CHAPTER IV

“PROTEOMIC STUDY REVEALS DOWN REGULATION OF APOLIPOPROTEIN A1 IN PLASMA OF POORLY CONTROLLED DIABETES”
IV.1. Introduction

The human plasma proteome analysis has the potential to ease disease diagnosis and therapeutic monitoring (Anderson and Anderson, 2002; Anderson, 2010). Plasma protein biomarkers are useful for diagnosis and prognosis of many diseases including diabetes. Inability to utilize blood glucose is the hallmark of diabetes leading to the development of complications including neuropathy, retinopathy, nephropathy and atherosclerosis (Taylor and Agius, 1988). Development of these complications is 2.5 times higher in patients with long-term poorly controlled glycemic levels than controlled glycemic levels (Chase et al., 1989). Further, on the basis of HbA1c (glycated haemoglobin) levels diabetic subjects can be categorized as controlled diabetics (CD) and poorly controlled diabetes (PCD) with HbA1c levels up to 8% and more than 8%, respectively, of total haemoglobin (Takahashi, 2007).

To understand molecular mechanisms of pathophysiology of diabetic complications, many studies have utilized proteomic approaches and have been reviewed in great detail (Sundsten and Ortsater, 2009) Pathology of diabetic complications is associated with increased generation of Reactive Oxygen Species (ROS) resulting in oxidative, glycoxidative and carbonyl stress (Brownlee, 2001). Advanced Glycation End products (AGEs) upon engagement with the receptor for AGE (RAGE) induce the generation of ROS and activation of transcription factor NF-kB causing changes in gene expression (Giacco and Brownlee, 2010).

AGEs are also known to affect the activity of several plasma proteins. For example, about fifty percent enzyme activity of asparte aminotransferase was inactivated as a result of glycation (Bousova et al., 2005). Similarly impaired activity of glycated alpha-1-antitrypsin was observed in diabetes thereby leading to protease-antiprotease imbalance (Hashemi et al., 2007). Glycated transferrin showed deterioration of antioxidant capacity in diabetic patients (Mohammad et al., 2010). Additionally, glycoxidative modification leads to protein aggregation resulting in protein instability. In order to prevent serious metabolic disturbances caused by accumulation of glycoxidatively modified proteins, these proteins are further degraded by the proteasomal system (Jung and Grune., 2008).
Glycoxidative modification of protein results in the elicitation of autoantibodies against several diabetic plasma proteins. These proteins include albumin, insulin, carbonic anhydrase and heat shock proteins, thereby resulting in their decreased levels in diabetic plasma (Winter and Schatz, 2011). However, any variation in insulin levels affects insulin regulated protein synthesis of several proteins. For example, decreased insulin synthesis and insulin resistance in diabetes affects gene expression of albumin and fibrinogen (Peavy et al., 1978; Tessari et al., 2006). All these factors contribute to the differential protein expression. It is possible that to compensate altered protein functions and protein loss; there could be enhanced or altered gene expression thereby resulting in varying levels of proteins in diabetes. Previous studies have reported differential expression of various proteins like alpha-1 antitrypsin, fibrinogen, vitamin D binding protein, complement C3 and apolipoprotein in diabetes (Blanton et al., 2011; Ceriello, 1997; Engstrom et al., 2005; Lapolla et al., 2008). However, it is important to study the differential protein expression in poorly controlled diabetes to understand the pathophysiology associated with the development of diabetic complications. Therefore, in this study for the first time we have analyzed differential protein expression in plasma of controlled and poorly controlled diabetic subjects by using proteomic methods, and validated by western and dot blot analysis.

IV.2. Materials and Methods

All chemicals were procured from Sigma-Aldrich (St. Louis, USA) otherwise mentioned. Antibodies for fibrinogen, haptoglobin, vitamin D binding protein, alpha-1-antitrypsin, and apolipoprotein A1 (Apo A1) were procured from Abcam, UK.

IV.2.1. Clinical Plasma Sample Collection

Blood samples were collected from diabetic patients through an informed consent from Maharashtra Medical Research Society (MMRS) and approved by the Joshi Hospital Ethics Committee. Fasting blood glucose and HbA1c levels were determined by using a glucometer (Bayer, Germany) and 'in2itTM' analyzer (Bio-Rad, CA, USA) respectively. Plasma was obtained by EDTA treatment, which was then centrifuged at 1500 g for 15 min and the supernatant was stored at -80°C until
further use. Serum creatinine, serum HDL, serum triglycerides and serum cholesterol were estimated using diagnostic kits that were procured from Agappe Diagnostics (Switzerland).

**IV.2.2. Plasma Sample Preparation**

Based on HbA1c levels, plasma samples were grouped into non-diabetic (<6.4%), controlled diabetic (7-8 %) and poorly controlled diabetes (8.8-12.3 %). Ten representative plasma samples with equal volume from each group were pooled and were used for proteomic analysis. In order to remove the high-abundant proteins like Albumin and Immunoglobulin G from plasma, the ProteoPrep® Blue albumin & IgG Depletion Kit, Sigma-Aldrich (St. Louis, USA), were used. 30 μl plasma sample was processed using 500 μl of blue slurry, and the depletion procedure was performed at room temperature according to manufacturer’s instructions. Protein concentration of the depleted plasma sample was determined by using a quick start Bradford protein assay kit (Bio-Rad, CA, USA).

**IV.2.3. 2DE, Western Blot and Image Analysis**

**IV.2.3.1. First Dimension- Isoelectrofocussing (IEF)**

In the first dimension, proteins were separated by IEF with precast IPG strips (nonlinear gradient pH 4-7, 7 cm, BioRad). 150 μg of protein was solubilized in 125 μl rehydration buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 70 mM DTT, 0.5% C7BzO, 1 μl ampholytes pH 3-10. IPG strips were passively rehydrated overnight at room temperature. IEF was performed using the PROTEAN IEF Cell (BioRad, CA, USA) at 20°C with a constant power (50 μA/ IPG-strip) at 250 V for 30 min. slow ramping; followed 4000 V for 2.5 hour, linear ramping; and finally 10000 Volt-hour with linear ramping.

**IV.2.3.2. Second Dimension- SDS PAGE**

After performing IEF, IPG strips were equilibrated in 0.375 mM Tris/ HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS and 2% DTT for 15 min. washed with 0.375 mM Tris/HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, and 2.5%
iodoacetamide for another 15 min. Then the IPG strip was transferred to the top of 12% SDS-PAGE gel, and overlayed with 0.5% low melting agarose. SDS-PAGE was carried out at constant voltage of 70 V for initial 10 min followed by 110 V/IPG-strip for the rest of the time. Resolved proteins were visualized by CBB-R250 staining or by western blotting.

IV.2.3.3. Western Blot Analysis

Proteins were transferred onto PVDF membrane and blocked overnight at 4°C using 5% skimmed milk in TBS. Blocked membranes were then incubated with goat anti-AGE antibody (Millipore, MA) in 1:3000 dilutions for 1 hour at room temperature. Membranes were then washed once with TBS-T (0.05% tween 20) for 3 mins followed by two washes with TBS 3 minutes each. Washed membranes are then incubated with 1:5000 dilution of rabbit anti-goat secondary antibody for 30 minutes at room temperature. Membranes were washed again as mentioned above and were then incubated with streptavidin conjugated horseradish peroxidase with a dilution of 1:5000 for 15 minutes.

Immunodetection on washed membranes was then performed by incubating membranes by using SIGMAFAST™ DAB substrate. Stained gels and developed blots were acquired by the calibrated densitometer (GS 800 Bio-Rad, CA, USA).

IV.2.3.4. Image Analysis

Image analysis was performed using PDQuest Advanced software version 8.0.1 (Bio-Rad, CA, USA). Acquired images were cropped and auto scaled using advanced crop settings. For all the cropped images same pre-processing parameters were used, including background correction which uses the ‘‘floating ball’’ method and the streak removal filter. After pre-processing by using spot detection wizard, total number of spots on the gel was determined. The unmatched spots were manually matched using manual spot editing tools. The spots showing fold change of more or less than 1.5 were considered as differentially expressed proteins.
IV.2.4. Trypsin Digestion

Spots were excised and destained using 50% ACN/50 mM ammonium bicarbonate. After destaining, gel pieces were dehydrated using 100% ACN followed by reduction of proteins with 10 mM DTT for 30 minutes at 56°C. Proteins were then alkylated with 55 mM of iodoacetamide for 45 minutes at room temperature in dark. Gel pieces were washed twice with 50% ACN/50 mM ammonium bicarbonate followed by dehydration using 100% ACN. To the dehydrated gel pieces trypsin was added in 1:10 (trypsin to protein ratio) and was incubated overnight at 37°C. Tryptically digested peptides were extracted with 5% formic acid in 50% ACN. Extracted peptides were vacuum concentrated and were then reconstituted in 5 µl of 0.1% formic acid in 3% ACN.

IV.2.5. LC-MS Analysis and Protein Identification

Two micro-liter digested peptides with final concentration of 100 ng/µl was analyzed by using nanoACQUITY UPLC online coupled to the SYNAPT HDMS system (MS²) (Waters Corporation, USA) as described by Cheng et al., 2009. The separation was performed on a BEH 130 C18 1.7 µM × 75 µM × 150 mm Peptide Separation Technology column at 40°C. Mobile phase A was aqueous 0.1% formic acid and B was 0.1% formic acid in acetonitrile. The protein digests were eluted with a 60-min gradient (0-50% B) and a 90-min gradient (0-50% B). The flow rate was 300 nl/min. An auxiliary pump was used to spray a solution of 500 femto mole/µl Glu1-fibrinopeptide B (GFP) in 50% ACN containing 0.1% formic acid for mass accuracy reference (lockmass channel), with a flow rate of 500 nl/min. MS acquisition was operated in the positive ion V-mode with scan time of 1 second. An alternating low-energy (collision cell energy 3 V) and elevated energy (collision cell energy ramped from 15 to 40 V) was used to obtain the precursor ions (MS) and their fragmentation data (MS²), respectively. A capillary voltage of 3.2 kV, source temperature of 80°C and cone voltage of 32 V were maintained during the analyses. Sampling of the lock spray channel was performed every 30s. The system was tuned for a minimum resolution of 10000 and calibrated using a 500 fmol/µl GFP infusion.
After MSE analysis, data was analyzed by using Protein Lynx Global Server software (PLGS. Version 2.4. Waters Corporation, Milford, MA, USA). For protein identification, processed samples were searched against the UniProt human database containing 44,987 protein entries. Search criteria included fixed and variable modifications as carboxymethylation and oxidation (M) respectively.

IV.2.6. Dot Blot Analysis

Ten plasma samples each from non-diabetic, controlled diabetic and poorly controlled diabetic patients were diluted with PBS in 1:1000 dilutions. 2 μl of the diluted sample was spotted onto nitrocellulose membranes. The membrane was air dried and blocked for two hours with 5% skimmed milk prepared in TBS at 37 °C. The membrane was then incubated with anti-Apo A1 antibody with a dilution of 1:7000 for one hour at room temperature. The membrane was washed twice with (0.05% tween 20) followed by incubation with biotinylated secondary antibody for 30 minutes. The membrane was washed twice with TBS-T followed by incubation with streptavidin conjugated horseradish peroxidase for 15 min at room temperature. Immunodetection was performed by SIGMAFAST™ DAB substrate.

IV.2.7. Statistical Analysis

All experiments were performed in triplicates. Statistical analysis was performed by student’s t test. Data are expressed as means ± SD. P value < 0.05 was considered as statistically significant.

IV.3. Results and Discussion

Diabetic plasma protein differential expression studies help in better understanding of pathophysiology of diabetic complications. Many such studies have been reported earlier with a comparison of ND and diabetic plasma samples (Sundsten and Ortsater, 2009). However, the pathophysiology of diabetic complication is better reflected in PCD than CD. In view of this, the differential plasma protein expression was studied in CD and PCD clinical plasma samples after monitoring fasting plasma glucose, HbA1c levels, serum creatinine, High Density Lipoproteins (HDL), triglycerides and cholesterol levels (Table 4.1).
Table 4.1: Parameters evaluated in ten controlled, ten poorly controlled diabetic patients and ten normal controls for 2DE, western blot, and LC-MS analysis. Data expressed are in mean ± SD; * P< 0.005 when compared with ND; †P< 0.05 when compared with CD.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Fasting plasma glucose (mg/dl)</th>
<th>HbA1c (%)</th>
<th>Serum creatinine (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non diabetic (ND)</td>
<td>87.9 ± 11.7</td>
<td>5.5 ± 0.3</td>
<td>0.7 ± 0.05</td>
<td>50.6 ± 5.6</td>
<td>91 ± 22.6</td>
<td>145.1 ± 15.4</td>
</tr>
<tr>
<td>Controlled diabetic (CD)</td>
<td>92.8 ± 11.55†</td>
<td>7.2 ± 0.6†</td>
<td>0.8 ± 0.02†</td>
<td>42.7 ± 6.2†</td>
<td>116.3 ± 19.2†</td>
<td>146.0 ± 14.0</td>
</tr>
<tr>
<td>Poorly controlled diabetic (PCD)</td>
<td>187.3 ± 23.7†</td>
<td>9.9 ± 1.0†</td>
<td>1.1 ± 0.17†</td>
<td>33 ± 2.1†</td>
<td>219.3 ± 16.8†</td>
<td>200.0 ± 30.3</td>
</tr>
</tbody>
</table>

Proteins were separated by using 2DE followed by staining with CBB-R250 as shown in Fig 4.1. PDQuest analysis disclosed six spots that were differentially expressed. The spots were excised and tryptically digested followed by protein identification by LCMS. Differentially expressed proteins are enlisted in Table 4.2.

The densitometric analysis of 2DE gels revealed upregulation of fibrinogen and haptoglobin and downregulation of vitamin D binding protein, alpha-1-antitrypsin, transthyretin and Apo A1 in diabetic plasma compared to non-diabetic plasma samples.

In diabetes, fibrinogen is upregulated to compensate protein loss in peritoneal dialysis fluid (Prinsen et al., 2003). Further, the elevated levels of fibrinogen are associated with lower platelet inhibition in patients with cardiovascular disease (Ang et al., 2008). Haptoglobin, by binding to hemoglobin prevents the loss of iron and parenchymal injury mediated by hemoglobin during vascular hemolysis (Langlios and Delanghe, 1996).
Figure 4.1: Differential protein expression study by 2DE. (A) 2DE analysis of non diabetic (ND), controlled diabetic (CD) and poorly controlled diabetic (PCD) clinical plasma samples. (B) Densitometric analysis of differentially expressed proteins from ND, CD and PCD.
Table 4.2: Protein identification and fold expression in diabetic and non-diabetic controls by using 2DE and LC-MS\textsuperscript{i} analysis. \textsuperscript{a} - Non-diabetic \textsuperscript{b} - Controlled diabetic \textsuperscript{c} - Poorly controlled diabetes. S- P value was found to statistically significant, \( P < 0.05 \).

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Name</th>
<th>Accession number</th>
<th>mW (Da)</th>
<th>pI (pH)</th>
<th>PLGS Score</th>
<th>Coverage (%)</th>
<th>Fold change (CD\textsuperscript{b}/ND\textsuperscript{a})</th>
<th>Fold change (PCD\textsuperscript{c}/CD\textsuperscript{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alpha 1 antitrypsin</td>
<td>P01009</td>
<td>46707</td>
<td>5.24</td>
<td>1130.88</td>
<td>34.92</td>
<td>0.78</td>
<td>1.05</td>
</tr>
<tr>
<td>2</td>
<td>Vitamin D binding protein</td>
<td>P02774</td>
<td>52929</td>
<td>5.23</td>
<td>7865.01</td>
<td>51.05</td>
<td>0.77</td>
<td>1.04</td>
</tr>
<tr>
<td>3</td>
<td>Fibrinogen gamma chain</td>
<td>P02679</td>
<td>51478</td>
<td>5.23</td>
<td>3187.94</td>
<td>31.78</td>
<td>1.87</td>
<td>1.08</td>
</tr>
<tr>
<td>4</td>
<td>Apolipoprotein A 1</td>
<td>P02647</td>
<td>30758</td>
<td>5.43</td>
<td>1290.93</td>
<td>32.20</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>Haptoglobin</td>
<td>P00738</td>
<td>25176</td>
<td>6.11</td>
<td>3442.24</td>
<td>48.52</td>
<td>1.64</td>
<td>1.03</td>
</tr>
<tr>
<td>6</td>
<td>Transthyretin</td>
<td>P02766</td>
<td>15877</td>
<td>5.39</td>
<td>9106.519</td>
<td>45.83</td>
<td>0.68</td>
<td>1.02</td>
</tr>
</tbody>
</table>
Haptoglobin polymorphism in human is implicated in inflammatory diseases including infections, atherosclerosis, and autoimmune disorders (Langlios et al., 1996). Additionally, haptoglobin is a marker for adiposity, virus-induced autoimmune diabetes and provides protection against the development of diabetic vascular complications (Chiellini et al., 2004; Kruger et al., 2010; Levy et al., 2000). However, in this study, there was no significant change in the levels of haptoglobin in CD and PCD was observed (Fig 4.1). Vitamin D-binding protein (VDBP) is a multifunctional plasma protein associated with bone development, actin scavenger system, binding of fatty acids and immune and inflammatory responses (White and Coke, 2000; Gomme and Bertolini, 2004). Urinary loss of VDBP in type 1 diabetic patients with albuminuria is quite clear (Thrailkill et al., 2010) and its downregulation is associated with the pathogenesis of type 1 diabetes (Blanton et al., 2011). The association of the reduced trypsin inhibitory capacity of diabetic plasma is due to the non-enzymatic glycation of alpha-1-anti-trypsin (Hashemi et al., 2007) and our previous study has shown the increased extent of glycated alpha-1-anti-trypsin in both diabetic clinical and mice plasma (Bhonsle et al., 2012). It was interesting to observe the prominent down regulation of apo A1 in PCD than CD as shown in Fig 4.1.B. Further Apo A1 identification by MS1 was validated by western blot analysis. Additionally, this result was supported by dot-blot analysis of 10 each clinical plasma sample from ND, CD and PCD using Apo A1 antibody as shown in Fig 4.2.

Figure 4.2: Dot blot analysis of ten ND, CD and PCD samples using anti-Apo A1 antibody. B- Represents a blank.

Lower levels of Apo A1 are associated with the development of diabetic vascular complications mediated by the reverse cholesterol transport system (Quintao et al., 2000). The decreased levels of Apo A1 in PCD could be due to several reasons...
including autoantibodies against Apo A1 (Vuilleumier et al., 2010; Montecucco et al., 2011); elevated levels of inflammatory molecules (Haas et al., 2003) and insulin resistance (Mooradian et al., 2004). Apo B 100/Apo A1 ratio represents the balance between atherogenic and anti-atherogenic particles and is a better parameter for the prediction of cardiovascular risk than the lipids, lipoproteins, and lipid ratios (Mallick et al., 2011). Low levels of HDL were found to be negatively correlated with HbA1c and triglycerides in PCD. However, the levels of total cholesterol were not found to be significant. A recent study has shown that Apo B 100/Apo A1 ratio was increased in poorly controlled diabetes plasma than the controlled diabetes plasma (Wagner and Ordonez-Llanos, 2002) suggesting the contribution of lower levels of Apo A1 to this increased ratio. In this study, elevated levels of serum creatinine were observed in poorly controlled diabetes, suggesting Apo A1 levels were inversely proportional to serum creatinine levels. As increased serum creatinine levels are implicated in improper kidney functioning, these results support the previous work, where lower plasma HDL-Cholesterol levels were associated with a greater incidence of chronic kidney disease (Zoppini et al., 2009). Therefore, lower levels of Apo A1 in diabetes could be associated with increased risk of cardiovascular disease and chronic kidney disease.

The 2DE analysis along with validation with dot blot strongly suggests the down regulation of Apo A1, which may serve as an early marker for diabetic complications unlike microalbuminuria which is known to be one of the late markers of diabetic complications.