

## Correlation between HDL Levels & Paraoxonase Activity in Serum of Patients with Type II Diabetes Mellitus

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**Abstract:** Type 2 diabetes mellitus is a hereditary disease, which has a close correlation with development of coronary artery disease. It has been shown that the HDL-associated enzyme, paraoxonase (PON1), is responsible for prevention of the oxidation of LDL, thereby protecting against complications like coronary artery disease and myocardial infarction in patients of diabetes mellitus. So, PON1 functions as an anti-oxidant. In this study, the paraoxonase (PON1) activity was correlated with the levels of HDL in serum of the patients of type II diabetes mellitus. There were 100 patients of type II diabetes mellitus, ranging from 40 to 80 years of age. A significant positive correlation was found between serum PON1 activity and HDL cholesterol levels. It appears that this association contributes to the protection conferred by HDL, against low density lipoproteins (LDL) peroxidation and subsequent development of coronary artery disease.

**Keywords:** Coronary Artery Disease, Diabetes Mellitus, High Density Lipoproteins, Low Density Lipoproteins, Paraoxonase Enzyme.

### INTRODUCTION

Type 2 diabetes mellitus is a hereditary disease, which has a close correlation with development of coronary artery disease. For several decades it was known that hypercholesterolemia is one of the major risk factors for coronary artery disease leading on to myocardial infarction.

HDL appears to decrease the accumulation of lipid peroxides on LDL by a mechanism that is, at least in part, enzymatic. It has subsequently been shown that the HDL-associated enzyme, paraoxonase (PON1), is one of the enzymes of HDL responsible for its ability to prevent the accumulation of lipid peroxides on LDL.

“Paraoxonase is located in the blood on HDL, the “good cholesterol”, and it can break down oxidized LDL to non-harmful products”. The discovery of this enzyme’s activity opens a possible new route to prevention of heart diseases. The real function of the enzyme has been something of a mystery since it was discovered more than 40 years ago. Its previous known function was to breakdown organophosphates, chemicals that are used as insecticides and poisonous gases. That activity was obviously not the complete story of paraoxonase, as humans do not normally contain these substances in their blood.

Researchers later found a very strong inverse relationship between the activity of paraoxonase in the blood and the risk of heart disease. Lower activity is associated with higher risk. The subsequent studies

helped to understand the mechanism behind that relationship.

The next step was to find out how to regulate the activity of paraoxonase and to increase its level in human blood. If we could find means of changing the enzyme activity, we could look for methods of intervention. This could have very strong implications on heart disease therapy.

Work has been done for the past decade on the mechanism by which blood cholesterol quantity (cholesterol levels), as well as blood cholesterol quality (cholesterol oxidation) affects atherosclerosis. It has been shown that patients with a high risk of coronary heart disease have increased LDL oxidation.

Plasma lipids are derived from food (exogenous) or are synthesized in the body. The lipids

which are generally insoluble in water are largely transported within the body in an aqueous medium. To accomplish this and thereby make lipids available for metabolism, the lipids are first complex with various specific proteins. The resultant lipoproteins are globular particles & consist of varying amounts of neutral lipids, (eg. Triglycerides or cholesterol esters), at the central core, surrounded by a coat of unesterified cholesterol, phospholipid and protein. Free fatty acids (FFA) are chiefly carried by albumin.

**Objectives of the Study**

Patients with diabetes mellitus have an increased risk for atherosclerotic vascular disease. One of the major contributing factors is the dyslipidemia, which is associated with the disease. Diabetes mellitus is usually associated with increased levels of total cholesterol and LDL cholesterol and decreased levels of HDL cholesterol. It is well known that LDL cholesterol is a risk factor whereas HDL cholesterol is protective factor for cardiovascular or atherosclerotic vascular disease.

Alterations in lipoprotein metabolism may also be a contributing factor, as diabetics are susceptible to atherosclerotic changes, despite having normal levels of plasma lipids. A lot of work has been carried out in order to delineate the mechanisms involved in the accelerated atherosclerosis in these patients.

Recent work has shown that the enzyme paraoxonase, which is associated with HDL, could function as an antioxidant. Paraoxonase prevents peroxidations of both LDL and HDL. It could therefore have an intimate role to play in the etiopathogenesis of atherosclerotic vascular disease.

**The following parameters were estimated:**

- Serum paraoxonase activity.
- Serum high-density lipoprotein levels
- The enzyme activity was studied in the patients using (I) CaCl<sub>2</sub> only and (ii) using CaCl<sub>2</sub> and NaCl.

The paraoxonase activity was correlated with the levels of HDL in the serum of the patients.

Sl.No.	PARAMETERS	METHODS
1.	HDL cholesterol	Lipoprotein separation by polyanion method, then cholesterol esterase-horse radish peroxidase using N, N-diethylaniline HCl/4-aminoantipyrine.

**Serum Paraoxonase (PON1)**

Serum paraoxonase activity was estimated by a spectrophotometric method using p-nitrophenyl acetate as the substrate.

**Principle**

The serum is incubated with buffered p-nitrophenyl acetate as a substrate. The rate of formation of p-nitrophenol was measured spectrophotometrically.

**MATERIALS AND METHODS**

This study was conducted during a period of one year in our hospital.

- 100 patients of type 2 Diabetes Mellitus were taken.
- The patients were based on the following inclusion and exclusion criteria:

**Inclusion criteria**

- Patients attending the Diabetic Clinic of our hospital formed the subjects of this study.
- Only patients with an established diagnosis of type II diabetes mellitus were included (known diabetics under treatment for more than 2 years)

**Exclusion criteria**

- Any patient on lipid lowering drug was excluded.
- Patients with type I diabetes mellitus were excluded.

**Subjects**

Selection of subjects for study was done as follows:

**Study groups**

Were patients of type II diabetes mellitus? There were 100 patients ranging from 40 to 80 years of age.

**SAMPLING**

Patients reported to the lab after 12 hours of fasting. 5ml of blood samples were collected in Vacutainer<sup>(R)</sup> gel tubes and subjected to centrifugation. Clear serum was separated and used for the following biochemical investigations using standard clinical chemistry methods (analysis was carried out within an hour after the collection of samples):

- Serum HDL cholesterol levels.
- Serum paraoxonase activity.

**METHODS**

These tests were carried out in auto-analyzer DADE-DIMENSION AR<sup>(R)</sup> clinical chemistry system.

**Reagents**

- **Tris – HCl Buffer:** To prepare 200mM solution, which then has to be diluted in 1 in 10 dilutions for use.  
Tris – 0.2 M is 24.2g/L
- 2.42 g/100ml – solution A.  
HCl – 0.2 M is 17.2 ml/ 1000ml.

- 1.72 ml in 100ml – solution B.

**Stock Buffer:** 50ml of solution A & 26.8ml of solution B and the volume made up to 200ml.

**Working Buffer:** 20m M/L – Dilute stock 1 in 10. This is used for diluting serum sample 1:1.

- Tris Buffer containing 1mM CaCl<sub>2</sub>
- Tris Buffer containing 1mM CaCl<sub>2</sub> + 1M NaCl
- **Substrate** – 0.5 ml of alcohol containing 15mg of p-nitrophenyl acetate (Sigma Chemical Co.) freshly prepared.

**Procedure**

• **Non-enzymatic hydrolysis**

3ml of buffer (maintained at 37°C) taken in a cuvette and 50µl of substrate added to it. The rate of change of OD (optical density) was monitored for 2 minutes at 412nm.

• **PON activity**

• **In presence of CaCl<sub>2</sub>:** 3ml of buffer +50µl of serum (1:1 diluted) initial absorbance was adjusted to 0.500. 50µl of substrate was added & the rate of change of absorbance was recorded for 0', 30', 60', 90', and 120' at 412nm.

• **In presence of CaCl<sub>2</sub> & NaCl:** Done as previously describe for CaCl<sub>2</sub>.

**CALCULATIONS**

- PON activity was calculated as n moles of p-nitrophenol formed per minute per ml (volume of assay mixture).
- The molar absorptivity of p-nitrophenol = 17,000 / M/ cm  
= 0.017 / n mol / cm / ml  
= 0.00567 / n mol / 3ml  
(As total volume was 3ml)

So, enzyme activity in 50µl of serum under assay conditions =  $\frac{\Delta OD / \text{min}}{0.00567}$

= ΔOD/minute X 176 µmol/ml/min

**Final activity** Activity expressed as n moles p-nitrophenol formed per minute per ml (volume of assay mixture).

[ΔOD is the corrected OD, i.e., Total OD–non-enzymatic hydrolysis (0.125)]

**OBSERVATIONS & RESULTS**

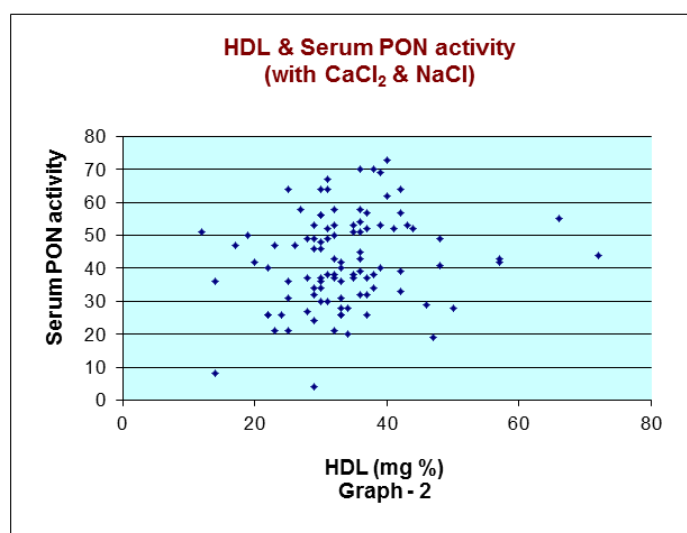
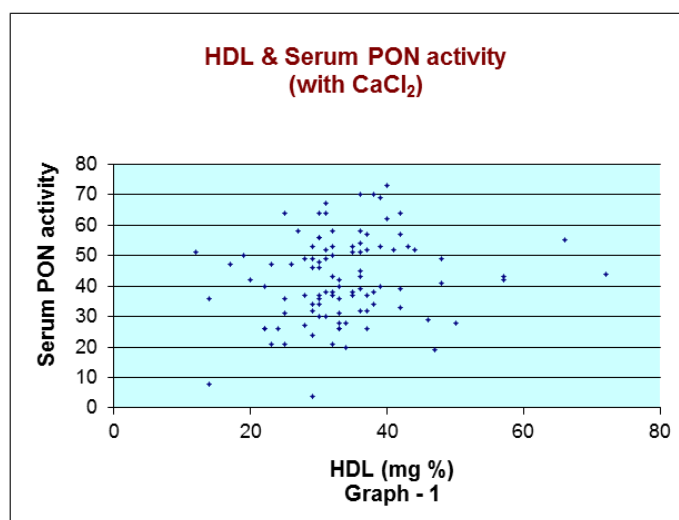
The major aim of the study was to investigate the correlation between serum HDL levels and PON activity in patients with type II diabetes mellitus.

There was a significant positive correlation between PON activity and serum HDL cholesterol levels.

**Serum HDL cholesterol: (Graphs – 1 & 2)**

Parameter	With CaCl <sub>2</sub>	With CaCl <sub>2</sub> & NaCl
PON activity	42.28 ± 13.88	38.14 ± 15.08
HDL Cholesterol	33.39 ± 9.18	33.39 ± 9.18

Parameter	PON activity With CaCl <sub>2</sub> Vs HDL Cholesterol	PON activity With CaCl <sub>2</sub> & NaCl Vs HDL cholesterol
PON activity	r = 0.2313 p < 0.02	r = 0.2015 p < 0.05



## DISCUSSION

In view of the complexity of the disease and its complications, it becomes mandatory to outline in brief, about type II diabetes mellitus, the normal lipid metabolism and the disorder caused by diabetes therein. Then a review of the atherosclerotic process needs to be highlighted with the protective role of HDL and the protection conferred by the antioxidant properties of the enzyme paraoxonase associated to it.

For several decades it was known that hypercholesterolemia is one of the major risk factors for coronary artery disease leading on to myocardial infarction [1]. With improvement in techniques of separation of lipid fractions, it was recognized that there are two major fractions involved in the transport of cholesterol in plasma. While LDL transports cholesterol from the liver to extrahepatic tissues, HDL has a role to play in the “reverse cholesterol transport”, i.e., HDL transports cholesterol from the extrahepatic tissues back to the liver. Based on this finding it was later on demonstrated that while LDL cholesterol was a risk factor HDL was considered to be protective [1]. Several

epidemiological studies have been carried out worldwide in order to substantiate this hypothesis. In the past 2 to 3 decades, the focus of attention has shifted from the lipid moiety of lipoproteins to the apolipoproteins associated with it. It has been recognized that apolipoproteins perform several crucial functions as shown below [2].

- They can form part of the structure of the lipoprotein.
- They can act as enzyme cofactors.
- They act as ligands for interaction with lipoprotein receptors in tissues.

Over the past few years, it has come to light that some of the protein fractions associated with lipoproteins exhibit enzymatic activity. HDL has been shown to be associated with an arylesterase / paraoxonase and also platelet-activating factor hydrolase or PAF hydrolase [1]. There was a resurgence of interest in the probable role that these enzymes could play during physiological and pathological conditions.

Paraoxonase was soon found to have hydrolytic effect on not only paraoxon but also a wide variety of arylesters including lipid peroxides [1, 3]. It was therefore, postulated that at least a part of the protective effect of HDL could be attributed to an antioxidant potential of this enzyme. Could variations in PON be looked upon as a reflection of variations in HDL? Do PON activities correlate with HDL? Would estimation of serum PON be a better index of protection afforded than the levels of HDL itself?

As a first step in this direction, it has to be established whether the values of PON correlate with the values of HDL.

**This study has shown a significant relationship between serum PON1 & HDL levels and has opened up a whole new concept**

As in this study it has been shown that there is significant positive correlation between serum PON1 and HDL cholesterol levels and it is well known that correlation need not necessarily mean causation. However, in this context it could be taken as, at least one of the causative factors because of the fact that PON1 is physically associated with HDL. However, this does not mean that estimation of PON1 activity could replace the estimation of HDL cholesterol as an estimate of its protective effects against CVD.

**Classification of lipoproteins**

Lipoproteins are classified on the basis of their densities which in turn reflect size. The greater the lipid / protein ratio in the complex, the larger it is and lower its density. There are four major classes of lipoproteins [4].

- Chylomicrons
- Very low density lipoproteins (VLDL)
- Low density lipoproteins (LDL)
- High density lipoproteins (HDL)

**High density lipoproteins (HDL)**

Cholesterol synthesized in cells would accumulate there if not removed. The only excretory route is in the bile [4]. The transport of cholesterol from extrahepatic tissues to liver involves HDL. HDL particles are synthesized in the liver and intestine and released into the blood stream by exocytosis.

Abnormalities in lipid metabolism may be genetically predisposed or may be a consequence of imbalance caused by a disease process in the body. Disorders of lipid metabolism may present as dyslipidemias, of which hyperlipidemia needs special consideration here. Diabetes mellitus is usually associated with hyperlipidemia.

Hyperlipidemia is acknowledged to be a major risk factor for atherosclerosis. Most of the evidence specifically implicates hypercholesterolemia,

hypertriglyceridemia plays a less significant role, but its effect may be greater in women than men. The major component of the total serum cholesterol that is associated with increased risk is low-density lipoprotein (LDL) cholesterol [5]. In contrast, there is an inverse relationship between symptomatic atherosclerosis and high-density lipoprotein (HDL) level; thus, the higher the levels of HDL, the lower are the risk of ischemic heart disease. HDL is believed to mobilize cholesterol from developing and existing atheroma and transport it to the liver for excretion in bile. Thus, HDL participates in reverse transport of cholesterol, thereby earning its designation as the good cholesterol [5]. There is thus great interest in dietary, pharmacologic, and behavioral methods of lowering serum LDL and raising serum HDL. Exercise and moderate consumption of ethanol both raise the HDL level, whereas obesity and smoking lower it.

**High – Density lipoprotein cholesterol in diabetics**

Low HDL cholesterol levels in patients with poorly controlled disease, especially those with NIDDM, [6, 7] could contribute to coronary heart disease in diabetes (particularly in women). Low levels of HDL may result from glycation, which accelerates its turn over [8], or from triglyceride elevation. The composition of HDL is abnormal in NIDDM [9] but returns to normal with rigorous metabolic control [10, 11]. HDL protects against atherosclerosis by two mechanisms: reverse cholesterol transport and inhibition of LDL oxidation by paraoxonase [12], which is carried by HDL in plasma [13]. Serum PON1 is tightly bound with high density lipoproteins (HDLs), and it appears that this association contributes to the protection conferred by HDL against low density lipoproteins (LDL) peroxidation.

A further study showed that PON1 not only protects the LDL molecule from lipid peroxidation but the HDL molecule as well [14]. The hypothesis of a protective role of serum PON1 against lipid peroxidation received strong support in the year 2000 when it was reported that combined PON1 / apoprotein E knockout mice present with an increased level of LDL peroxidation and of atherosclerosis. Whether PON1 genetic polymorphisms affect the capacity of PON1 to protect LDL from peroxidation is still a contentious issue. Mackness *et al.* [15] reported that HDL from Q subjects had a higher protective capacity than that from QR and RR subjects. Aviram *et al.* [16] demonstrated that purified PON1 Q was more effective than PON1 R in hydrolyzing cholesteryl linoleate hydroperoxides and cholesteryl linoleate hydroxides from coronary carotid lesions. However, Cao *et al.* [17] showed that PON1 form QQ or RR diabetic patients' decreased LDL peroxidation to a similar extent, suggesting that the Gln/Arg genetic polymorphism has no effect on the enzymes antioxidative capacity.



As the HDL is a very complex molecule with its lipid & protein fractions derived from different sources. It is possible that these fractions could vary independently. The apolipoproteins are under genetic control and probably under regulatory affects of environmental factors. PON could decline despite HDL cholesterol being normal.

Therefore it stands to reason that the atherogenic potential of HDL could be reflected as

- A fall in PON accompanied by a fall in HDL cholesterol.
- A fall in PON with normal HDL cholesterol.
- A normal PON with decreased HDL cholesterol.

Before going into the details of clinical significance of serum PON activities in DM, it would be appropriate to justify the choice of substrate in this particular study.

#### **Cardiovascular disease and diabetes arise from common genetic and environmental antecedents.**

The study of PON is being carried out extensively all over the world. Its relationship to DM, CVD, MI, liver disease etc., is being studied. The common underlying causes are being explained in relation to one another.

It seems reasonable to expect that a complication of a disease should not occur in the absence of the disease itself, nor should it precede the disease that it supposedly complicates. The microvascular complications of diabetes follow these straightforward rules. The macrovascular complications however do not [18]. Large vessel atherosclerotic disease clearly can occur in the absence of diabetes, and there is little doubt that it sometimes precedes it, although how often this occurs has not been well quantified.

#### **SUMMARY & CONCLUSION**

- Serum paraoxonase (PON1) activity was studied in patients with type II diabetes mellitus.
- Serum HDL levels were also determined in these patients with type II diabetes mellitus.
- The paraoxonase (PON1) activity was correlated with the levels of HDL in serum of these patients of type 2 diabetes mellitus.
- A significant positive correlation was found between serum PON1 activity and HDL cholesterol levels in these patients.
- It appears that this association contributes to the protection conferred by HDL against low density lipoproteins (LDL) peroxidation and subsequent damage to capillary endothelium by LDL.
- The paraoxonase (PON1) acts as an anti-oxidant, thus preventing the oxidation of LDL thereby protecting against complications like coronary artery disease in patients of diabetes mellitus.

- So, the results of this study support the theory that HDL protects against atherosclerosis by two mechanisms: reverse cholesterol transport and inhibition of LDL oxidation by paraoxonase (PON1), which is carried by HDL in plasma.
- So, despite normal levels of plasma lipids, diabetics are at an increased risk for atherosclerosis due to qualitative changes in their lipids or dyslipidemia.
- These changes are ultimately manifested in the form of the well-known complications of diabetes mellitus, like cardiovascular disease, nephropathy, neuropathy etc.
- Dietary modifications to include antioxidants would to some extent retard the process.

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