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 (assymptometic 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
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 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 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-1 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-nitrophenol 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-thiobutil 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 more sensitive and specific substrate in identifying patients with chronic hepatic disease than did paraoxon [17].

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 PON1AREase 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 the 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 (µLs) 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 µL 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 rs834573 between diabetic patients and healthy controls.

| Disease Status | Genotype | Comparison of genotype proportion (p value) | Major Allele frequency | Minor allele frequency | Comparison of allele frequency |
|---------------|----------|---------------------------------------------|-----------------------|-----------------------|-------------------------------|
| Case          | AA 63    | Co-dominant p<0.0001** OR=3.46, OR=0.0002** OR=3.49, 95% CI=1.77-2.69 | A 0.149               | G 0.02                | p value=0.0001, chi-square 17.5, OR=8.688, 95% CI=1.066-70.79 |
|               | AG 30    |                                            |                       |                       |                               |
|               | GG 8     | Dominant p=0.0001** OR=3.46, OR=0.0002** OR=3.49, 95% CI=1.77-2.69 |                       |                       |                               |
| Control       | 87       | 95% CI=1.88-6.35                           |                       |                       |                               |
|               | 14       | GG vs GG                                   | 0.92                  | 0.08                 |                               |
|               | 1        |                                            |                       |                       |                               |

* 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 CaCl2 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 µL diluted serum sample (1: 20 in TRIS-HCl buffer) to 288 µL working reagent followed by 72 µL 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 µL volume of sample was loaded into each well (equivalent to 125 µL 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 reb 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 ± 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 regressiona t statistic (p value) |
|-----------------------------|-----------------------------|----------------------|---------------------------------|
| Age                         |                             | <0.001               | -0.144 (0.886) |
| Sex                         | 52±11.89 Male, 49 Female    | 64±7.24 Male, 49 Female | -0.922 (0.357) |
| BMI                         | 25.25 ± 3.17                | 23.97± 3.181         | 0.933 (0.316) |
| Fasting blood glucose level (mg/dl) | 160.31 ± 56.43 | 53 Male, 43 Female | 0.977 (0.333) |
| Postprandial blood glucose level (mg/dl) | 201.501 ± 74.49 | 23.97± 3.181 | 0.977 (0.333) |
| Serum PON1 AREase activity  | 1.54 ± 0.96                 | 3.897 ± 2.33         | 9.0 (0.34) |

All the data were represented as mean ± SE, *p value<0.05 in Student’s t test. 

*Multiple 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 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). Serum 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).

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 PON1AREase activity in diabetic group. Serum PON1AREase 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).
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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 PON1A>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 asymptometric<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 Ferre• 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
acidi 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 hepatic cellular 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
expression 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
PON1192, PON155, 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
generically 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 PON1 | Controls (102) | Patients (101) |
|----------------|---------------|---------------|
| A>G            | (Mean ± S. E) | (Mean ± S. E) |
| PON1 AREase activity (kU/L) | Range | Range |
| No | | |
| AA | 87 | 3.98±1.23 | 1.01-9.99 | 63 | 1.67±0.974 | 0.561-5.25 |
| AG | 14 | 3.57±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 |
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 (Homzygous major allele carrier) rather than people having two G (Homzygous 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. Paraoxonase, 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 consequent 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 cowokers 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 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 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 and pentosidine and compared them with the nonglycated models.

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                           | 0.138              | 0.163   |
|                                   | Healthy controls            | 0.321                           |                    |         |
| 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.
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 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 activity levels in DM patients and controls as well between different genotypic group. Data are represented as Mean ± S. E. Differences are indicated in p value. 2B. 1. Diagram represented the distribution of serum log-PON1 activities across DM patients and controls. 2B. 2. Diagram represented the distribution of log- serum PON1 activities across homozygous AA genotypes of DM patients and controls. 2B. 3. Diagram represented the comparison of log-serum PON1 activities between heterozygous AG genotypes. 2B. 4. Difference represented distribution of log serum PON1 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 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 GCCGGG 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 PON1 AREase 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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