Results were expressed as mean ± SD. Comparisons between groups were made using Student's *t*-test for continuous variables. χ^2 and Fisher's exact test were performed to compare the frequencies of PON1 phenotypes between subjects with and without retinopathy. Initially risk variables of diabetes were assessed in univariate analysis and then they are modeled using stepwise multivariate logistic regression analysis to determine the independent determinants of DR. Model 1 included age, duration of diabetes, BMI, blood glucose level, total cholesterol (TC), triglycerides (TG), HDL cholesterol, LDL cholesterol and TOS. Model 2 included model 1 variables plus arylesterase activity. Model 3 included model 1 variables plus PON1 allele frequencies. OR and 95% CI was calculated. Naglekerke R^2 was compared for each of the logistic regression models. Statistical significance was considered at the level of $P < 0.05$. All tests were performed with the statistical software Mystat 12 for Windows.

3. Results

3.1 Clinical, Laboratory and Demographic Characteristics

The main clinical, anthropometric and biochemical data of diabetics is shown (Table 1). Fundus examination revealed that 23 diabetic patients had no signs of DR, 26 diabetics had mild to moderate DR while 6 diabetics had severe non-proliferative DR. Patients with DR were found to have longer duration of disease and statistically significant difference in fasting blood glucose, HDL cholesterol and total oxidant status (TOS). Age, sex, TC, TG, very low density lipoprotein (VLDL) cholesterol, LDL cholesterol was statistically insignificant in the two groups.

Table 1: Clinical and biochemical data of type 2 diabetic patients with and without retinopathy

Parameter	DM without DR (n=23)	DM with DR (N=32)	*P* value
Age	55.39 ± 15.51	55.43 ± 11.74	0.99
Sex (M/F)	7/8	16/14	0.673
Smoking status	1/15	5/30	0.352
Duration of DM (yrs)	8.52 ± 3.13	11.34 ± 4.3	0.007**
BMI	23.03 ± 3.53	23.79 ± 4.0	0.472
BSL (mmol/L)	7.17 ± 2.32	10.5 ± 2.05	0.001**
TC (mmol/L)	4.42 ± 1.1	4.48 ± 1.27	0.85
TG (mmol/L)	1.64 ± 0.4	1.67 ± 0.45	0.796
HDL-C (mmol/L)	0.96 ± 0.22	0.8 ± 0.23	0.017*
VLDL-C (mmol/L)	0.75 ± 0.18	0.76 ± 0.21	0.8
LDL-C (mmol/L)	2.71 ± 1.04	2.89 ± 1.19	0.54
TOS (μmol H_2O_2	14.07 ± 7.45	19.25 ± 7.71	0.016*
equivalents/L)			
Basal PON 1 activity (IU/L)	137.95 ± 55.04	112.78 ± 48.37	0.086
Arylesterase activity (IU/L)	96.78 ± 35.42	71.00 ± 34.09	0.01*
Frequency of PON alleles (PON1Q / PON1R)	32/14	28/36	0.007**

BSL= Blood sugar level, TC=Total cholesterol, TG= Total triglyceride, HDL-C= High density lipoprotein cholesterol, LDL-C= Low density lipoprotein cholesterol, VLDL-C= very low density lipoprotein cholesterol, TOS= Total oxidant status, $P < 0.05$, ** $P < 0.01$.

Table 2: Correlation studies

	BSL	HDL-C	PON1 activity	Arylesterase activity	TOS
TOS	0.464	-0.296	-0.364	-0.181	1.000
Arylesterase activity	-0.222	0.207	0.084	1.000	-
PON1 activity	-0.097	0.172	1.000	-	-
HDL-C	-0.265	1.000	-	-	
BSL	1.000	-	-	-	-

3.2 PON1 activity and polymorphism-

Serum PON1 activity was lower in diabetic patients with DR than those without DR but not statistically significant ($P = 0.086$). However, arylesterase activity of PON1 was found significantly lower in diabetics with DR than the diabetic patients without DR ($P = 0.01$). PON1 activity and arylesterase activity of PON1 is significantly negatively correlated with blood glucose level and TOS (Table 2).

The three PON1 phenotypes (PON1 QQ, PON1 QR and PON1 RR) in diabetic patients were easily distinguished by two substrate hydrolysis assay. The distribution of allele frequencies of PON1 phenotypes were in Hardy-Weinberg equilibrium ($\chi^2 = 0.06$, P = not significant). We found no significant difference between the PON1 phenotypes for any of the diabetes risk factors as determined by both univariate analysis or by multivariate logistic regression analysis (data not shown).

Table 3. Occurrence of diabetic retinopathy among different Paraoxonase phenotypes

Grades of retinopathy	PON phenotypes		Total
	QQ Homozygotes	R carriers	
No retinopathy	12(21.81%)	11(20.00%)	23(41.81%)
Mild to moderate NPDR	4(7.27%)	22(40.00%)	26(47.27%)
Severe NPDR	1(1.81%)	5(9.09%)	6(10.9%)
Total	17(30.9%)	38(69.09%)	55(100.00%)

($P = 0.015$*, significant)

Table 4: **Multivariate models of predictors of retinopathy in diabetic patients**

Independent variables	Z value	SE	OR(95% CI)	*P* value
Model 1 (R^2= 0.667, *P* = 0.000)				
Age (years)	-1.786	0.042	0.928(0.855-1.007)	0.074
Smoking status	1.428	0.518	8.732(0.446-170.98)	0.153
Duration of DM (years)	1.496	0.168	1.286(0.925-1.788)	0.135
BMI (Kg/m^2)	0.813	0.143	1.123(0.849-1.486)	0.416
BSL (mmol/L)	2.953	0.018	1.055(1.018-1.092)	0.003**
TC (mmol/L)	0.228	0.582	1.256(0.402-3.93)	0.392
TG (mmol/L	-0.218	0.117	0.975(0.776-1.225)	0.827
HDL-C (mmol/L)	-0.491	0.585	0.75(0.238-2.363)	0.623
LDL-C (mmol/L)	-0.418	0.582	0.784(0.25-2.455)	0.676
TOS (μmol H_2O_2 equivalents/L)	-0.365	0.071	0.974(0.848-1.120)	0.323
Model 2 (R^2= 0.723, *P* = 0.000)				
Age (years)	-2.127	0.044	0.911(0.837-0.993)	0.033*
Smoking status	1.414	1.678	10.72(0.40-287.47)	0.157
Duration of DM (years)	1.424	0.23	1.387(0.884-2.117)	0.155
BMI (Kg/m^2)	0.707	0.172	1.129(0.806-1.582)	0.48
BSL (mmol/L)	3.016	0.019	1.060(1.021-1.101)	0.003**
TC (mmol/L)	0.323	0.502	1.176(0.44-3.145)	0.746
TG (mmol/L)	0.092	0.104	1.01(0.824-1.238)	0.926
HDL-C (mmol/L)	-0.406	0.505	0.815(0.303-2.191)	0.684
LDL-C (mmol/L)	-0.354	0.502	0.837(0.313-2.239)	0.723
TOS (μmol H_2O_2 equivalents/L)	-0.947	0.079	0.928(0.795-1.083)	0.08
Arylesterase activity (kU/L)	-1.798	0.024	0.958(0.915-1.004)	0.072
Model 3 (R^2= 0.726, *P* = 0.000)				
Age (years)	-1.685	0.045	0.933(0.860-1.011)	0.092
Smoking status	1.181	1.625	6.817(0.282-164.8)	0.238
Duration of DM (years)	1.256	0.170	1.239(0.887-1.729)	0.209
BMI (Kg/m^2)	1.172	0.153	1.196(0.887-1.613)	0.241
BSL (mmol/L)	2.768	0.018	1.051(1.015-1.089)	0.006**
TC (mmol/L)	0.706	0.716	1.657(0.408-6.738)	0.48
TG (mmol/L)	-0.555	0.142	0.924(0.700-1.221)	0.579
HDL-C (mmol/L)	-0.833	0.729	0.545(0.131-2.274)	0.405
LDL-C (mmol/L)	-0.733	0.719	0.591(0.144-2.415)	0.464
TOS (μmol H_2O_2 equivalents/L)	0.151	0.090	1.014(0.850-1.208)	0.08
PON1 R carriers	1.905	1.390	14.12(0.926-215.2)	0.057

SE= Standard error, OR= Odds ratio, CI= Confidence Interval.

Table 3 summarizes the distribution of the PON1 phenotypes in the patients studied according to DR status. Among the 55 diabetics included in the study, 5 out of 17 diabetic patients (29.41%) with PON1 QQ phenotype had evidence of DR while 18 of 26 diabetic patients (69.23%) with PON1 QR and 9 out of 12 diabetic patients (75.00%) with PON1 RR phenotype had evidence of DR. The difference in the occurrence of DR between patients with PON1 QQ homozygotes and PON1 R carriers was statistically significant (*P* = 0.015). DR was associated with statistically significant higher frequency of R allele (Table 1). Moreover the frequency of patients with PON1 RR phenotype was greater in diabetics with severe forms of DR. As determined by univariate logistic regression, diabetic PON1 R allele carriers were more likely to have had DR than those with diabetic PON1 QQ homozygotes. (OR 5.891, [95% CI (1.676-20.705)], *P* value = 0.006). This data suggests greater risk associated with PON R allele in the development of DR in patients of DM. Out of the established biochemical and clinical characteristics, blood glucose level was the only independent predictor of DR in our study group (Model 1). The logistic regression model 2 prepared for arylesterase activity of PON1 as a predictor of increased risk of DR, the significant association between arylesterase activity and DR identified in univariate regression remained significant in multivariate testing (OR 0.958, [95% CI (0.915-1.004)], *P* value = 0.023). The increased risk of DR predicted by the PON1 R carriers is estimated in univariate analysis (OR 5.891 [95% CI (1.676-20.705)], *P* = 0.004). However it loses its significance in multivariate testing. (OR 14.12, [95% CI (0.926-215.2)], *P* = 0.057). However Naglekerke R^2 value increased (from 0.692 to 0.726) after addition of PON1 phenotyping in the model prepared by the conventional risk factors of DM, indicating contribution of PON1 polymorphism for the development of DR.

4. Discussion

DR is the most common and serious microvascular complication in both type 1 and type 2 DM.[25] Duration of diabetes and degree of glycemic control are the two major determinants in the development of retinopathy.[3,26] But these are not the only sufficient conditions which explain the development of chronic microvascular complications. The occurrence of retinopathy even in strict glycemic control and short duration of diabetes evokes possibility of non-modifiable genetic factors which contribute to the development of DR.

Genetically determined differences in antioxidant protection could contribute to differential susceptibility of diabetic patients to microvascular complications. One such hepatically synthesized protein is PON1 enzyme which shows genetic variability determined by two alleles, PON1 Q and PON1 R, which manifests as three phenotypes viz. PON1 QQ, PON1 QR and PON1 RR. [27] We performed two substrate hydrolysis assays for individual PON1 phenotype determination. In our study, PON1 activity between diabetics with and without DR was not found significantly different. However, arylesterase activity of PON1 enzyme in cases of DR was significantly decreased than the diabetics without DR (*P* value = 0.01). Paraoxon hydrolytic activity of PON1 is trimodal as compared to unimodal distribution of arylesterase activity of PON1. This indicated that the arylesterase activity of the enzyme, which is representative of enzyme concentration, may be more important than the paraoxon hydrolytic activity especially in the pathogenesis of DR. Oxidative stress plays important role in pathogenesis of DR as evident in

our study. Furthermore, among the factors which may be associated with increased oxidative stress, low HDL and poor glycemic status emerge as first candidates. PON1 has been shown to metabolise lipid soluble radicals.[18] As PON1 activity as well as arylesterase activity of PON1 is inversely associated with oxidative stress, this findings strongly support the role of PON1 in modulating free radical injury. Our findings are consistent with the observations of Kao *et al* (1998), which demonstrated that PON1 levels are low in diabetics with microvascular complications.[28]

In vitro, PON1 polymorphism evokes differential hydrolysis of substrate paraoxon, while phenylacetate is hydrolysed approximately at the same rate.[29] No physiological substrate for PON1 has been identified till today. We found R allele more associated with development and progression of retinopathy. Moreover PON1 R carriers had more severe forms of DR. This relationship persisted even after adjustment of conventional DM characteristics. Thus our results demonstrate that both PON1 activity and polymorphism has mechanistic link with prevalence of DR in DM. PON1 R allele has been found to be an additional risk predictor for the development of retinopathy in diabetic individuals. Our findings are consistent with the study conducted by Mackness *et al* (2000) which demonstrated a graded risk relation between presence, as well as severity, of DR and PON1 R allele. [30] In the majority of studies, persons with PON1 R carriers were found to be at increased risk of microvascular and macrovascular complications. [31-33] However, Flekac *et al* (2008) compared diabetic patients with and without DR and found no difference in PON1 phenotype distribution in two groups.[34] The reasons for these discrepancies may be due to differences in type of diabetes, ethnic groups and other genetic contributors.

Cytotoxicity of oxidised LDL to retinal cells and pericytes is proposed to be the major cause of retinopathy.[6,9] Several studies have shown HDL associated PON1 to retard oxidation of LDL by reducing hydroperoxides. Moreover, PON1 activity is reduced in poor glycemic status and high oxidative stress. So our findings support an idea that the PON1 induced protection of lipid peroxidation may be reduced in diabetic patients because of lower enzymatic activity.

Mackness *et al* (2001) have demonstrated that PON1 QQ phenotype possesses greater ability to reduce oxidized lipids than PON1 RR phenotype.[35] Moreover Regieli *et al* (2009) stated that PON1 RR have relatively low peroxidase activity towards copper induced oxidized LDL than PON1 QQ phenotype.[36] These unfavourable characteristics of PON1 R allele may explain the significant association of retinopathy in diabetic patients with PON1 R carriers. However, the limitation to our study is the small sample size which is not enough to establish the predictive value of PON1 polymorphism in DR. PON1 genotyping, which is more definitive, is not performed in the present study. So the wrong classification of few individuals among three phenotypes can be possible. Also the actual concentration of PON1 is not directly estimated in the present study and larger studies are therefore required to serve this purpose. Moreover, residual confounding by the variables cannot be accounted.

This study, therefore gives us an alarm for more energetic treatment for retinopathy in diabetic patients with PON1 RR phenotype. Furthermore PON1 status can be considered as an additional potential candidate in the algorithm followed for the management of cases of DR.

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