

Correlation Between Serum PON1 Arylesterase Activity and Rs 854573 *PON1* A<G Polymorphism with Type 2 Diabetes in an Eastern Indian Cohort

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Abstract: Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder of glucose and lipids and characterized by defect in insulin secretion or action. Oxidative imbalance has also been implicated in the etiology of diabetes. Paraoxonase-1 (PON1) is an esterase and lactonase which is found in the circulation bound to high-density lipoproteins (HDL). Alterations and associations of circulating PON1 levels with a variety of diseases including diabetes encourages us to investigate the possible association between *PON1* A/G rs854573 polymorphism and serum PON1 activity with T2DM. The study essentially follows a population based case-control format with 101 diabetic and 102 healthy controls. The findings revealed association of polymorphism with the diseased status (p value 0.0002, OR 3.49, 95% CI 1.77 to 6.9). With significantly higher range of mean serum PON1 Arylesterase (AREase) activity in control (9.99 – 0.96 kU/L) than in diabetic patients (5.25 - 0.508 kU/L) (p value, <0.001), a large difference between common diabetic AA genotype and combined diabetic heterozygous and homozygous genotypes (AG+GG) for risk allele G (asymptomatic p value, <0.001), or in between two AA genotypes (Diabetic/Non diabetic, p<0.001), was explored by parametric and non parametric statistical pairwise comparison. Serum PON1 activity was found to be independent of other clinical factors such as plasma glucose levels. Western blot analysis of serum samples detected a significant difference of PON1 proteins in diabetic patients and control subjects (p value 0.008). In conclusion serum PON1 AREase activity which to an extent correlated with *PON1* promoter polymorphism might be a good predictor of the disease risk.

Keywords: AREase Activity, Oxidative Stress, Polymorphism, PON1, T2DM, Western Blot

1. Introduction

Diabetes mellitus (DM) is a chronic disorder caused by impaired metabolism of glucose and lipids due to defect in insulin secretion or action and characterized hyperglycemia which eventually leads to microvascular and/or macrovascular pathologies affecting more than 17.5 million

deaths worldwide [1]. According to International Diabetes Federation (IDF), 371 million people have been reported of having DM and this number is expected to rise to 552 million by 2030. Type 2 diabetes mellitus (T2DM) is a multifactorial metabolic disorder which is growing in epidemic proportions throughout the world and the greatest increase in prevalence is predicted to occur in Asia and Africa by 2030 [2]. Several lines of evidences suggest that the aetiopathogenesis of the

common form of T2DM includes a strong genetic component contributed by several common genetic variants, each with relatively modest effect, acting in combination with each other and with environmental and lifestyle triggers [3]. Growing body of evidence also suggests that oxidative stress plays a key role in the pathogenesis of micro- and macrovascular diabetic complications [4].

Human paraoxonase (*PON*) gene family consists of three members, *PON1*, *PON2* and *PON3*. *PON1* possesses paraoxonase (PONase), arylesterase (AREase) and lactonase activities; while *PON2* and *PON3* primarily display lactonase activity. *PON1* is found in the circulation bound to high-density lipoproteins (HDL) [5]. *PON1* degrades oxidized phospholipids in lipoproteins and plays an important role in the maintenance of organism's antioxidant system [6, 7]. Alteration in circulating *PON1* level has been found to be associated with a variety of diseases that involves oxidative stress [8]. Following the introduction of the oxidative stress hypothesis in the aetiopathology of atherosclerosis and the discovery of antioxidant effect of HDL [9], *PON1* has attracted significant interest as a protein responsible for the most of antioxidant properties of HDL [10]. Purified *PON1* protects HDL and low-density lipoprotein (LDL) from oxidation catalyzed by copper ions [11]. *PON1* inhibits copper-induced HDL oxidation by prolonging lag phase of oxidation and reduces peroxide and aldehyde contents in oxidized HDL [12]. Liver is the principal tissue for *PON1* gene expression. It is likely that *PON1* stays associated with endoplasmic reticulum through its hydrophobic N-terminus until it is released from the hepatocytes. The mechanism of *PON1* secretion is not well investigated. A study on cultured hepatocytes and transfected Chinese hamster ovary (CHO) cells indicates *PON1* accumulates on the plasma membrane of hepatocytes and slowly dissociate into extracellular medium [13]. Dissociation is promoted by HDL, very-low-density lipoprotein (VLDL), and in much lesser extent by protein-free phospholipids particles or ApoA-I protein [14].

PON1 activity can be evaluated by using its different substrates such as paraoxon (paraoxonase), 4 (p)-nitrophenyl acetate (AREase) and dihydrocoumarin (lactonase) [15]. In a human study evaluating *PON1* activity between patients with non-end-stage chronic renal failure and healthy individuals showed 4-nitrophenyl to undergo significant changes. This substrate showed higher activities between narrower ranges of population values that could probably reflect less analytical errors [16]. In other study, phenyl acetate was found to be more sensitive and specific substrate in identifying patients with chronic hepatic disease than did paraoxon [17]. A stronger association between decreased serum *PON1* activity and metabolic syndrome was detected in childhood obesity when 5-thiobutyl butyrolactone was used as substrate compared to paraoxon [18]. Serum *PON1*-HCTLase and *PON1*-AREase activities of the enzyme were reported to be significantly lowered in diabetic patients [19, 20]. *PON1* activity was also found to be decreased in diabetic nephropathy and neuropathy [21-23].

Several studies have also investigated the relationship

between genetic variability of *PON1* and risk of diabetes and related disorders. *PON1* gene harbors nearly 200 single nucleotide polymorphisms (SNPs) of which -909 G>C (rs854572), -162 A>G (rs705381), -108 C>T (rs705379) located in the promoter and Q192R (rs662) and L55M (rs854560) located in the coding region are the most commonly studied. The frequency of *PON1* polymorphisms differ with ethnic backgrounds, such that the incidence of the RR genotype is 42.7% in Japan, about 20% in Caucasians, and 33% in China [24]. The combined genotypes of RR/LL increase the risk of coronary artery disease (CAD) [25, 26]. It has been reported that the QR genotype was the most common in patients with coronary artery disease. In addition, a positive family history of coronary artery disease was found to be associated with the R allele [27]. RR genotype has been significantly associated with coronary artery disease in a north Indian population [28]. Based on these findings, the RR genotype may be considered to be a risk factor for cardiac complications in type 2 diabetes patients in the Turkish population. Unfortunately, most of these studies have examined simple association of *PON1* SNPs with disease susceptibility. There are very few reports which have jointly evaluated *PON1* SNPs and serum *PON1*AREase activity. More importantly no such study has been conducted in Indian population which is estimated to bear the largest burden of DM by 2030. Inspired by all these facts and reports, the present study aims to investigate genetic relation of *PON1* rs854573 A>G polymorphism with serum *PON1* concentration and activity in T2DM patients in an Eastern Indian T2DM cohort.

2. Materials and Methods

2.1. Study Subjects

A total of 101 T2DM patients and 102 healthy controls participated in this case control association study. Blood samples were collected from 45 to 55 years old unrelated individuals suffering from T2DM for the period of 1–5 years as well as from sex and age matched healthy volunteers from Calcutta National Medical College and Hospitals (CNMC) hospital, Kolkata, India. The study was guided by World Health Organization criteria and University and Hospital ethical committees' guidelines. All the clinical data were obtained from outpatient departments of CNMC. All T2DM patients represented body mass index (BMI) of 18–35 kg/m². The 2 h post-glucose tolerance test of blood sugar level (after a 75 g glucose load) for diabetic patients was greater than 200 mg/dL (11.11 mmol/ L) while the value is less than 140 mg/dL (7.8 mmol/ L) for control healthy subjects (without any family history of diabetes or hypertension). None of the subjects (both control and patients) were smokers. Complete clinical and demographic characteristics of the study population have been reported in Table 1.

2.2. Sample Preparation and Genotyping

Following salting out [29] of genomic DNA from 5 mL of

venous blood, the yield and purity of DNA were confirmed by spectrophotometric analysis by estimating the absorbance at 260 and 280 nm. Human serum samples from total blood were isolated and divided into aliquots and stored at -70°C before DNA isolation and thawed a single time for analysis of AREase activity and PON1 protein. Approximately 100–150 ng of genomic DNA from each individual was amplified using specific primer pairs. Briefly, the PCR was performed in a Veriti-thermocycler (Applied Biosystem), following a standard protocol. Eight to ten microliters (μ Ls) of PCR product (143 bp) was digested with 5 U of respective restriction endonucleases from New England Biolabs (NEB)

(Table 2) in a final volume of 20 μ L following the manufacturer's instructions. The resulting fragments were separated on 2–3% agarose gel, visualized in Gel-Documentation System 2000 (Bio-Rad Laboratories (UK) Ltd). Genotype assignment was based on size discrimination of PCR-digested products. The presence of A allele was confirmed by 143 bp fragment where as G allele was specified by two fragments of 132 bp and 11 bp sizes in Gel-Documentation System 2000. Samples with known genotypes were included in each set of digestion to ensure that the observed genotypes were not due to partial/incomplete digestion.

Table 1. Comparison of genotype and allele frequencies of rs854573 between diabetic patients and healthy controls.

Disease Status	Genotype Frequency			Comparison of genotype proportion (p value)				Major Allele frequency	Minor allele frequency	Comparison of allele frequency
	AA	AG	GG	Co-dominant	Dominant	Independent				
Case	63	30	8	p<0.0001**	P=0.0002**	AA vs AA	0.149	0.77	0.23	p value<0.0001, chi-square 17.5, OR=8.688, 95% CI=1.066- 70.79
Control	87	14	1	95% CI=1.88-6.35	OR 3.49, 95% CI=1.77-6.9	AG vs AG	0.02	0.92	0.08	
						GG vs GG	0.02			

* indicates P value<0.05; ** indicates P<0.01.

2.3. *PON1* Arylesterase (AREase) Activity Analysis

All the chemicals for enzymatic analysis were obtained from Sigma. Para-nitrophenyl (p-nitrophenyl) acetate was obtained from Sigma also. Serum AREase activity was determined using p-nitro phenyl acetate as the substrate [30]. The working reagent consisted of 25 mmol/L triethanolamine-hydrochlorine buffers (TRIS-HCl), pH 7.4, with 1.0 mmol/L CaCl_2 with or without 1 mmol/L phenyl acetate. The start reagent consisted of 2.5 mmol/L p-nitrophenyl acetate in water. The reaction was initiated by addition of 20 μ L diluted serum sample (1: 20 in TRIS-HCl buffer) to 288 μ L working reagent followed by 72 μ L of start reagent. The rate of formation of p-nitrophenol was determined at 405 nm in a Carry UV vis spectrophotometer at 25°C over 225 s after a 100 s lag time. The activity, expressed in katal unit/L (kU/L), was based on the molar absorptivity (14000) of p-nitrophenol at 405 nm, at pH 7.4 [31].

2.4. Western Blot Analysis of Serum *PON1* Protein

For western blot analysis previously isolated serum sample as obtained from venous blood, was used. SDS-electrophoresis Polyacrylamide gels (8% Acrylamide, 29:1 ratio of Acrylamide to Bis-acrylamide) containing 12 wells, prepared in biorad cassettes, were used for the separation of components of serum. An 80-fold dilution of serum was made with 60 mM Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 50 mM Dithiothreitol (DTT) and heated at 95°C for 5 min. A 10 μ L volume of sample was loaded into each well (equivalent to 125 μ L of serum). Protein bands were transferred onto nitrocellulose membranes (Chromous Biotech) using the bio-rad semidry electrophoresis transfer apparatus. Membranes were then blocked for 1 h at room temperature in blocking buffer [Tris-

buffered saline with 0.1% Tween 20 and 5% BSA (Sigma-Aldrich, St Louis, MO, USA)]. The membranes were then incubated overnight at 4°C in a blocking buffer containing a mouse monoclonal primary antibody against human PON1 (catalogue no. Sc59646; Santa Cruz biotech, USA) at 1:1000 dilutions. The membranes were then washed three times for 5 min in a wash buffer (Tris-buffered saline with 0.1% Tween 20) and incubated for 1 h at room temperature with secondary antibody (rabbit anti-mouse horseradish peroxidase) at 1:5000 dilutions in blocking buffer, followed by three 5-min washes in wash buffer. The protein bands were detected using Luminol reagent (sc-2048, Santa Cruz, USA) and quantified using a CCD image sensor (ChemiDoc XRS; Bio-Rad) and software (Quantity One; Bio-Rad). Following detection, the membranes were stripped in strip buffer (25 mmol/l glycine, 1% SDS, pH 2) for 1–2 h and then blocked in blocking buffer for 1 h at room temperature. In order to normalize for equal protein, the membranes were re-probed overnight at 4°C with goat Glyceraldehyde-3-Phosphate-Dehydrogenase (GAPDH) (Biobharati, life science) antibody at 1:5,000 dilution in blocking buffer, followed by washing and incubation for 1 h with secondary antibody (anti-mouse horseradish peroxidase, P0260; Dako) at 1:2,000 dilution in blocking buffer, followed by 5 min washing in buffer. The protein bands were detected using enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, Bucks, UK) and quantified using a CCD image sensor (ChemiDoc XRS) and software (Image J). The content of serum PON1 protein was expressed as arbitrary units relative to GAPDH protein content.

2.5. Statistical Analysis

The clinical variables were represented as mean \pm SE (Table 1). All the clinical parameters were log transformed for achieving normal distribution and subjected to Pearson

correlation test in pair-wise combinations. Hardy–Weinberg equilibrium (HWE) was tested using the goodness-of-fit test in cases and controls. Strength of association was tested by odds ratio estimates at 95% confidence interval. P values<0.05 were considered significant. Simple linear regression analysis was carried out on significantly correlated clinical parameter. Considering one specific parameter as dependent and other correlated parameters as predictor variable, standardized residuals were obtained and clarified for association analyses. Association between serum PON1 AREase activity and genotypes of *PON1* (rs854573) A>G promoter polymorphism was analyzed by parametric independent t test and ANOVA to examine equality of means and non-parametric Kruskal–Wallis H test which makes no assumption of normal distribution or equality of means/variances of the quantitative index under different genetic model (Table 2). A multilinear regression analysis was done among different clinical parameters and serum PON1 activity. Densitometric data of western blot analysis regarding serum PON1 mass obtained from image J software. Excepting western blot data analysis, all other statistical clarifications were performed by SPSS version16 software package.

Table 2. Demographic and clinical characteristics T2DM patients and non-diabetic controls and multiple linear regression analysis with respect to serum paroxonase activity.

	T2DM (Mean ± Std error) n=101	Controls (Mean ± Std error) n=102	Significance (p value)	Multiple regression ^b t statistic (p value)
Age	52±11.89	64±7.24	<0.001 ^a	-0.144 (0.886)
Sex	58 Male, 43 Female	53 Male, 49 Female	-	0.977 (0.333)
BMI	25.25 ± 3.17	23.97± 3.181	0.01 ^a	0.933 (0.316)
Fasting blood glucose level (mg/dl)	160.31 ± 56.43	80.33 ± 6.63	<0.001 ^a	0.922 (0.357)
Postprandial blood glucose level (mg/dl)	201.501 ± 74.49	128.30 ± 11.71	<0.001 ^a	0.9 (0.34)
Serum PON1 AREase activity	1.54 ± 0.96	3.897 ± 2.33	<0.001 ^a	Dependent Variable

All the data were represented as mean ± SE, ^ap value<0.05 in Student's t test.

^bMultiple regression was performed using serum PON1 AREase activity as a dependable variable.

3.2. Genetic Association of A>G *PON1* (Rs854573) Polymorphism with T2DM

The major allele frequencies of A>G *PON1* (rs854573) polymorphism were 0.77 and 0.92 in case and control populations respectively. HWE prevailed for both the groups. Significant differences in the proportion of genotype frequencies rs854573 SNP with the disease were observed between T2DM and healthy controls following both codominant (p value<0.0001, Odds Ratio 3.46 95% CI 1.88 to 6.35) and dominant (p value 0.0002, OR 3.49, 95% CI 1.77 to 6.9) patterns of inheritance (Table 2). Allele frequencies of two groups were also found to vary significantly (p value<0.0001, chi-square 17.5, OR of risk allele 8.6882, 95% CI 1.0662 to 70.7996) (Table 2).

3.3. Serum *PON1* in the Study Population

The densitometric analysis of the bands obtained in Western blot analysis using the same amount of pooled serum samples (n=13 in cases and n=17 in controls) and monoclonal *PON1* primary antibody (sc-59646, Santa Cruz; 1:1000) identified significantly lower level of serum *PON1*

3. Results

3.1. Clinical Demography

A total no of 203 individuals (Cases=101 and Controls=102) participated in the present study. Relevant demographic and clinical parameters have been collected from the study participants (Table 1). The data presented for the patients corresponded to that collected prior to any medical interventions. Since log transformation for clinical variables approximated the features of normal distribution correlation between different clinical parameters was explored using log transformed data (Table 1). The crude and log transformed values of clinical parameters were found to differ significantly between cases and control groups. Significant correlations were observed between BMI and fasting sugar level, fasting and Post Prandial (PP) sugar levels in the patients. None of the clinical parameters was found to be related with serum *PON1*AREase activity in diabetic group. Serum *PON1*AREase activity was found to be dependent only on *PON1* A/G (rs854573) (P=0.0001) polymorphism as revealed by multiple linear regression analysis (Table 1).

protein concentration in diabetic patients compared to that control subjects (p value 0.008, Figures 1B. 1, 1B. 2). In accordance with this observation, mean serum *PON1* AREase activity (1.54 kU/L/ SD ± 0.96) in T2DM patients was found to be decreased significantly (p value=0.00001, Table 4, Figure 1A) compared to that normo-glycemic group (3.897 kU/L/ SD ± 2.33) (Tables 1, Figure 2). *PON1* AREase activities were found to differ significantly between AA, AG and GG genotypic classes in the T2DM but not in the normal healthy controls (Table 4). Mean serum *PON1* AREase activity in AA genotypic class was significantly higher than mean enzyme activity obtained by pooling AG and GG genotypes in the diseased group using both parametric and non-parametric tests (Tables 3, 4, Figure 2). Pairwise comparison by parametric t-test revealed significant differences between diabetic and non diabetic homozygous (AA) for major allele (p value<0.001, Table 4, Figure 2) and between diabetic and non diabetic heterozygotes (AG) (p value<0.001, Table 4, Figure 2). A highly significant non parametric asymptomatic difference was also obtained for the same comparison (p<0.001, Table 4, Figure 2).

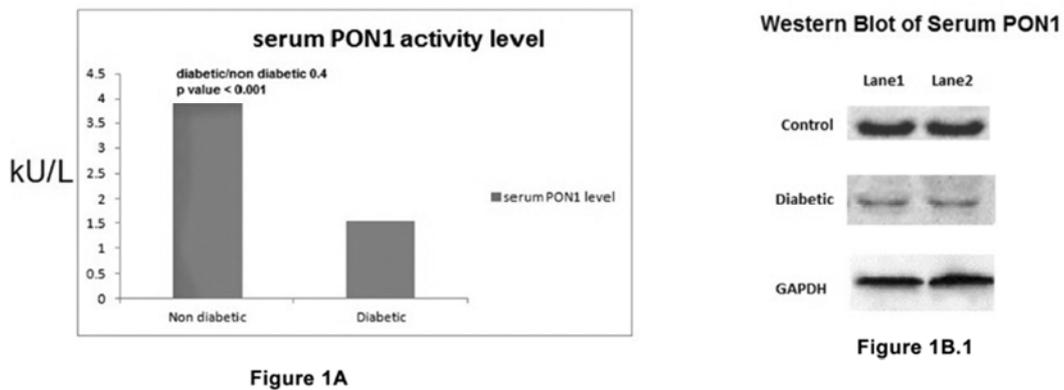


Figure 1A

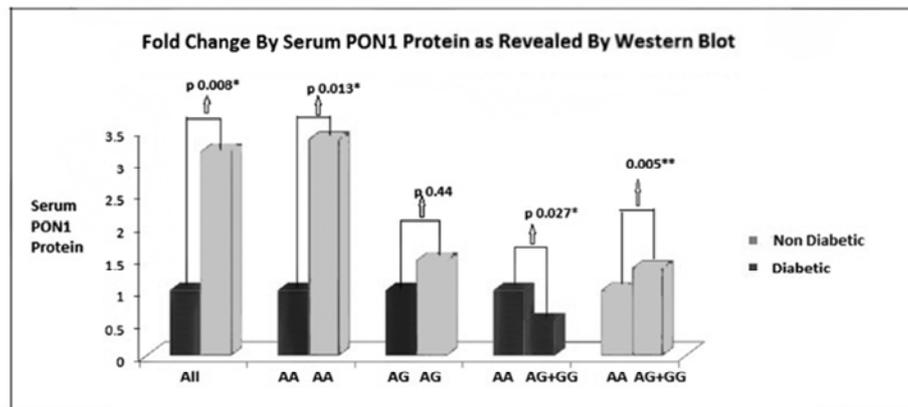


Figure 1B.2

Figure 1. 1A Serum PON1 Arylesterase activities in DM control and patients. Data are represented as Mean \pm S. E. Significant difference between two groups are indicated as p value < 0.001. Figure 1B Decreased level of serum PON1 protein in diabetic patients. Figure 1B. 1: Western blot analyses were performed with stored serum isolated from venous blood of DM patients and controls. The controls were free from diabetic complications. The blots were probed for mice anti human PON1 primary antibody (Santa Cruz, USA) and re-probed with goat GAPDH. The ratio of PON1 to GAPDH was measured. Representative results were shown. Figure 1B. 2 Densitometry of the Western blot results. The signals of the PON1 from thirteen DM samples and seventeen controls were normalized with that of the GAPDH signals. Data are represented as mean \pm SEM. The fold differences of serum PON1 protein levels in the signals between DM patients and controls, and the P values of non parametric Kruskal Wallis tests are indicated.

4. Discussion

4.1. Observations of Present Study

This study presented a statistical correlation between different clinical parameters relevant to T2DM including BMI, fasting and postprandial blood glucose levels with *PON1 A>G* (rs854573) polymorphism. Several rigorous statistical tests such as non-parametric Kruskal Wallis, residual analysis of clinical parameters with single logistic regression, parametric t Test and ANOVA were employed to detect genetic association of *PON1 A>G* (rs854573) with T2DM. The genetic association between *PON1 A>G* (rs854573) genotypes and T2DM followed both codominant and dominant models of inheritance, with AG and GG genotypes were remarkably enriched among the patients (Table 2) [32]. Keeping in line, allele frequency of G was found to be high in T2DM patients (Table 2). The study presented significantly higher serum PON1 protein concentration in diabetic and non diabetic participants (Figure 1B). A significant difference in the level of serum PON1 AREase activity was

detected between the healthy control and the diabetic individuals (Mean 3.897 kU/L vs 1.54 kU/L, p value 0.00001, Table 1, Figure 1A) and this difference to an extent was found to operate in a genotype dependent manner such as AG and GG genotypes in the T2DM group have lower enzyme activity than that of AA genotype (p value asymptomatic < 0.01, Table 4, Figure 2). The serum PON1 AREase level did not show any relation with other clinical parameters (Table 1). The unobserved correlation of PON1 enzyme activity with other clinical parameters corroborated with that presented by Ferré et al., 2013 (Ferré et al., 2013, 18). On the other hand reduced PON1 activity in subjects with T1DM and T2DM was inversely associated with glucose concentrations [33, 34]. This inconsistency could be attributed to ethnic differences between the study populations.

4.2. PON1 Promoter SNPs Contribute in Diseases with Oxidative Stress

PON1 SNPs have strong contribution on worldwide association studies especially in diabetes and oxidative stress related diseases. A numbers of different SNPs like an amino

acid substitution at position 192 (Q192R) polymorphism or coding region L55M polymorphisms have been found to be associated with DM and CAD patients in different Indian populations [19, 35], or in Turkish DM populations [36, 37]. T2DM patients with LL genotype (rs 854560 L55M) were found to have an increased risk of CAD and retinopathy [38, 39]. The contributions of PON1 promoter polymorphisms; -107 C/T (rs705379), -909G/C (rs854572) in atherosclerosis and DM are well established [40]. Genotyping of *PON1* locus identified promoter polymorphisms (-108 T>C, -832 G>A, -1741 G>A) to be associated with hepatocellular and cholangiocellular carcinoma with decreased PON1 and PON3 expression [41]. The protective effect of the *PON1* rs662 AA genotype on lung cancer risk was limited to non-smokers in a Korean population [42]. However a limited no of studies conducted on rs 854573 *PON1* A>G polymorphism in Indian T2DM cohort failed to find any association with disease [43]. In this respect the observed association in our present study, is 1st report on Eastern Indian T2DM cohort.

4.3. *PON1* Enzyme Status: Its Potential Role for Developing Diabetes and Other Diseases with Oxidative Stress

The enzyme PON1 of *PON* gene family is a calcium dependent 45-kDa protein coded by chromosome 7q21-22. PON1 and PON3 are associated with serum HDL [44]. Determination of PON1 activity has been done not only in different oxidative stress related diseases but also in different ethnic groups (Gupta *et al.*, 2011, 19). Serum PON1-HCTLase and PON1-AREase activity were reported to be significantly lowered in DM (Boemi *et al.*, 2001; 21; Hampe and Mogarekar, 2014; 36; MacKness *et al.*, 2000; 22; Sonoki *et al.*, 2009; 23; Wheeler *et al.*, 2004, 20). Similar reduction of serum PON1 activities in asthma patients with elevated PON1 protein expression of lung tissue in control experimental mice, were reported [45]. Level of serum PON1 activity found to be inversely correlated with serum PON1 concentration and hepatic PON1 protein expression in chronic liver impairment [46]. On contrary, positive correlation of both parameters are evident in pediatric non alcoholic steatohepatitis [47], atherosclerotic heart disease [48], rheumatoid arthritis [49], age related macular degeneration [50], and cataract [51]. In context of different ethnic groups, serum PON1 activity severely affected Turkish [52], Egyptian or Brazilian T2DM cohort [53].

4.4. Polymorphism Influence of *PON1* SNP on Its Activity and Serum Protein Level

Polymorphism influence of *PON1* has been widely studied in human medicine with excellent reviews produced by different research groups [54]. Several polymorphisms in the coding and promoter regions of the *PON1* gene including *PON1*192, *PON1*55, *PON1*-162, *PON1*-832, *PON1*-909, *PON1*-1076 and *PON1*-1741 have been associated with changes in the enzyme's activity and/or concentration [18, 55]. Not only in diabetes, PON1 enzyme level can range widely, even between individuals with the same PON1 phenotype [56] in different diseases. In early studies PON1 phenotypes did not influence changes in PON1 associated with some clinical conditions such as hepatic disease [57].

However selection of substrates to measure PON1 activity is very crucial, since substrate specificity becomes highly influenced by polymorphisms. Paraxon as substrate in humans, has a potential for obtaining false high values of PON1 in populations with high frequency of *PON1R* when compared with populations with high frequency of *PON1Q* and vice versa [58]. The assays using p-nitrophenyl acetate as substrate had higher analytical variability than did paraoxon and phenyl acetate (PA) assays in other studies [59]. However lack of investigations of polymorphic study involving PON1 AREase activity using p-nitrophenylacetate as substrate in India encourages us to compile a pilot measurement of PON1 activity. The codominant significance as revealed by one way ANOVA in present study is 0.002 in T2DM patients (AA vs AG vs GG genotypic classes) (Table 4). Result of ANOVA separating the sources of variation into analytical variance, either within group or between groups variance indicate majority of difference were contributed by differences among individuals. Therefore it could be hypothesized that the non enzymatic and non-paraoxonase-specific hydrolysis of p-nitrophenylacetate in the reaction is the major contributor to this variability. In addition PON1 AREase activity shows significant non parametric asymptomatic differences (p value<0.001) between different genotypic groups (AA vs AA, AG vs AG, AA vs AG+GG) of diabetic and non diabetic participants (Tables 3, 4, Figure 2). The PON1 AREase activity is therefore thought to be genetically determined and has marked racial and inter individual variation, which might be the reason for apparent inconsistency among different studies reporting serum PON1 AREase activity [60, 61].

Table 3. *PON1* AREase activities in three genotypic classes (rs854573) of T2DM cases and healthy controls.

Genotypes <i>PON1</i> A>G	Controls (102)			Patients (101)		
	No	<i>PON1</i> AREase activity (kU/L) (Mean ± S. E)		No	<i>PON1</i> AREase activity (kU/L) (Mean ± S. E)	
		Range			Range	
AA	87	3.981±2.39	1.01-9.99	63	1.676±0.974	0.561-5.25
AG	14	3.579±1.947	1.425-6.92	30	1.402±0.957	0.666-5.25
GG	1	0.96	0.96	8	0.754±0.17	0.508-0.99
AA+AG	101	4.02± 2.32	1.425-9.99	93	1.589±0.97	0.666-5.25
AG+GG	15	3.42± 1.99	0.96-6.92	38	1.29±0.907	0.508-1.40
All	102	3.897 ± 2.33	0.96-9.9	101	1.54 ± 0.96	0.508-5.25

Table 4. Analysis of serum PON1 AREase activity in relation to rs854573 genotypes.

Combination of different genotypes		Parametric tests (p values)		Non-parametric tests (p values)
		ANOVA	Independent sample t-Test	Kruskal Wallis test
AA vs GG	T2DM	0.003**	0.001**	<0.001**
	Healthy controls	0.380	0.073	0.211
AA vs AG	T2DM	0.232	0.083	0.019*
	Healthy controls	0.063	0.609	0.599
AG vs GG	T2DM	0.057	0.017*	0.003**
	Healthy controls	0.321	0.138	0.163
AA	T2DM vs Healthy controls	<0.001**	<0.001**	<0.001**
AG	T2DM vs Healthy controls	<0.001**	<0.001**	<0.001**
Diabetic AA vs Diabetic AG+GG		0.009**	0.009**	0.001**
Control AA vs Control AG+GG		0.380	0.375	0.359
Diabetic AA+AG+GG vs Control AA+AG+GG		<0.001**	<0.001**	<0.001**

* indicates P value<0.05; ** indicates P<0.01.

In the present scope of investigation, immunoblotting of serum PON1 protein level explored marked significant differences in diabetic and non diabetic populations (Figure 1B. 1 & 1B. 2), with enriched protein levels in people containing two A alleles (Homozygous major allele carrier) rather than people having two G (Homozygous minor allele carrier) or one A and one G alleles (Heterozygous carrier) both in patients and controls (Figure 1B). However, an inverse relationship of decreased serum enzyme activity with elevated expression of PON1-mRNA and PON1 protein had been found in patients of chronic liver impairment [62]. Chronic liver diseases are associated with increased oxidative stress, MCP-1 synthesis, and inflammation [63]. Therefore the protective role of over-expressed PON1 protein or mRNA is evident against development of chronic liver diseases since liver plays a key role in the synthesis of PON1. Recently, it was reported that PON1 mass inversely predicts mortality in patients on hemodialysis. Thus, the indication for the measurement of the mass of PON1 by immunoblotting, continue to expand and facilitates its use as a biomarker to predict risk for various vascular diseases and diabetes [64].

Not only between total diabetic and healthy participants, a significant lower range of serum PON1 AREase activity also has been found in diabetic AA genotypes with respect to healthy AA genotype (0.561-5.25 kU/L vs 1.01-9.99 kU/L, p<0.001, Figure 2). This indicates disease status to be contributor of the lower serum PON1 activities in the present investigation. It has been previously shown that the presence of diabetes severely affects PON1 activity and concentration; independently of a genetic effect on the PON1 phenotypic distribution [52]. The cause of the lower AREase activity in diabetic patients is still to be understood. Paraonase, a protein, with 369 amino acids, is believed to be anchored to the HDL by its hydrophobic N-terminal end. There are reports of significantly decreased levels of HDL-C and PON1 AREase activity in diabetic patients with or without complications [65]. However, in some cases absence of a correlation between AREase activity and HDL cholesterol suggests that the lower AREase activity in diabetes is caused by factors other than the lower HDL cholesterol level. DM is characterized by, oxidative stress related consequence of glucose autoxidation, production of AGEs, and/or activation

of the polyol pathway [54]. As PON1s are involved in the protection of LDL oxidation, a lipid peroxidation biomarker malondialdehyde (MDA), routinely analyzed to evaluate the degree of oxidative stress, has been found to be increased in condition of decreased serum PON1 activity [51]. The poor glycemic control in T2DM may lead to increased glycation of proteins and other biomolecules resulting in altered conformation and/or function of enzymes and structural proteins [66; 67]. Furthermore, the glycation, or nonenzymatic glycosylation, of several structural and functional proteins has been described previously in diabetes [68]. An earlier study has indicated that PON1 is susceptible to glycation and PON1 activity is reduced by 40% upon glycation [69]. To examine the effect of glycation on PON1 structure, Hashim and coworkers used a glycated model of human PON1 using carboxy methyl-lysine (CML) and pentosidine and compared them with the nonglycated models. It was already been suggested that substitutions or small changes in the amino acid side chain of N168, N224, and D269 of human PON1 protein may cause perturbation in neighboring residues or may affect the size and orientation of the calcium-binding site [70]. The orientation of catalytically active dyad H115 and H134 remained unchanged in glycated model but the presence of pentosidine/ CML molecules at Lys70 and Lys75 affected the binding of substrate since the backbone of these residues cover the top of the active site to some extent that continues in the loop region (residues 72–79) forming an active-site lid [71]. Furthermore the glycation of PON1 at Lys70 and Lys75 made some conformational changes in the residues D269, N168, and N224, which might contribute toward the lowering of PON activity [51]. Therefore condition like oxidative stress that could lead to enhanced glycation of proteins; increase the risk of disease status by lowering the PON1 activity level.

4.5. PON1 Shows Selective Modulation in Specific Drugs Against T2DM Therapy

Several studies are being conducted using a no of different antioxidative drugs such as simvastatin, rosiglitazone, in T2DM therapy. Simvastatin treatment shows no effects on PON1 activity modulation in *Q192R* and *M55LPON1* polymorphisms [72] but rosiglitazone showed favorable

effects on improvement in glycemic control and to an increase in paraoxonase activity and HDL-C levels [73]. Although the present study is devoid of any therapeutic management, the excessively low levels of PON1 AREase

activity give some lights into pharmacogenomic aspect of the study implying the need to either rosiglitazone like drug treatment in patients or need of thorough care in patient groups using a combination of drug therapies.

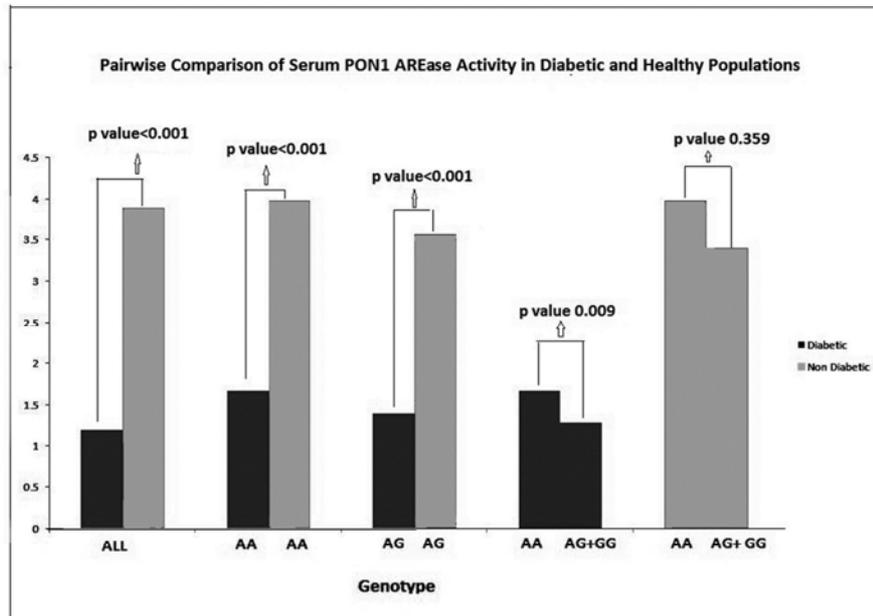


Figure 2A

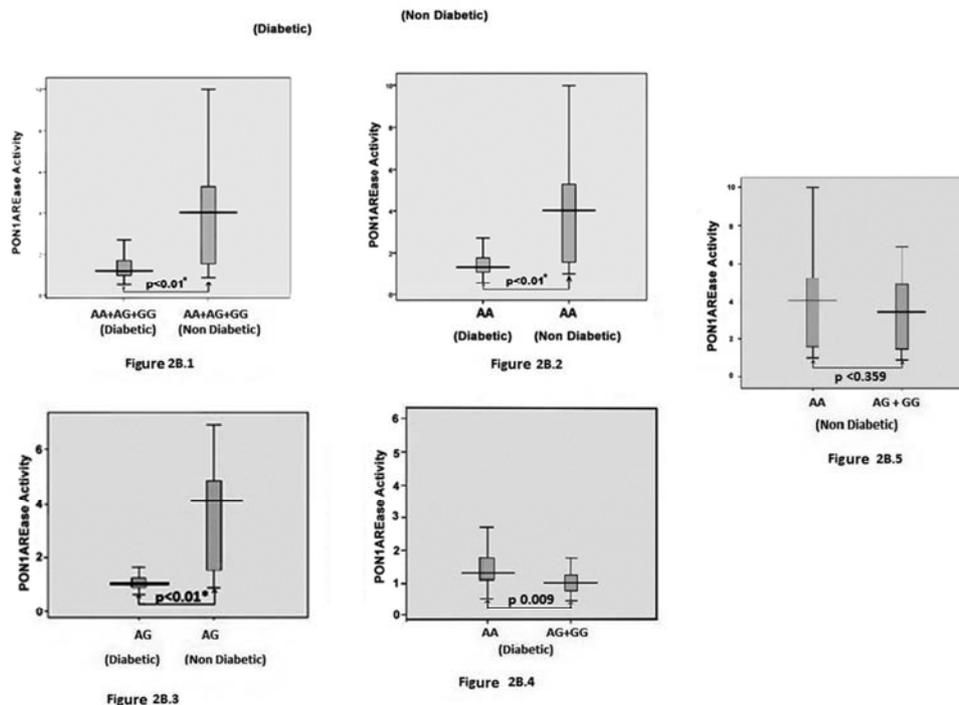


Figure 2. Association between rs 854573 *PON1 A>G* genotypes with serum *PON1 AREase* levels represented in histograms and boxplots. 2A. Histogram showing difference between serum *PON1 AREase* activity levels in DM patients and controls as well between different genotypic group. Data are represented as Mean \pm S. E. Differences are indicated in p value. 2B. 1. Diagram represented the distribution of serum log-*PON1 AREase* activities across DM patients and controls. 2B. 2. Diagram represented the distribution of log- serum *PON1 AREase* activities across homozygous AA genotypes of DM patients and controls. 2B. 3. Diagram represented the comparison of log-serum *PON1 AREase* activities between heterozygous AG genotypes. 2B. 4. Difference represented distribution of log serum *PON1 AREase* activity in minor homozygous genotype (GG) with AA and AG genotypic groups pooled in DM patients. 2B. 5. Difference represented distribution of log serum *PON1 AREase* activity in minor homozygous genotype (GG) with AA and AG genotypic groups pooled in healthy donors. Statistical significance between pairwise comparisons was mentioned. Statistical significance was determined by Parametric t-Test and non-Parametric Kruskal Wallis tests. * indicates p value significance in 95% level. The bottom, middle line, and top of each box correspond to the 25th percentile, median, and the 75th percentile, respectively. Bars extend to the lowest value and to the highest value of each group.

4.6. Limitation of the Study and Future Scope

Finally, it is important to highlight the limitation of the present study. The present study conducted only on PON1 activity and protein level as disease prediction marker but studies on other PON family member proteins such as PON2 or PON3 could give some more focus on its expression level. However our study size is quite competent for predicting role of human PON1 protein in genotype based risk assessment in T2DM. Our study failed to predict any transcription factor (TF) binding sites in sequence of rs 854573 *PON1 A>G* SNP site. *PON1* promoter polymorphism has a significant effect on expression of *PON1* gene as *-108C>T* polymorphism lies within GGCGGG consensus sequence, which is the binding site for the Sp1 transcription factor (TF) [74], or other transcription factors like p53, Irf1, GATA1 binding sites at different *PON1* promoter polymorphic regions [54]. Mammalian TF Specificity Protein 1 (Sp1) plays an essential role in regulation of PON1 expression. High glucose level activates protein kinase C (PKC), which activates Sp1, and stimulates *PON1* transcription in human hepatoma cell lines HepG2 and HuH7 [75]. A potent PKC activator phorbol 12-myristate 13-acetate (PMA) also stimulates *PON1* transcription in HepG2 through activation of Sp1. Therefore the need of more expression studies including large polymorphic zones of *PON1* in future perspective is hypothesized.

5. Conclusion

In conclusion type II diabetic patients have much lower serum PON1AREase activities compared to healthy controls in Eastern Indian T2dM cohorts. They also have some decreased serum PON1 protein level in comparison to control populations. The association of *PON1 A>G* polymorphism predicts the risk in case samples. Lower serum PON1 AREase activities therefore imply the use of described methods for measurement of PON1 activity and accurate genotype assignment are rapid and have potential to facilitate the efficient investigation of PON1 status in clinical and epidemiological studies since PON1 AREase activity is a good biomarker for predicting risk of diabetes prevalence.

Conflict of Interest

The authors declare no conflict of interests.

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