Summary of the final report of the project entitled "Relevance of Adiponectin Metabolism in type 2 Diabetes: A biochemical and Genetic Perspective" Sanction no. UGC- MRP-MAJOR-BIOC-2013-15475

Diabetes mellitus, a major multifactorial disorder, is closely associated with impaired lipid metabolism, dyslipidemia and obesity, emerging as a major health challenge of this millennium. Rapid urbanization and changing lifestyle combined with genetic susceptibility results in metabolic syndrome like dyslipidemia and obesity. The characteristic features of dyslipidemia are increased plasma triglyceride, decreased HDL cholesterol and increased small dense LDL-cholesterol particles. Though, dyslipidemia in diabetes is an established risk factor for cardiovascular diseases, yet there is dearth of identifying appropriate signatures to address this issue. Adiponectin, is actively involved in lipid metabolism and plays crucial role in dyslipidemia and vascular complications.

To investigate the underlying genetic and molecular pattern of adiponectin metabolic pathway in diabetic dyslipidemia we performed a case-control study in a total 264 individuals belonging to 3 categories such as diabetes with dyslipidemia (n=88), diabetes without dyslipidemia (n=86) and normal healthy controls (n=90).The study was designed to correlate biochemical and oxidative stress parameters in T2DM and indices for antioxidant defence were evaluated. Results indicated that , patients with diabetes and dyslipidemia had significantly lower antioxidant defence status reflected by reduced plasma free thiols, glutathione (GSH), vitamin C, vitamin E and PBMCs catalase activity. Significantly low vitamin C level in diabetic dyslipidemic patients infers that oxidative stress in diabetes might have contributed to the depletion of such antioxidant levels. Hyperglycemic and hyperlipidemic condition induce apoptosis through mitochondrial membrane depolarization by generating free radicals. Increased oxidative stress in diabetic patients leads to protein oxidation. The pro-thrombotic state in diabetic dyslipidemia reflects increased activity of platelets coagulation cascade and impaired fibrinolysis which in turn hyperactivates platelet von willebrand factor (vWF) and P-Selectin (CD62P) progressing atherosclerosis. Impaired calcium homeostasis has also been witnessed in diabetic dyslipidemia.

Adiponectin, an adipose tissue derived hormone plays protective role against the development of T2DM, dyslipidemia and CVD. Expression of adiponectin (ADIPOQ) and its receptors (ADIPOR1 and ADIPOR2) were measured in visceral and subcutaneous adipose tissues. Significant downregulation of adiponectin receptors (ADIPOR1 and ADIPOR2) in

diabetic-dyslipidemic condition was observed in peripheral blood mononuclear cells (PBMCs), visceral (VAT) and subcutaneous (SAT) adipose tissues. A decreased plasma adiponectin level in diabetic dyslipidemic conditions indicates impaired adiponectin metabolism. In this study an increase in the expression of inflammatory cytokine TNFα and NF-κβ were found to be associated with diabetic conditions with and without dyslipidemia. The expression of TNFα in PBMCs was even greater when diabetes is coupled with dyslipidaemia. Downregulation of PPARγ, increased plasma lipid peroxidation products (4-hydroxynonenal adducts, malonyldehyde and lipid hydroperoxides) and C-reactive protein (CRP) reveal greater risk in diabetic-dyslipidemic conditions culminating into CVD. Genetic polymorphisms in ADIPOQ gene and the genes of its receptors have been the major reasons for functional defect of novel adipokine, thus prompting the progression of insulin resistance, dyslipidemia and atherogenesis. A multiple linear regression followed by MDR analysis implicated elevated plasma malondialdehyde and decreased adiponectin level to be correlated with diabetic dyslipidemia. A strong correlation between ADIPOQ rs2241766 T>G and ADIPOQ rs1501299 G>T polymorphisms in diabetic and dyslipidemic condition was observed. SNP in ADIPOR1 (rs1342387 G>A) showed a correlation with diabetes but not with dyslipidemia and no association was found for rs4766415 A>T. Other biochemical factors including plasma C-reactive protein and 4-hydroxynonenal adducts found to be increased in diabetic dyslipidemic conditions. In summary, our results reveal interesting complex interplay of genetic and biochemical parameters in diabetic dyslipidemia which is significant from the perspective of risk stratification and therapeutic strategy development.

Final Report

of

UGC- MRP-MAJOR-BIOC-2013-15475

Relevance of Adiponectin Metabolism in type 2 Diabetes: A biochemical and Genetic Perspective

Submitted to

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG

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Annual/Final Report of the work done on the Major Research Project

1.	Project report No. 1st /2nd /3rd/Final:	Final.
2.	UGC Reference No:	F.No.- 43-42/2014(SR); Dated: 08.12.2015
3.	Period of report:	01/07/2015 to 30/06/2018 (3 years).
4.	Title of research project	Relevance of Adiponectin Metabolism in type 2 diabetes; A biochemical and genetic perspective
5.	Name of the Principal Investigator	Prof. Maitree Bhattacharyya
6.	Department	Department of Biochemistry.
7.	University/College where work has progressed	University of Calcutta.
8.	Effective date of starting of the project	01/07/2015 (The fund was credited in the university account only on 06.04.2016)
9.	Total amount approved	Rs. 15, 66, 000.00
10.	Total grant received	Rs. 13,23,332.00
11.	Total expenditure	Rs.14,91,283.00

c. Report of the work done: Attached in a separate sheet.

Report of the work done

TITLE OF THE PROJECT:

Relevance of Adiponectin Metabolism in type 2 diabetes; A biochemical and genetic perspective

INTRODUCTION

Global emergence of type 2 Diabetes mellitus (T2DM) and its association with dyslipidemia and other metabolic disorders has become a major health challenge in this millennium. Dyslipidemia i.e, presence of irregular lipid profile may be hyperglycemia dependent or independent. When dyslipidemia is induced by hyperglycemia it leads to defective insulin action changing the plasma lipoproteins. The worldwide prevalence of diabetes is increasing rapidly and expected to reach an astounding 7.7% by 2030, affecting approximately 439 million people, of which India is projected to share one-third of the total disease burden [1,2]. Type 2 diabetes mellitus (T2DM) is the most common form of diabetes and is attributed to various physiological and socio-behavioral factors in addition to genetic predisposition [3]. T2DM is often accompanied by dyslipidemia which is characterized by an elevated plasma total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL) and reduced high-density lipoprotein cholesterol (HDL) levels that eventually lead to development of an atherogenic metabolic profile and cardiovascular complications [4, 5, 35]

Of the different explanatory paradigms underlying T2DM and dyslipidemia, the role of adipose tissue as an endocrine organ has recently gained much popularity [6]. Adipose tissues present in subcutaneous (SAT) and visceral (VAT) areas differ in their molecular, physiological, clinical characteristics and prognostic significance [7]. Adiponectin, a major adipokine, is exclusively synthesized in the adipose tissues of adult humans and possesses insulin-sensitizing, antiapoptotic and antiinflammatory properties [8-10]. Adiponectin presumably functions by increasing glucose uptake by binding to its receptors (ADIPOR1 and ADIPOR2) present in adipose tissues, peripheral blood mononuclear cells (PBMCs), skeletal muscles and liver. Low serum adiponectin level is considered to be a risk factor for the development of T2DM [11]. Current research suggests that adiponectin may have a potential role in obesity management and dyslipidemia by improving insulin sensitivity,

glucose utilization, lowering total cholesterol, triglycerides, LDL and increasing HDL [12-14]. In addition to quantitative alteration in serum, polymorphisms located on genes encoding adiponectin (ADIPOQ) and its receptors (ADIPOR1 and ADIPOR2) have been shown to affect the performance of adipokine in T2DM associated metabolic complications [15-19].

Furthermore, the metabolic and humoral influences of adipose tissue, oxidative stress and inflammatory pathways also play a role in the pathogenesis of diabetic dyslipidemia [20-23]. Of the different ROS induced cellular damages, peroxidation of lipids particularly the poly-unsaturated fatty acids present in the cell membrane is considered to be a key degradative event. While CRP is a classical marker of inflammation, plasma malondialdehyde and 4-HNE protein adducts reflect accelerated rate of lipid damage [24-26].

PBMCs have been reported to show altered membrane structure-function in diabetes mellitus [14-16] but no such extensive study has been done so far to find out their relationship with membrane dielectric properties, especially in type 2 diabetes and associated dyslipidemia.

In this study the dielectric behavior of PBMCs plasma membrane has also been studied from diabetic subjects with and without dyslipidemia compared to healthy controls has been studied. To find out the profile of membrane capacitance and impedance of diseased groups we investigated the morphology of PBMCs isolated from diabetic subjects with and without dyslipidemia and healthy controls under scanning electron (SEM) and atomic force microscopy (AFM). SEM and flow cytometric (Forward scatter, FSC) platform were also utilized to reveal whether there are any differences in cell size in control and diseased cases.

The unprecedented upsurge in the incidence of T2DM and frequent co-existence of hyperglycemia and dyslipidemia urge us to investigate possible association different risk factors of cardiovascular diseases including BMI, HbA1c, lipid profile, oxidative stress and inflammatory markers, adiponectin and host genotypes in Indian T2DM patients to identify representative pathophysiological markers and predict the risk of diabetic dyslipidemia. To the best of our knowledge, this kind of multi-level data analysis has not yet been elaborated in Indian population, which represents the largest burden of diabetes in the world [27].

BRIEF OBJECTIVE OF THE PROJECT

The objectives of the project are:

1. To correlate the biochemical parameters pertaining to adiponectin and lipid metabolism pathways with diabetes and consequent risk of CVD.
2. To investigate any statistical association between genetic polymorphisms underlying adiponectin pathway and the biochemical parameters measured in objective 1 as well as risk of diabetes and consequent CVD.
3. To carry out functional analyses of SNPs which may be found associated with the disorder.

METHODS

2.1. Clinical subjects

In this case-control study sampling was performed in 264 subjects recruited from Institute of Post-Graduate Medical Education and Research and Seth Sukhlal Karnani Memorial Hospital (IPGMER and SSKM) Hospital, Kolkata, India. Selection criteria of the participants included 35-70 years of age, BMI of 21-32 kg/m^2 and diabetic patients with HbA1c (%) > 6.0. Diabetic dyslipidemic subjects (n=88) were newly diagnosed with dyslipidemia hence during the sampling period they were not into anticholesterol (such as statins) medications (Table 1). A total number of 86 diabetic subjects without any history of dyslipidemia were also recruited along with 90 metabolically healthy individuals without any history of diabetes, dyslipidemia and cardiovascular disorders as controls. Total cholesterol, LDL and HDL levels were higher than 200mg/dl and 130 mg/dl and lower than 40mg/dl respectively in dyslipidemic individuals. Each enrolled diabetic patient had a history of diabetes for at least 3 years, however, they did not suffer from any other health complications. This observational study was designed by careful selection of sex and age-matched controls and patients. All subjects were without any histories of tobacco and alcohol intakes or any other addictions. Visceral (VAT) and subcutaneous (SAT) adipose tissues were collected from diabetic subjects without dyslipidemia (n=10), diabetic subjects newly diagnosed with dyslipidemia (n=10) and healthy controls (n=9), who underwent cholecystectomy at SSKM hospital during this course of study following the ethical guidelines of IPGMER-SSKM and Calcutta University. Blood samples were also collected from the subjects who provided us with adipose tissue samples. An informed consent for participation in the study was obtained from

each individual participating in this study who contributed us with blood and tissue samples. The research program was approved by Calcutta University Biosafety and Ethics Committee.

2.2. Isolation of plasma, PBMCs and platelets from venous blood

6 ml venous blood samples were collected from both control and patient subjects 2 hours after light meal with their prior consent. 4 ml blood was kept in heparinized vial. 3.2% Sodium-citrate buffer was used as an anticoagulant for remaining 2ml blood.

1ml blood from heparinized vial was immediately utilized to separate plasma by centrifugation at 900 x g for 15 minutes at room temperature and preserved at -80^{o} C for future analysis. PBMCs (lymphocytes and monocytes) were isolated within one hour (Figure 1.1) of sampling from the remaining 3ml heparinized blood by density gradient centrifugation using histopaque-1077 (SIGMA) by centrifuging at 400 x g for 30-40 minutes at room temperature. The middle layer or 'buffy coats' contains the PBMCs which were collected carefully and washed twice with HBSS (pH 7.4) and resuspended in the same buffer. Observed cell count under haemocytometer was $3x10^{6}$/ml.

Blood in 3.2% Sodium-citrate buffer was centrifuged at 180 x g for 15 minutes to isolate platelet rich plasma (PRP). 0.6 ml PRP was transferred into a polypropylene tube following addition of 1 ml acidic citrate dextrose (ACD, 39 mM citric acid, 75 mM sodium citrate, 135 mM dextrose, pH 4.5) with 100 ng/ml prostaglandin E1 (PGE1) [SIGMA] and centrifuged at 650×g for 15 minutes at room temperature and supernatant was discarded. Platelets were suspended in 2 ml of Jamieson's buffer (5.5 mM dextrose, 1.28 mM NaCl, 4.26 mM Na_2HPO_4, 7.46 mM NaH_2PO_4, 4.77 mM sodium citrate, 2.35 mM citric acid, 0.35% BSA [SIGMA] pH 6.5) and centrifuged at 500×g for 15 min at room temperature. Supernatant was discarded and pellet was resuspended in Ca^{2+}-free Tyrode-HEPES buffer. Observed cell count under haemocytometer was $2x10^{7}$/ml.

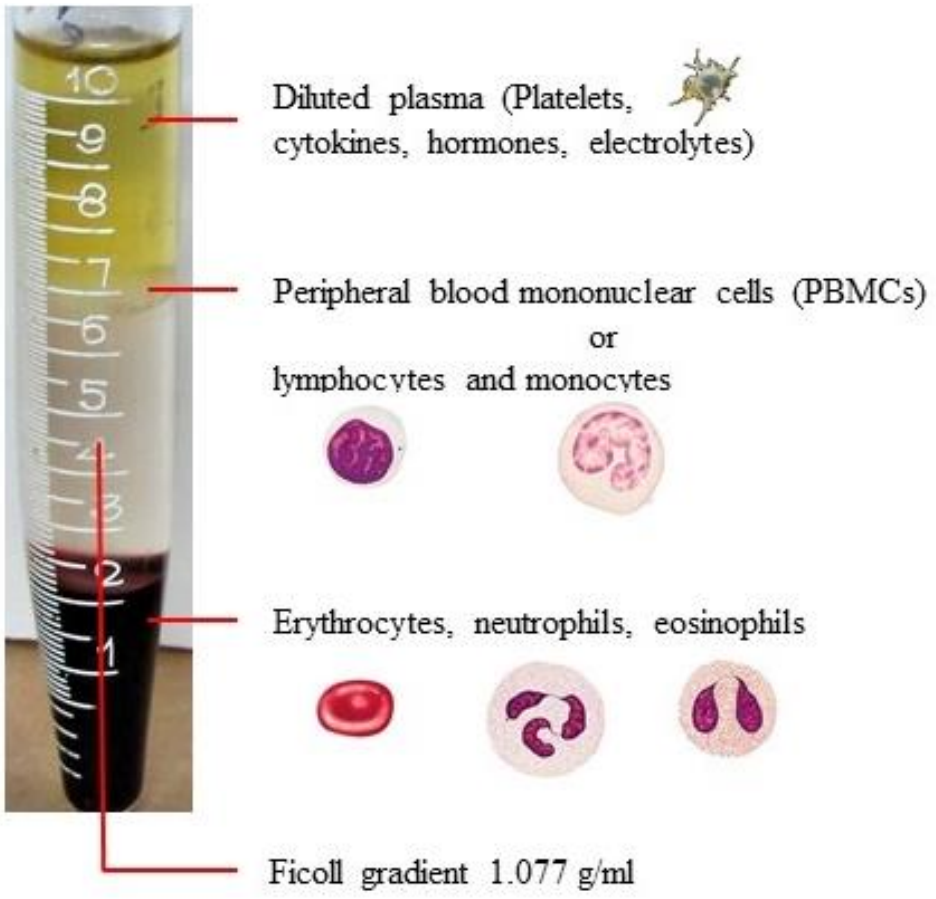


Figure 1: Isolation of different components of blood.

2.3. Estimation of plasma antioxidants glutathione (GSH), Vitamin E and Vitamin C

Plasma glutathione level was measured using DTNB/GR Enzyme Recycling Method [28]. A 50-µl aliquot of plasma was precipitated with 4 % (w/v) sulfosalicylic acid in a 1:1 ratio. The samples were kept at 4 °C for 1 h, centrifuged at 3,500 rpm for 15 min, and the supernatant was collected. The reaction mixture contained 50 µl of the supernatant, 50 µl of phosphate buffer (0.1 M, pH 7.4), and 10 µl of 0.4 % (w/v) DTNB. Formation of the yellow color derivative was measured at 412 nm. Plasma glutathione level was expressed as µmol/l.

Vitamin E (α tocopherol) level in plasma was measured by the method described by Martinek, 1964 [29]. 600µl plasma sample was mixed with ethyl alcohol and xylene. After centrifugation at 3000rpm for 10 minutes 400µl of xylene supernatant was taken and mixed with 400µl of TPTZ solution. The absorbance of the mixture is taken at 460nm, along with the blank setting it to zero. Then 0.13ml of $FeCl_3$ reagent was added, and after 1:30 minute

the absorbance is measured at 520nm. Concentration of Vitamin E (α tocopherol) is measured using the following equation;

$$\textbf{Vitamin E (mg/100ml)} = \frac{\textbf{OD}_{520}\textbf{ of sample} - (0.29 \times \textbf{OD}_{460})}{\textbf{OD}_{520}\textbf{ of standard}}$$

A standard solution of α tocopherol was prepared with concentration 1mg/100ml.The assay of Vitamin C was carried out by the reduction of 2,6 - Dichlorophenolindophenol (DCIP) by ascorbic acid. The intensity was measured at 520 nm [30]. 500µl of plasma was deproteinized by adding 1ml of trichloroacetic acid (TCA) and after mixing, centrifuged at 14,000g for 20 minutes at 4^{o}C. 0.6ml of aliquot of the supernatant was taken and 0.33ml of citrate/acetate buffer (pH-4.15) was added. 0.33ml of DCIP was added to the sample and after 30 seconds it was read against distilled water at 520nm. A few crystals of ascorbic acid were added to bleach the dye by reducing it completely and the sample was read again. This value serves as a blank for the same. A standard curve, including a reagent blank, was constructed with standards ranging between 0 and 20µg of ascorbic acid per ml of 5% TCA. The change in absorbance (ΔA) due to the reduction of the dye by ascorbic acid in the sample was calculated from the following equation:

$\Delta A = (RB - RB_b) – (S - S_b)$ where RB is the absorbance of the reagent blank; RB_b is the absorbance of the reagent blank after bleaching with ascorbic acid; S is the absorbance of the sample; and S_b is the absorbance of the sample after bleaching with ascorbic acid. The concentration of ascorbic acid in the sample was obtained by comparing ΔA with the standard curve and expressed as mg/dl.

2.4 .Estimation of catalase activity in PBMCs

PBMCs were suspended in lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM EDTA, pH 8.2) and lysed by sonication (Hielscher UP200S) giving 3 bursts of 15 seconds in ice (cycle 0.5, amplitude 50%). The activity of enzyme was assayed according to Aebi *et.al*. [31] in which the disappearance of hydrogen peroxide (H_2O_2) was followed spectrophotometric ally at 240nm. 0.05M H_2O_2 in phosphate buffer (PB) was added to PBMCs lysate (containing 20-40µg protein) and adding 0.05M PB the volume was made up to 3ml. The set up was scanned at 240nm for 1 min to note the breakdown of H_2O_2 at an interval of 15 seconds. The concentration of H_2O_2 was calculated using ε (H_2O_2) = 43.6 $mol^{-1}cm^{-1}$. Specific activity of

catalase was expressed as micromoles of H_2O_2 decomposed/min/mg of PBMCs lysate protein.

2.5. Estimation of total thiol in plasma

Plasma total thiol was estimated by method described by Hu ML, 1994 [32]. Thiol groups in blood plasma proteins were determined using 5,5′-dithio-bis (2-nitro-benzoic acid) (Ellman's reagent, DTNB). The thiol-disulfide interchange reaction between DTNB and thiol is the basis of this spectrophotometric assay. The sample was mixed with DTNB and at the end of the incubation (1 h, 37°C) period the absorbance was recorded at 412 nm. The concentrations were calculated by using $\varepsilon = 13600\ M^{-1}\ cm^{-1}$ and total thiol level was expressed as µmol/l.

2.6. Estimation of protein carbonyl content

15 µl plasma was mixed with 85 µl PBS. This was followed by addition of 100 µl 20% cold TCA, kept in ice for 10 minutes, centrifuged at 10000g for 10 minutes and supernatant was discarded. The process was repeated again. To the precipitate 500 µl (10mM DNPH in 2M HCl) was added. The system was incubated at 37^o C for 1 hour with regular shaking. Then equal volume of 20% cold TCA was added. The system was kept in ice for 10 minutes, centrifuged at 10000g for 5 minutes and the supernatant was rejected. The pellet was washed with 500 µl 10% TCA, centrifuged at 10000g for 5 minutes and supernatant was rejected. Now 1ml 1:1 ethylacetate: ethanol mixture was added to the pellet, vortexed and centrifuged at 10000g for 5 minutes and supernatant was rejected. This step was repeated for 2 more times. 1 ml 6M Guanidine Hydrochloride (pH 2.3) was added to the pellet and incubated at 37^o C for 15-20minutes with vortexing in every 5 minutes. The absorbance of 2,4 DNPH derivatives were measured at 370nm wavelength. ($\varepsilon = 22{,}000\ M^{-1}cm^{-1}$)

2.7 .Estimation of plasma Ca^{2+}

Plasma Ca^{2+} was measured using a kit obtained from Cayman chemicals. The absorbance was recorded at 580nm.

2.8. Intracellular Ca^{2+} concentration in PBMCs

Isolated PBMCs were washed in Hanks' Balanced Salt Solution (HBSS), pH 7.4 and incubated with 5µM Fluo-3, AM with 2.5mM probenecid for 20 minutes at 37^oC in dark. Cells were washed with HBSS and again incubated for 30 minutes at 37^oC in dark.

Intracellular Ca^{2+} concentration was measured in the fluorescence emission at 530 nm in FACS Verse equipped with an argon-ion laser (15mW) tuned to 488 nm. Data was analyzed by BD FACSuite application software. Endogenous Ca^{2+} concentration was also detected under fluorescence emission at 525 nm by confocal fluorescence microscope (Olympus IX81). High resolution images were analyzed by Olympus FV1000 software.

2.9. Estimation of mitochondrial membrane potential in PBMCs (ΔΨm)

Mitochondrial membrane potential (ΔΨm) was estimated using cationic dye 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzamidazol-carboncyanine (JC-1,Sigma Aldrich). PBMCs obtained from whole blood were loaded with JC-1 (1μg/ml) in HBSS at 37^{o}C for 1 hour. Cells were excited at 488 nm when emission was detected at 585 nm (JC-1 aggregates) and 516 nm (JC-1 monomers) using Hitachi (F-7000) fluorescence Spectrometer and also under confocal fluorescence microscope (Olympus IX81) [33]. High resolution images (60X) were analyzed by Olympus FV1000 software. Membrane potential (ΔΨm) was presented as emission ratios (585/516).

2.10.Estimation of total Reactive Nitrogen Species (RNS) generated by PBMCs

Nitric Oxide production in PBMCs was assessed after incubating with DAF-2 DA (Cayman chemical co) followed by its intracellular de-esterification to DAF-2 [34]. In that process, PBMCs were incubated with 10 μmol/l of DAF-2DA at 37^{o}C for 1 hour, fluorescence intensity was measured by using a confocal fluorescence microscope (Olympus IX81). NO provided the third nitrogen to form a tri-azo ring with the two amino groups of the non-fluorescent DAF-2 and converted it into DAF- 2T (Diamino-triazolo-fluorescein), which was monitored at 490 nm excitation and 530 nm emission. High resolution images were analysed by Olympus FV1000 software. Intracellular RNS generation was also measured in the fluorescence emission at 530 nm in FACS Verse equipped with an argon-ion laser (15 mW) tuned to 488 nm. Data was analysed by BD FACSuite application software.

2.11. Estimation of plasma nitric oxide

250µl plasma sample was incubated for 45 min at room temperature with 250µl of substrate buffer (imidazole 0.1 mol/l, NADPH 208 µmol/L, flavineadenine dinucleotide 3.8 µmol/l: pH 7.6) in presence of nitrate reductase (*Aspergillus niger*, Sigma) to convert NO_3^- to NO_2^-. Excess amount of reduced NADPH, which interferes with the chemical detection of NO_2^-, was oxidized in continuation with the incubation of 5 µl (1mg/ml) of LDH, 0.2 mmol/l (120 µl) pyruvate and 75 µl of water. Total nitrite was then analyzed by reacting the samples with Griess reagent (1% sulfanilamide, 0.1% *N*- (1-naphthyl)-ethylenediamine dihydrocholoride in 5% H_3PO_4 [35]. Reaction samples were treated with 500 µl of trichloroacetic acid (20%), centrifuged for 15 min at 8000 × *g* and the absorbance at 548 nm was compared with $NaNO_2$ standard (0–100 µmol/l). Total nitrite level was expressed as µmol/l.

2.12. Estimation of platelet vWF and soluble P- Selectin level by Western blotting

Qualitative estimation of vWF and soluble P-Selectin was performed separately with the help of Western Blotting technique (BIO-RAD instrument with ChemidocTM XRS image lab software) using activated platelets with ADP (10µM) [36]. Briefly, the platelet pellet was dissolved in 100 µl ice-cold PBS with a protease inhibitor cocktail (P-8340, SIGMA), homogenized by using an ultrasonic device (Hielscher UP200S) giving 3 bursts of 20 seconds in ice (cycle 0.5, amplitude 60%), centrifuged at 16000 × g for 10 minutes at 4° C and the pellet was discarded. Then platelet extracts (approximately 30–50 µg) were loaded onto 12% Bis-Tris polyacrylamide gel (Invitrogen) and electrophoresed for 45 minutes at 200 V. Samples were electro transferred to nylon PVDF Immobilon-PSQ membranes (Millipore) for 90 minutes at 30 V with 20% methanol blotting buffer (Invitrogen). For detection, the Western Breeze Chemiluminescent System (Invitrogen) was used. Blots were blocked for 30 minutes with blocking buffer, and then incubated overnight at 4°C with the primary antibodies, anti-β actin (0.5 µg/ml) (housekeeping gene antibody), anti-P-Selectin (1:1000, Santa-Cruz, sc-6943) and anti-vWF (1:2000, Santa-Cruz, sc-14014). The membrane blocking was done with PBS-3% BSA solution for overnight. After conjugating with HRP conjugated secondary antibody repeated steps of washing with PBS and PBST buffers were followed by. Developer was used as per Western Blotting Detection System and membrane was detected by densitometry.

2.13. PBMCs size and complexity analysis by flow cytometry

To assess size and complexity of lymphocytes and monocytes events were gated to the population according to their forward scatter (FSC) and side scatter (SSC).

2.14 Isolation of visceral and subcutaneous adipose tissue (VAT and SAT)

Two types of white adipose tissue [visceral (VAT) and subcutaneous (SAT)] were obtained from patients who underwent abdominal surgery in SSKM hospital with their informed consent. The bioethical guidelines were approved by SSKM hospital. SAT was obtained approximately 2 cm deep to the abdominal skin. VAT was obtained from the omental apron several centimeters from the transverse colon. All adipose tissue biopsies were obtained after completion of the bypass procedure and right before the closing phase. 100 mg adipose tissues were digested in HBSS containing 5.5 mM glucose, 5% (w/v) fatty acid free BSA and 3.3 mg/ml type II collagenase for 30 min in a 37° C water bath. The digestion mixture was passed through a tissue sieve. The adipocyte-containing fraction was collected and washed several times by centrifugation at 585 g for 5 min.

2.15. Isolation of total RNA from PBMCs, VAT and SAT

PBMCs pellet obtained from 3 ml of blood were washed twice and 500µl of Trizol (Invitrogen) was added to it. It was mixed thoroughly and kept at room temperature for 15 minutes. After that 100 µl chloroform was added into it and the solution was kept at 4^{o}C for 10 minutes. Then it is centrifuged at 12000 rpm for 10 minutes at 4^{o}C. The upper clear solution contains the RNA which was taken precisely at a separate tube and 250 µl of isopropanol was added into it. After keeping the solution for 10 minutes at 4^{o}C centrifugation was performed at 12000 rpm for 10 minutes at 4^{o}C. The supernatant was discarded and the pellet was washed with 70% ethanol. The alcohol was removed by air-drying. The RNA pellet was finally dissolved in 20µl of DEPC treated water and kept in -20^{o}C for future analysis.

To isolate RNA from adipocytes 1ml of Trizol reagent is added to 100 µl adipocyte cell fraction and kept at room temperature for 15 mins. Then it was centrifuged at 12000 rpm for 10 minutes at room temperature. The fat monolayer was carefully removed and RNA was then extracted following the above mentioned method.

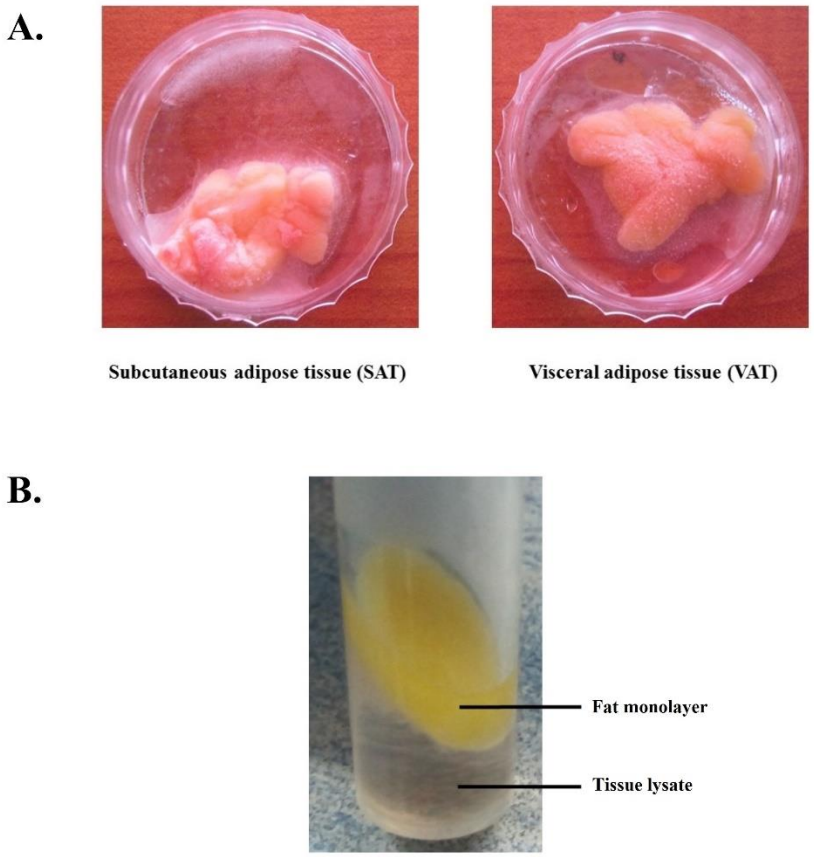


Figure: 2 (A) Subcutaneous and visceral adipose tissue samples prior to RNA extraction, (B) Preparation of adipocyte tissue lysate for RNA extraction

2.16. Synthesis of cDNA from total RNA

1µg of total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc.) in a thermal cycler (Applied Biosystems GeneAmp PCR System 9700) and stored at -20°C. The reaction con

dition for reverse transcription was kept as 25^{o}C for 10 minutes, 37^{o}C for 120 minutes and 85^{o}C for 5 minutes.

2.17. Expression of Adiponectin (ADIPOQ), adiponectin receptor 1 (ADIPOR1) and adiponectin receptor 2 (ADIPOR2) in VAT and SAT

cDNA obtained from VAT and SAT were amplified using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) in Applied Biosystems 7900HT Fast Real-Time PCR System. The primers and PCR condition for adiponectin (ADIPOQ), adiponectin receptor 1

(ADIPOR1) and adiponectin receptor 2 (ADIPOR2) are given below (Table 1). β actin was kept as a housekeeping gene. Details of β actin primer is given in table 2.

Table 2: Primers and PCR conditions for ADIPOQ, ADIPOR1 and ADIPOR2

Genes	**Primers**	**PCR conditions**
Adiponectin	Forward: 5'GGTGAGAAAGGAGATCCAGGT3' Reverse: 5' TCCTTTCCTGCCTTGGAT 3‘	95°C for 5 min, 1 cycle 95°C for 30s, 62°C for 30s, 72°C for 30s, 40 cycles
ADIPOR1	Forward: 5’TTCTTCCTCATGGCTGTGATGT3’ Reverse: 5’ AAGAAGCGCTCAGGAATTCG 3’	95°C for 5 min, 1 cycle 95°C for 30s, 58°C for 30s, 72°C for 30s, 40 cycles
ADIPOR2	Forward:5’ATAGGGCAGATAGGCTGGTTGA 3’ Reverse: 5’ GGATCCGGGCAGCATACA 3’	95°C for 5 min, 1 cycle 95°C for 30s, 60°C for 30s, 72°C for 30s, 40 cycles

2.18. Expression of ADIPOR1, ADIPOR2, PPARγ, NF-κβ and TNFα in PBMCs

cDNA obtained from PBMCs were amplified using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) in Applied Biosystems 7900HT Fast Real-Time PCR System. The primers and PCR conditions for adiponectin adiponectin receptor 1 (ADIPOR1) and adiponectin receptor 2 (ADIPOR2) are given in table 4.1. The primers and PCR condition for PPARγ, NF-κβ and TNFα are given in table 3. β actin was kept as a housekeeping gene (Table 3).

Table 3: Primers and PCR conditions for PPARγ, NF-κβ and TNFα

Genes	Primers	PCR conditions
PPARγ	Forward: 5' ATGACAGACCTCAGACAGA 3'	95°C for 3 min, 1 cycle
	Reverse: 5' AATGTTGGCAGTGGCTCAGG 3'	
		95°C for 30s, 64°C for 30s, 72°C for 30s, 40 cycles
NF-κβ	Forward: 5'ACACCGTGTAAACCAAAGCC 3'	95°C for 3 min, 1 cycle
	Reverse: 5'CAGCCAGTGTTGTGATTGCT 3'	
		95°C for 30s, 57°C for 30s, 72°C for 30s, 40 cycles
TNFα	Forward: 5' CCCTGGTATGAGCCCATCTATC 3'	95°C for 3 min, 1 cycle
	Reverse: 5' AAAGTAGACCTGCCCAGACTCG 3'	
		95°C for 30s, 60°C for 30s, 72°C for 30s, 40 cycles
β-actin	Forward: 5' GCGGGAAATCGTGCGTGACATT 3'	According to the PCR conditions of respective gene.
	Reverse:5'GATGGAGTTGAAGGTAGTTTCGTG 3'	

2.19. Estimation of plasma adiponectin

Plasma level of adiponectin was measured using sandwich ELISA at an absorbance of 450 nm. Plasma values are expressed as µg/ml. ELISA were performed using commercial ELISA kit.

2.20. Estimation of plasma C reactive protein (CRP)

Plasma CRP, an inflammatory stress marker was measured using sandwich ELISA at an absorbance of 450 nm. Plasma values are expressed as ng/ml.

2.21. Quantitation of lipid peroxidation by estimating plasma 4 hydroxynonenal (4-HNE) and malonyldehyde (MDA) content

The most abundant form of 4-HNE in living system is the 4-HNE-protein adducts which can be detected by ELISA using the method adapted from Weber et.al, 2013. In this method, 4-HNE-BSA standards were utilized which range from 0–250 pmol/mg. Standards and plasma samples were diluted with a final concentration of 10mg/ml of protein. Prepared standards/plasma samples were further diluted 10-fold in 50mM carbonate binding buffer (pH 9.6; 15mM sodium carbonate, 35 mM sodium bicarbonate) on ELISA plates for protein adsorption for 5 hours at 4^{o}C. Following blocking and successive washing steps plates were incubated at 4^{o}C overnight for binding of primary anti 4-HNE (ab46545, abcam) antibody. After several rounds of washing (by 0.1% Tween 20 in PBS) the plate was incubated with goat anti-mouse secondary antibody (Invitrogen) followed by incubation with chromogenic substrate solution (Thermo Fisher Scientific). The absorption was measured at 450 nm. Amounts of 4-HNE-protein adducts measured by the ELISA were expressed as pmol 4-HNE per µg of proteins (µM).

The levels of lipid peroxidation were determined through the measurement of substances that are reactive to thiobarbituric acid (TBARS). Malondialdehyde (MDA) is a by-product of lipid peroxidation cascade and it reacts with thiobarbituric acid (TBA) at a 2:1 proportion. This reaction results in a pink chromogen that can be quantified using a spectrophotometer. Thus, to a 200 µl of plasma, a 400 µl stock solution containing trichloroacetic acid (TCA 10%; v:v); (TBA 0.375% w:v) and hydrochloric acid (HCl 0.25N) were added. The mixture was homogenized incubated in a water bath for 15 minutes at 100^{o}C. The tubes were cooled in ice for 5 minutes and following addition of 500 µl of *n*-butanol the mixture was again homogenized for about 30s and the tubes were centrifuged for 15 minutes at 2500 g at 15^{o}C.

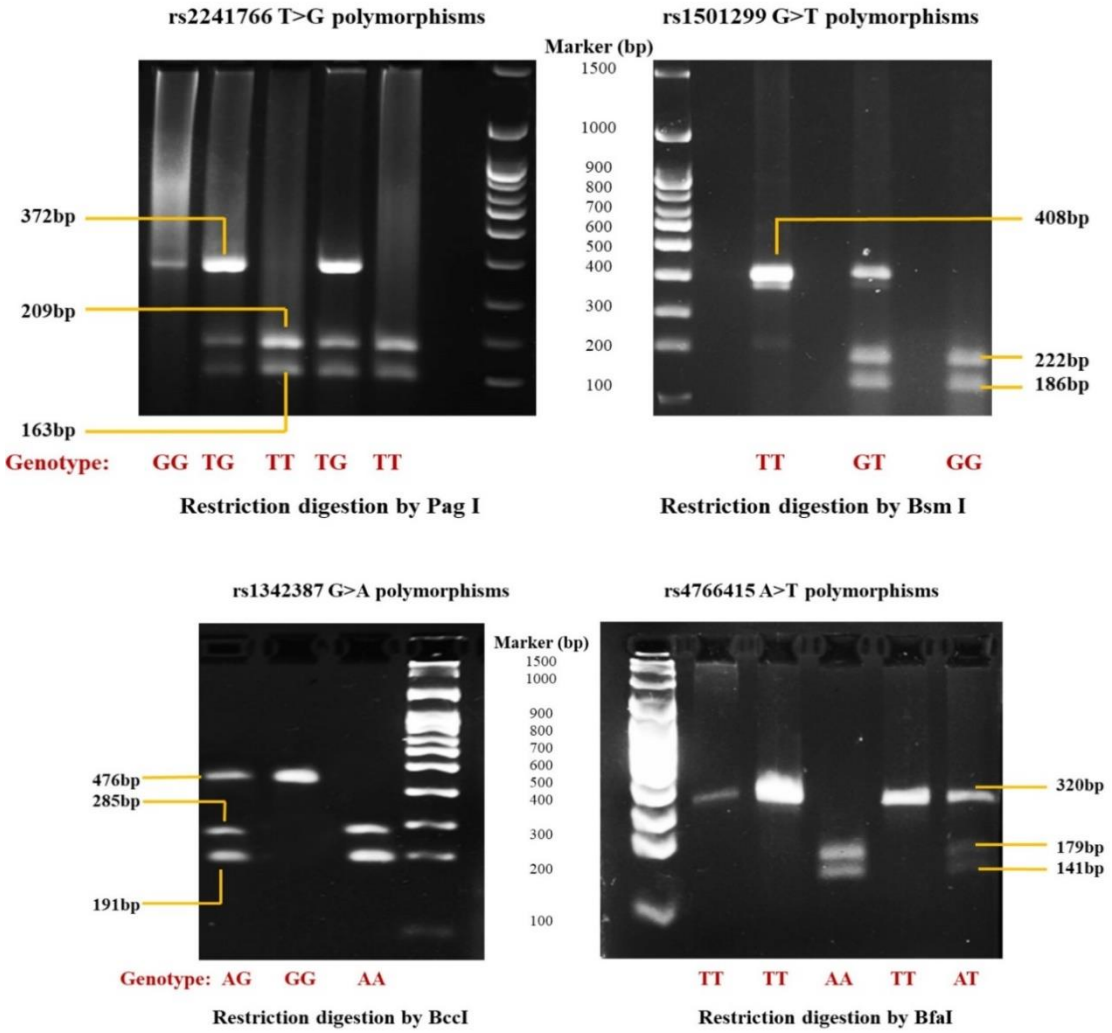
The supernatant was carefully collected and transferred in duplicate to a 96-wells microplate. TBARS molecules were quantified through a spectrophotometer at a wavelength of 532 nm. Results were compared to a standard curve previously prepared with a stock solution of MDA. The levels of lipid peroxidation were expressed in nmol of TBARS per ml of sample.

2.22. Measurement of lipid hydroperoxides (LOOH) in plasma

Plasma lipid hydroperoxides were measured by oxidation of ferrous iron in the presence of xylenol orange. In this method, ferrous iron is oxidized to ferric iron by hydroperoxides. This product then forms a complex with xylenol orange to yield a chromophore that can be detected spectrophotometrically at 560 nm. 50 µl of plasma were made up to 150 µl with methanol. 1350 µl of FOX2 reagent (0.22 mM Ammonium iron (II) sulfate, 22.5 mM H_2SO_4 and 90 µM xylenolorange) were added. The reaction mixtures were mixed and then incubated in darkness at room temperature for 50 min. Their absorbances were read at 560 nm against a blank (150 µl methanol and 1350 µl FOX2 reagent). Plasma LOOH was measured in µmol/l.

2.23. Genotyping of SNPs

Genomic DNA was isolated from venous blood following salting out method [28]. Single nucleotide polymorphisms (SNPs) present in adiponectin [rs2241766 (exon 2) and rs1501299 (intron 2)] and adiponectin receptors [rs1342387 (intron 4) and rs4766415 (intron 2)] genes were genotyped by PCR coupled with restriction fragment length polymorphisms analysis (RFLP) in all study participants (Table 4).94 insertion/deletion ATTG (rs28362491) polymorphism located in the promoter regulatory region of the NFKB1 gene were also genotyped by PCR.



2.24. Statistical analysis

The clinical variables such as age, sex, BMI were represented as mean ± standard deviation for both control and patient groups (Table 4). The parameters were log transformed to approximate normal distribution and subjected to Pearson correlation in pair-wise combinations using SPSS. The study subjects have been classified in four groups for pairwise association study. These include, (a) diabetic subjects without dyslipidemia vs healthy control, (b) diabetic subjects with dyslipidemia vs healthy control, (c) total diabetic subjects vs healthy control and (d) in between diabetic subjects with and without dyslipidemia. Hardy–Weinberg equilibrium was tested using the χ^2 goodness-of-fit test in cases and controls by SNPStats. Strength of association for each SNP was tested by odds ratio estimates

at 95% confidence interval by VassarStats. Odds ratio (OR) was calculated between groups. Logistic regression was used to calculate OR and 95% confidence intervals (CIs) for both recessive and co-dominant models for each SNP. *P* values <0.05 were considered significant. Haplotypes (rs2241766 and rs1501299) and their frequencies were estimated using haploview. Multiple linear regression analysis was carried out between parameters that were found to be significantly correlated using Pearson's correlation analysis by SPSS (Figure 1). Correlation matrix and dendrogram were prepared using MATLAB and SPSS respectively for visual inspection of correlated parameters (Figure 3). Multiple linear regression analysis was carried out between diabetic subjects without dyslipidemia and normoglycemic healthy controls keeping HbA1c as dependant variable (Table 3). A subset of diabetic subjects developed dyslipidemia over a span of 3 years. Thus another multiple linear regression analysis was carried out between two groups of diabetic subjects (with and without dyslipidemia) keeping total cholesterol as dependant variable (Table 3). Results of multiple linear regression analysis enables us to select possible risk predicting clinical, biochemical and genetic factors of diabetes and associated dyslipidemia for further gene-gene and gene-phenotype interaction analysis using multifactor dimensionality reduction (MDR) analysis. To run MDR, six parameters namely plasma adiponectin level, plasma 4-HNE adduct level, plasma MDA level and rs2241766, rs1501299 and rs1342387 were assembled as they showed statistical correlation with total cholesterol in the multiple regression analysis and SNP based association study [Method S5]. The models with P value <0.05 and >95% average cross-validation consistency (CVC) were regarded as the best predictive models. For MDR analysis, the "low", "moderate" and "high" risk classes for each parameter were designated as "0", "1" and "2". For each SNP locus the presence of double, single and no risk alleles have been denoted as '2'. '1' and '0' respectively. In the same way the plasma biochemical concentrations associated with high (plasma concentrations of <6.5µg/ml, >4nmol/ml and >0.9 pmol/µg for adiponectin, MDA and 4-HNE adducts respectively), moderate (plasma concentrations of 6.5-8.5 µg/ml, 2-4 nmol/ml and 0.7-0.9 pmol/µg for adiponectin, MDA and 4-HNE adducts respectively) and no risks (plasma concentrations of >8.5 µg/ml, <2 nmol/ml and <0.7 pmol/µg for adiponectin, MDA and 4-HNE adducts respectively) were assigned as '2', '1' and '0' respectively.

RESULTS

Characteristics of the study participants

The baseline demographic and clinical characteristics namely age, sex, BMI, levels of fasting blood glucose, HbA_1c, systolic and diastolic blood pressures, total cholesterol, LDL, HDL, triglycerides and non-HDL-Cholesterol of diabetic subjects with and without dyslipidemia and healthy controls were recorded (Table 4). Subjects in three categories were age and sex matched and there was no significant difference in BMI between groups ($p > 0.05$). A significant ($p < 0.05$) increase in systolic and diastolic blood pressures was noted in both diabetic groups compared to healthy controls. The systolic blood pressure differed in two diabetic groups, as well. Indicators of hyperglycemic stress such as HbA1c, fasting and postprandial blood glucose were significantly elevated in both diabetic groups ($p < 0.05$) compared to normoglycemic controls. Increased serum level of non-HDL-Cholesterol and decreased HDL level was found to be associated in dyslipidemic subjects compared to other two groups ($p < 0.05$). A significant increase in plasma level of CRP, 4-HNE adducts and MDA, and decrease in plasma adiponectin level were observed in diabetic patients with dyslipidemia compared to those without dyslipidemia and healthy controls ($p < 0.05$) (Table 4).

Table 4: Demographic, clinical and plasma biochemical parameters of study participants

Characteristics	**Healthy controls** ***n*=90 (M=44, F=46)**	**Diabetic without dyslipidemia** ***n*=86 (M=42, F=44)**	**Diabetic dyslipidemic** ***n*=88 (M=46, F=42)**	***p value***
Age (Years)	50.16 ± 7.14	49.23 ± 7.11	50.74 ± 6.89[a]	0.391[b], 0.64[c], 0.184[d]
BMI (Kg/m^2)	23.071 ± 3.85	23.695 ± 2.932	23.761 ± 2.808	0.167, 0.114, 0.873
Fasting blood glucose level (mg/dl)	89.75 ± 6.65	134.53 ± 35.761	153.45 ± 44.42	<0.0001[b,c], 0.0023[d]
Post Prandial blood glucose level (mg/dl)	112.35 ± 7.93	205.8 ± 58.54	209.15 ± 46.67	<0.0001[b,c],0.676[d]

Glycated hemoglobin (HbA1c%)	5.101 ± 0.45	7.891 ± 1.17	7.91± 1.298	<0.0001[b,c], 0.913[d]
Blood pressure (mm Hg)	118.66 ± 5.92, 78.71 ± 4.856	123.42 ± 11.83, 81.48 ± 6.36	129.2 ± 15.26, 83.15 ± 7.86	<0.0001[b,c], 0.006[d]/ 0.0013, <0.001, 0.126
Total cholesterol (mg/dl)	145.54 ± 26.092	150.08 ± 27.346	261.25 ± 53.345	0.2616[b], <0.0001[c,d]
Triglycerides (mg/dl)	145.54 ± 26.092	139.4 ± 31.93	218.03 ± 52.53	0.529[b], <0.0001[c,d]
HDL (mg/dl)	42.4 ± 9.217	43.24± 9.753	35.67± 7.318	0.556[b], <0.0001[c,d]
LDL (mg/dl)	76.78 ± 23.762	80.68± 23.81	178.52± 45.096	0.277[b], <0.0001[c,d]
Non-HDL-Cholesterol (mg/dl)	103.14 ± 25.81	106.84 ± 25.19	225.58 ± 50.82	0.338[b], <0.0001[c,d]
Adiponectin(µg/ml)	11.047 ± 2.338	7.841 ± 2.064	5.88 ± 1.953	<0.0001[b, c, d]
C-reactive protein (mg/l)	7.325 ± 2.358	14.222 ± 5.525	20.627 ± 7.268	<0.0001[b, c, d]
Malondialdehyde (nmol/ml)	1.384 ± 0.725	3.021 ± 0.825	4.974 ± 1.081	<0.0001[b, c, d]
4 hydroxynonenal adducts (pmol/µg)	0.4606 ± 0.2145	0.7706 ± 0.1892	0.9068 ± 0.2301	<0.0001[b, c, d]

[a] Results are given in mean ± standard deviation.

[b] *p* value between healthy control and diabetic without dyslipidemia

[c] *p* value between healthy control and diabetic dyslipidemic.

[d] *p* value between diabetic and diabetic dyslipidemic.

Decrease in plasma antioxidants glutathione (GSH), Vitamin E and Vitamin C

A decrease in antioxidants such as glutathione (GSH), Vitamin E and Vitamin C among diabetic dyslipidemic subjects indicates pronounced risk of oxidative stress. The data have been tabulated in Table 5 and Figure 2

Decrease in catalase activity

Catalase catalyzes the breakdown of H_2O_2 to H_2O and O_2. PBMCs catalase activity is found to be significantly lower among diabetic dyslipidemic subjects compared to diabetic and healthy subjects (p <0.05) [Table 5, Figure 2].

Reduction of plasma free thiols

During glycation free thiol groups of protein are captured by reactive dicarbonyls and affects protein antioxidant property by conformational changes. In this study it was observed that free thiol groups were lower in diabetic patients (p <0.05) as compared to healthy controls (Figure 2, Table 5). In diabetic dyslipidemic patients it was significantly lower as compared to both controls (p <0.05) as well as diabetic subjects (p <0.05).

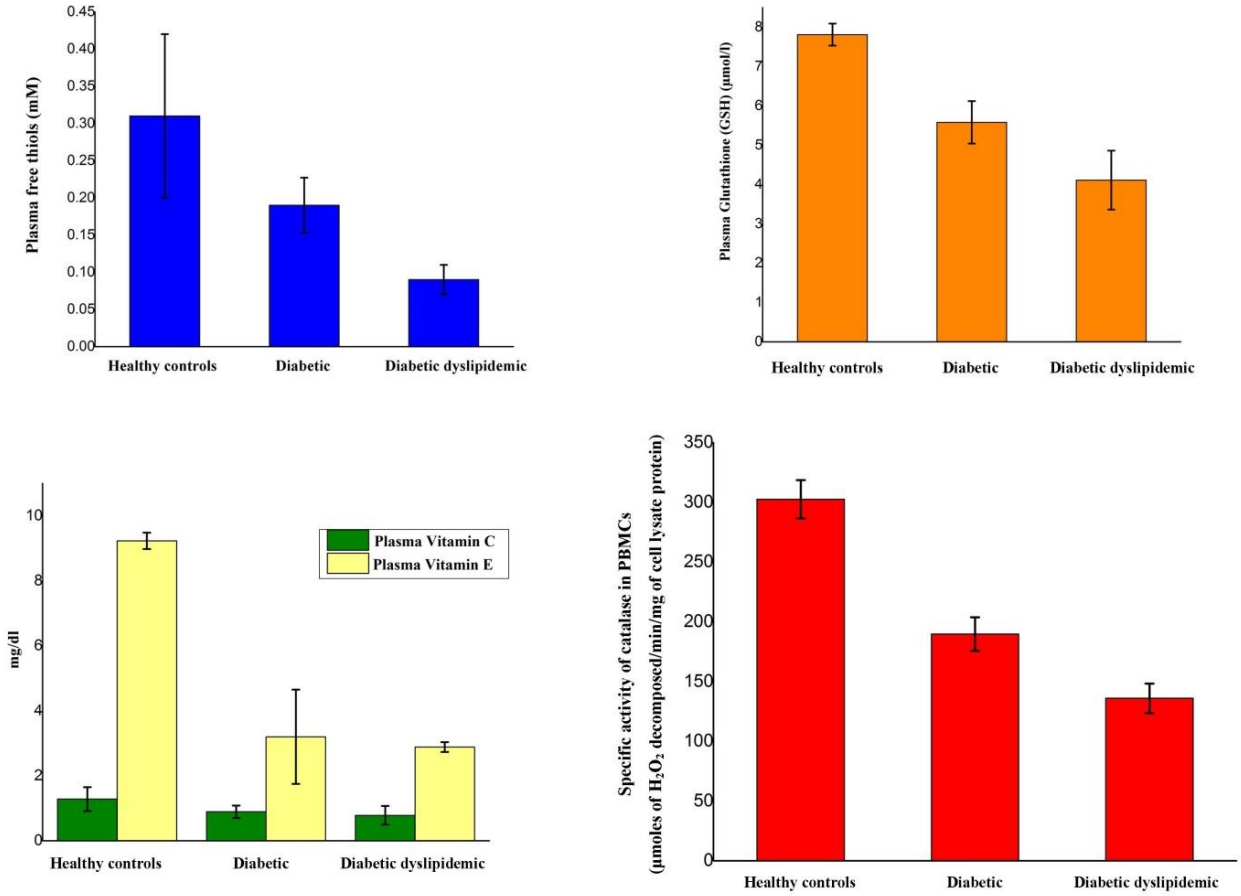


Figure 2: Level of antioxidants in study subjects; Plasma level of free thiols, glutathione (GSH), vitamin C, vitamin E and PBMCs catalase activity

Table 5: Level of antioxidants in study subjects

	Healthy controls	Diabetic	Diabetic dyslipidemic	*p* value
Plasma free thiols (mM)	0.31 ± 0.11	0.19 ± 0.037	0.09 ± 0.02	<0.05*
Plasma Glutathione (GSH) (μmol/l)	7.81 ± 0.28	5.58 ± 0.54	4.11 ± 0.75	<0.001
Plasma Vitamin C (mg/dl)	1.28 ± 0.37	0.89 ± 0.19	0.78 ± 0.287	<0.05
Plasma Vitamin E (mg/dl)	9.23 ± 0.25	3.2 ± 1.453	2.881 ± 0.15	<0.001
Specific activity of catalase in PBMCs (μmoles of H_2O_2 decomposed/min/mg of cell lysate protein)	302.55 ± 16.01	189.67 ± 14.11	135.94 ± 12.33	<0.01

**p value* between diabetic dyslipidemic and healthy controls; diabetic dyslipidemic and diabetic; diabetic and healthy controls.

Increase in protein carbonyl content

Protein carbonyl content level was higher in the diabetic (0.207 ± 0.13 nmol/mg) as well as in the diabetic dyslipidemic (0.32 ± 0.11 nmol/mg) patients as compared to healthy controls (0.16 ± 0.09 nmol/mg, p <0.05). In the diabetic group protein carbonyl content was significantly higher (p <0.05) as compared to diabetic dyslipidemic subjects (Figure 3).

Hyperpolarization of mitochondrial membrane potential in PBMCs (ΔΨm)

Membrane potential for PBMCs mitochondria was detected by the ratio of fluorescence emission values at 585 nm/516 nm. The shift from green monomers to red aggregates in mitochondrial membrane to in the mitochondrial cytosol of diabetic patients was monitored and found to be higher (3.22) compared to the control subjects (1.47) by calculating the red: green fluorescence intensities. Diabetic dyslipidemic subjects showed the highest values of ΔΨm (4.23), with the lowest values monitored in control subjects (Figure 4).

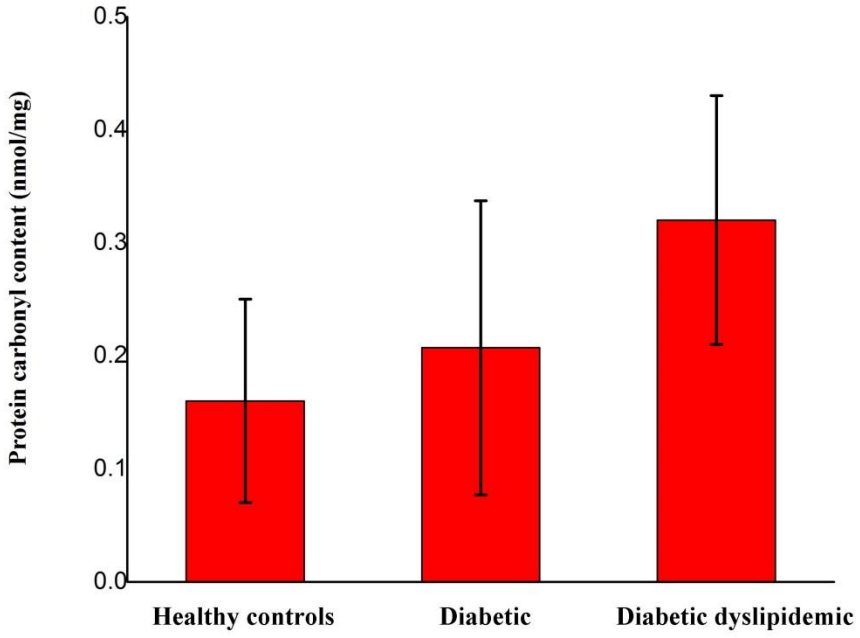


Figure 3: Concentration of plasma protein carbonyl content in study subjects.

Estimation of total Reactive Nitrogen Species (RNS) generated by PBMCs

Nitrosative stress was measured among diabetic subjects with and without dyslipidemia. Reactive nitrogen species (RNS) tend to be high among diabetic subjects with dyslipidemia compared to other two groups. Endogenous nitrosative stress or RNS have been also measured under confocal fluorescence microscopy. Images (60X) of PBMCs stained with DAF-2DA reveals (Figure 5) high endogenous RNS content among diabetic dyslipidemic group compared to other two groups.

Estimation of plasma nitric oxide

Plasma nitric oxide concentration (µmol/l) also have been found slightly increased among diabetes patients with dyslipdemia (21.78 ± 3.1) when compared to diabetic subjects (20.8 ± 2.15) and healthy controls (19.7 ± 2.9) [Figure 6]. Results are given in mean ± standard deviation (p value between diabetic dyslipidemic and healthy controls is 0.1738 and p value between diabetic and healthy controls is 0.4162 which is not statistically very much significant).

Estimation of platelet vWF and soluble P- Selectin level by Western blotting

vWF and soluble P- Selectin, important factors for platelet hyperactivity in diseased condition were up regulated in case of diabetic as well as diabetic-dyslipidemic subjects compared to the healthy controls (Figure 7).

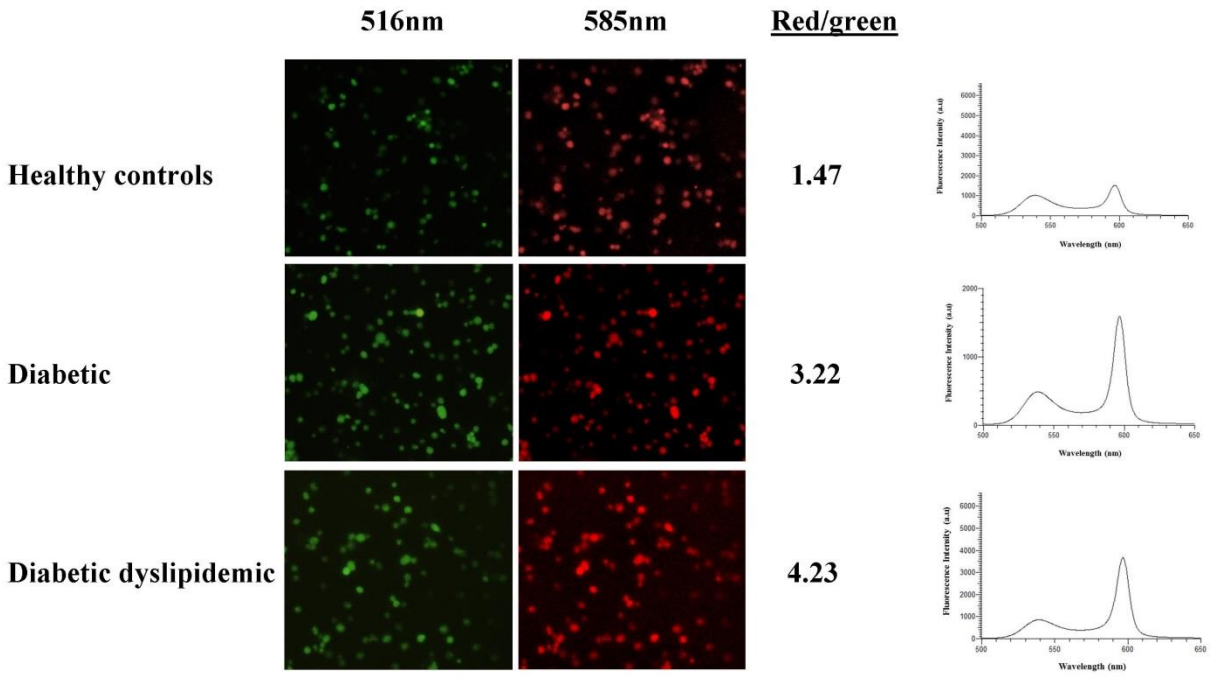


Figure 4: Mitochondrial membrane potential (ΔΨm) estimated by JC-1 in PBMCs of diabetic and diabetic dyslipidemic subjects indicating hyperpolarization compared to control subjects observed under Hitachi (F-7000) fluorescence Spectrometer (right).

Confocal fluorescence images of PBMCs (60X) reflecting JC-1 localization in diabetic subjects with and without dyslipidemia and healthy control. Strong accumulation of red color indicates hyper polarization of PBMCs of type 2 diabetic patients with and without dyslipidemia compared to healthy controls.

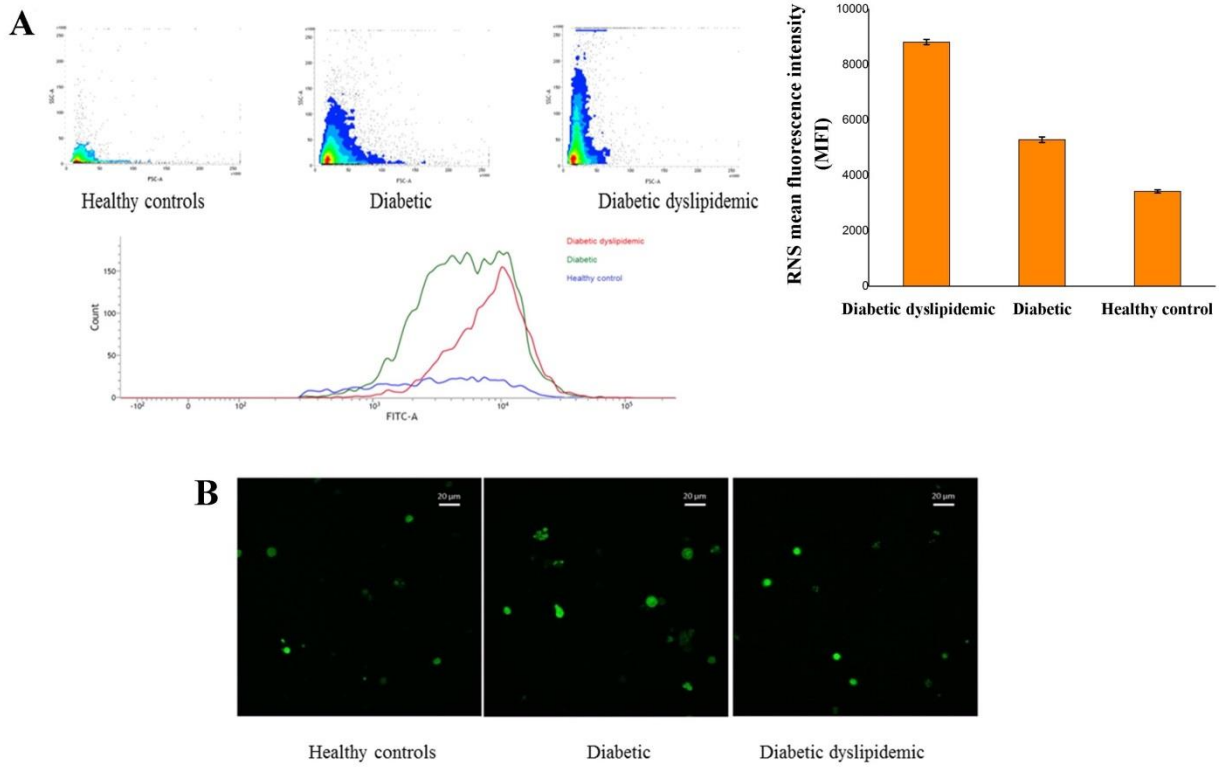


Figure 5: Endogenous reactive nitrogen species (RNS) concentration estimated by DAF-2DA under Flow cyrometry. FITC signal detects DAF-2DA signal. [A] Diabetic dyslipidemic [8,810 ± 93.66] group shows highest RNS mean fluorescence intensity (MFI) compared to diabetic subjects without complications [5,285 ± 99.29] and healthy controls [3,420 ± 56.16]. Results are given in mean ± standard deviation (p <0.001). Confocal fluorescence microscopy images (60X) of PBMCs isolated from healthy

controls, diabetic subjects and diabetic subjects with dyslipidemia stained with DAF-2DA to measure RNS intensity.

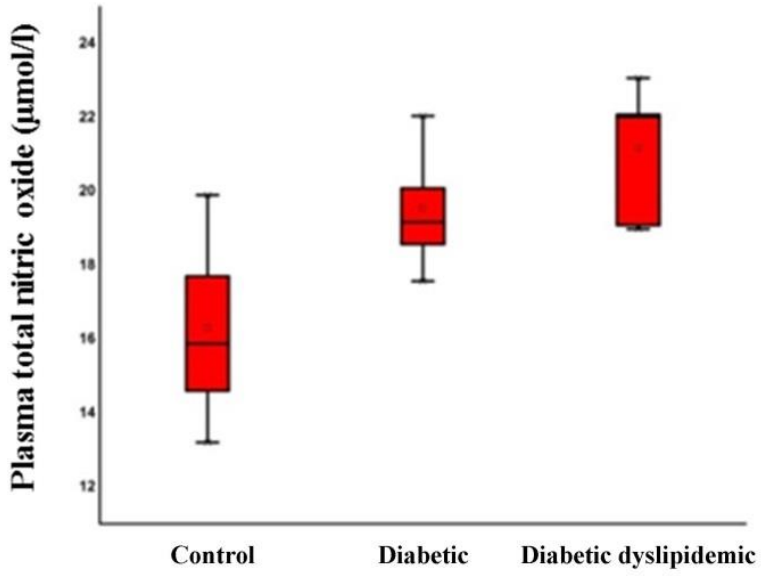


Figure 6: Box-Whisker plot showing plasma total nitric oxide in diabetic subjects with and without dyslipidemia and healthy controls

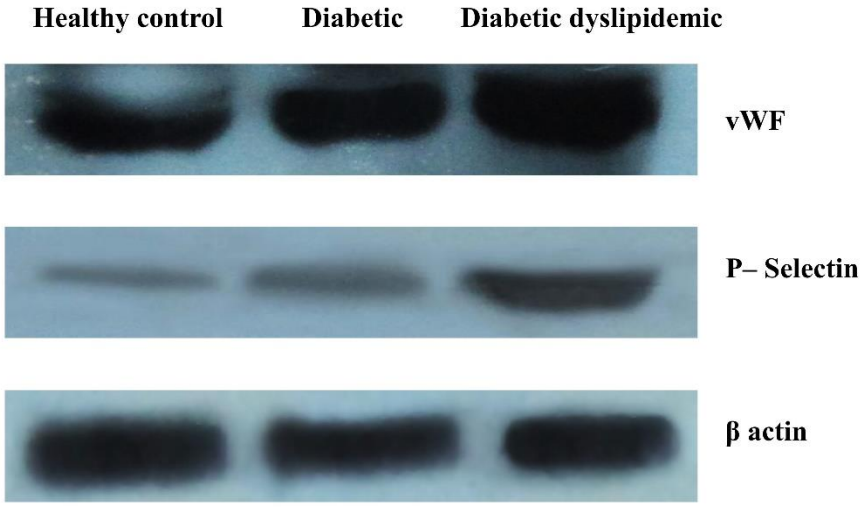


Figure 7: Western blot of vWF and soluble P- Selectin as obtained from platelet lysates of healthy control, diabetic and diabetic dyslipidemic subjects. β actin was used as a housekeeping gene.

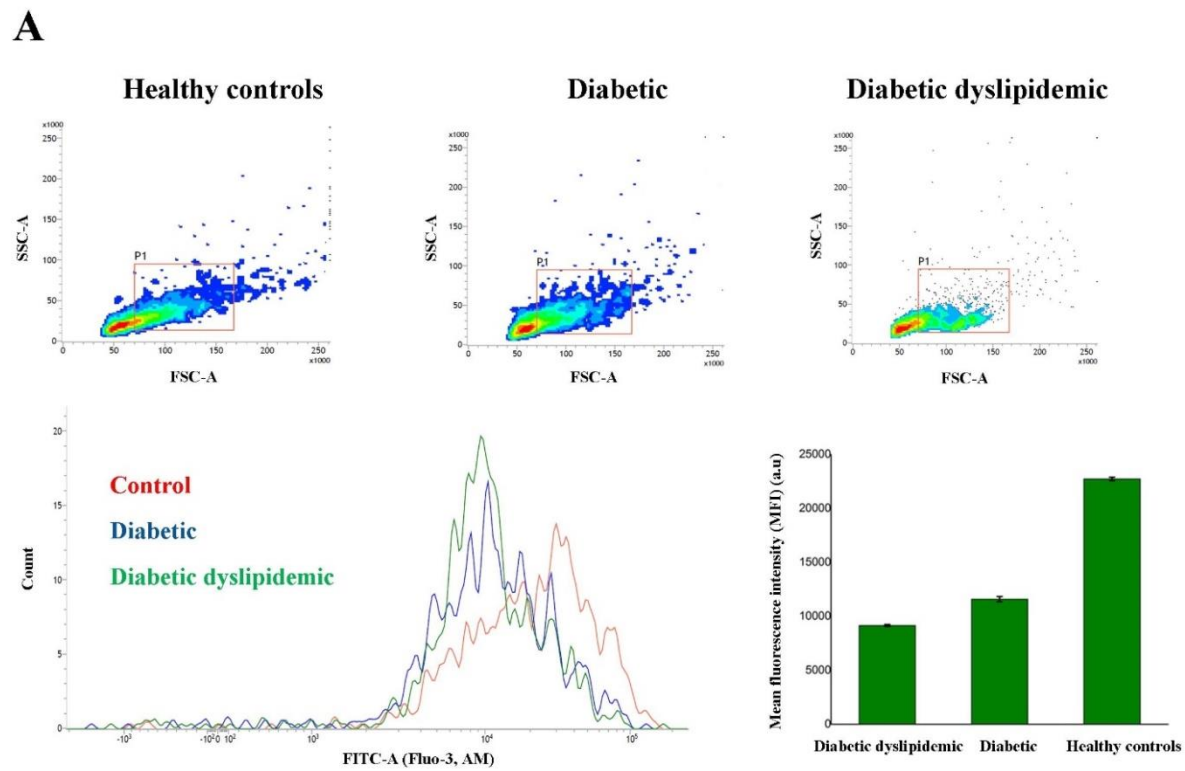
Alterations in plasma Ca^{2+} concentration

Significant increase in plasma Ca^{2+} concentration is associated diabetic dyslipidemic subjects followed by diabetic and healthy controls (p <0.05) which indicate impaired calcium signaling among diabetic conditions as shown in the below table.

	Healthy controls	Diabetic	Diabetic dyslipidemic	*p* value
Plasma Ca^{2+} (mg/dl)	8.031 ± 3.395	11.562 ± 5.393	16.136 ± 6.35	[a]0.005, [b]<0.001, 0.0014

Changes in intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was estimated by measuring the intensity of fluorescent dye Fluo-3, AM at 530nm among the three subject groups. Intracellular Ca^{2+} concentration was significantly lower in diabetic dyslipidemic subjects compared to diabetic and healthy subjects as observed under flow cytometry. Confocal fluorescence microscopy data also reveals decreased endogenous Ca^{2+} concentration in diabetic dyslipidemic group compared to other two subject groups (Figure 8).



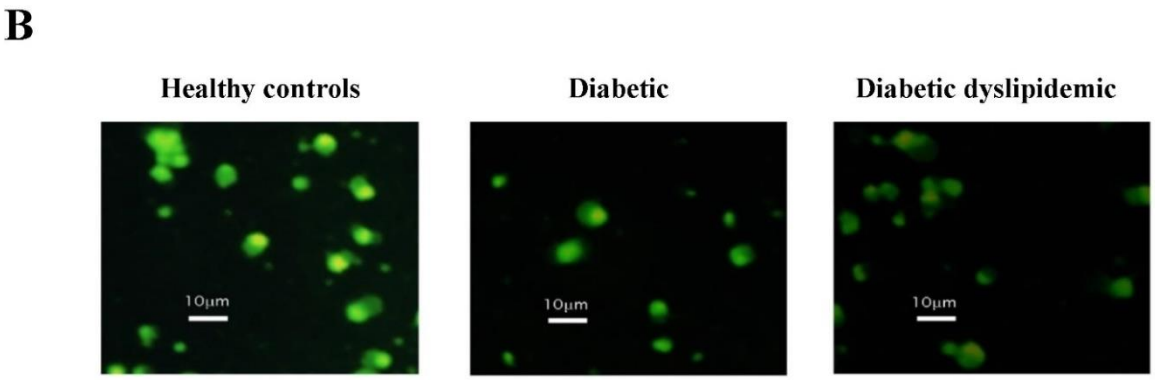


Figure: 8. Endogenous Ca^{2+} concentration estimated under Flow cytometry (A). FITC signal detects Fluo-3AM signal. Diabetic dyslipidemic group shows lowest intracellular Ca^{2+} compared to diabetic subjects without complications and healthy controls. Confocal fluorescence microscopy images of PBMCs (60X) (B) isolated from three subject groups stained by Fluo-3 AM to detect endogenous Ca^{2+} concentration. Diabetic dyslipidemic group shows lowest endogenous Ca^{2+} concentration compared to other two groups.

Changes in PBMCs size and complexity

PBMCs size and complexity have been observed under flow cytometry where Forward Scatter (FSC) and Side Scatter (SSC) represent those factors respectively. PBMCs isolated from three subject groups show no significant differences in their size. But PBMCs isolated from diseased groups show much complexity than healthy control group where diabetic dyslipidemic subjects show highest complexity in their PBMCs (Figure 9)

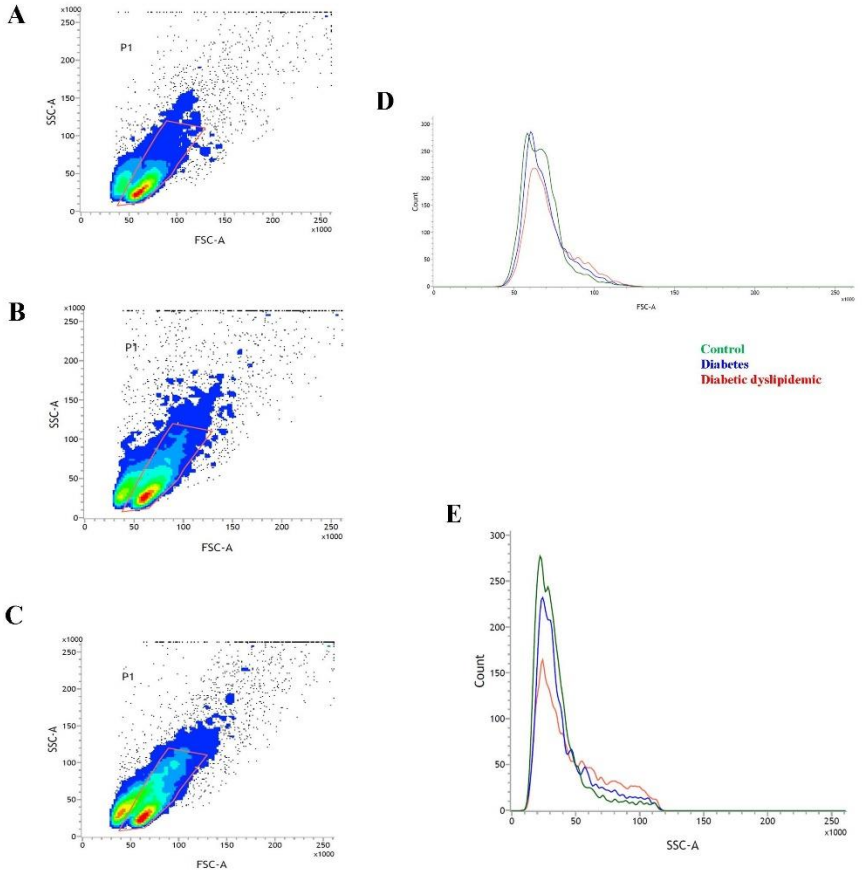


Figure: 9. Forward scatter (FSC) and Side scatter (SSC) of PBMCs isolated from control (A), diabetic (B) and diabetic dyslipidemic (C) subjects under flow cytometry.

Downregulation of ADIPOQ, ADIPOR1 and ADIPOR2 in visceral (VAT) andsubcutaneous (SAT) adipose tissue

Gene expression level have been explained by relative fold change in comparison with the control group. 2-ΔΔct value 0>1 explains a downregulation whereas 2-ΔΔct value greater than 2 explains upregulation. The healthy control group represents 2-ΔΔct valueas 1 indicating no significant fold change. The accuracy of the PCR is monitored by melt curve analysis (Figure 11). Expression of ADIPOQ, ADIPOR1 and ADIPOR2 are much greater in subcutaneous (SAT) than visceral (VAT) adipose tissue in general. Significant downregulation of ADIPOQ, ADIPOR1 and ADIPOR2 (Figure 10 B-D) have been observed in diabetic dyslipidemic group compared to diabetic group with no report of dyslipidemia. ADIPOQ expression was found to be 0.2915 in SAT and 0.1899 in VAT in diabetic dyslipidemic group and 0.5107 in SAT and 0.2341 in VAT in diabetic group without dyslipidemia ($P < 0.05$). Compared to control diabetic dyslipidemic subjects showed a downregulation of 0.2852 in SAT and 0.2398 in VAT whereas diabetic subjects without dyslipidemia showed a downregulation of 0.572004 in SAT and 0.3211 in VAT ($P < 0.05$) for ADIPOR1. For ADIPOR2 a downregulation of 0.2274 in SAT and 0.1709 in VAT for diabetic dyslipidemic group and 0.4122 in SAT and 0.3012 in VAT for diabetic subjects without dyslipidemia have been observed ($P < 0.05$).

Downregulation of ADIPOR1 and ADIPOR2 in PBMCs

Significant downregulation of ADIPOR1 and ADIPOR2 (Figure 10 C) is associated in diabetic dyslipidemic group compared to diabetic group with no report of dyslipidemia in PBMCs. Compared to control diabetic dyslipidemic subjects showed a downregulation of 0.0547 whereas diabetic subjects showed a downregulation of 0.1466 ($P < 0.05$) for ADIPOR1. For ADIPOR2 a downregulation of 0.1163 for diabetic dyslipidemic group and 0.1558 for diabetic subjects without dyslipidemia have been observed ($P < 0.05$). The accuracy of the PCR is monitored by melt curve analysis (Figure 12).

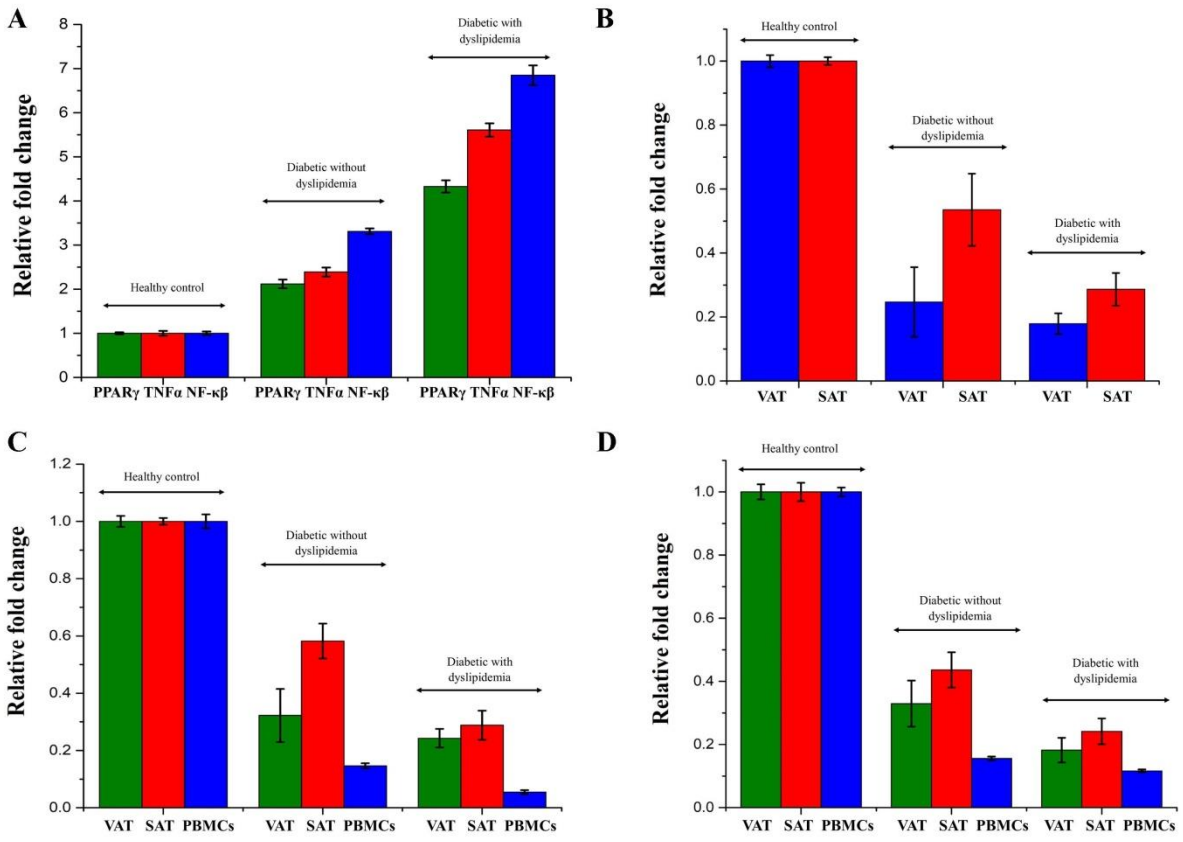


Figure: 10. [A] PPARγ, TNFα, NF-κβ expression in PBMCs, [B] Adiponectin expression in visceral (VAT) and subcutaneous (SAT) adipose tissue, [C] Adiponectin receptor 1(ADIPOR1) expression in VAT, SAT and PBMCs, [D] Adiponectin receptor2 (ADIPOR2) expression in VAT, SAT and PBMCs.

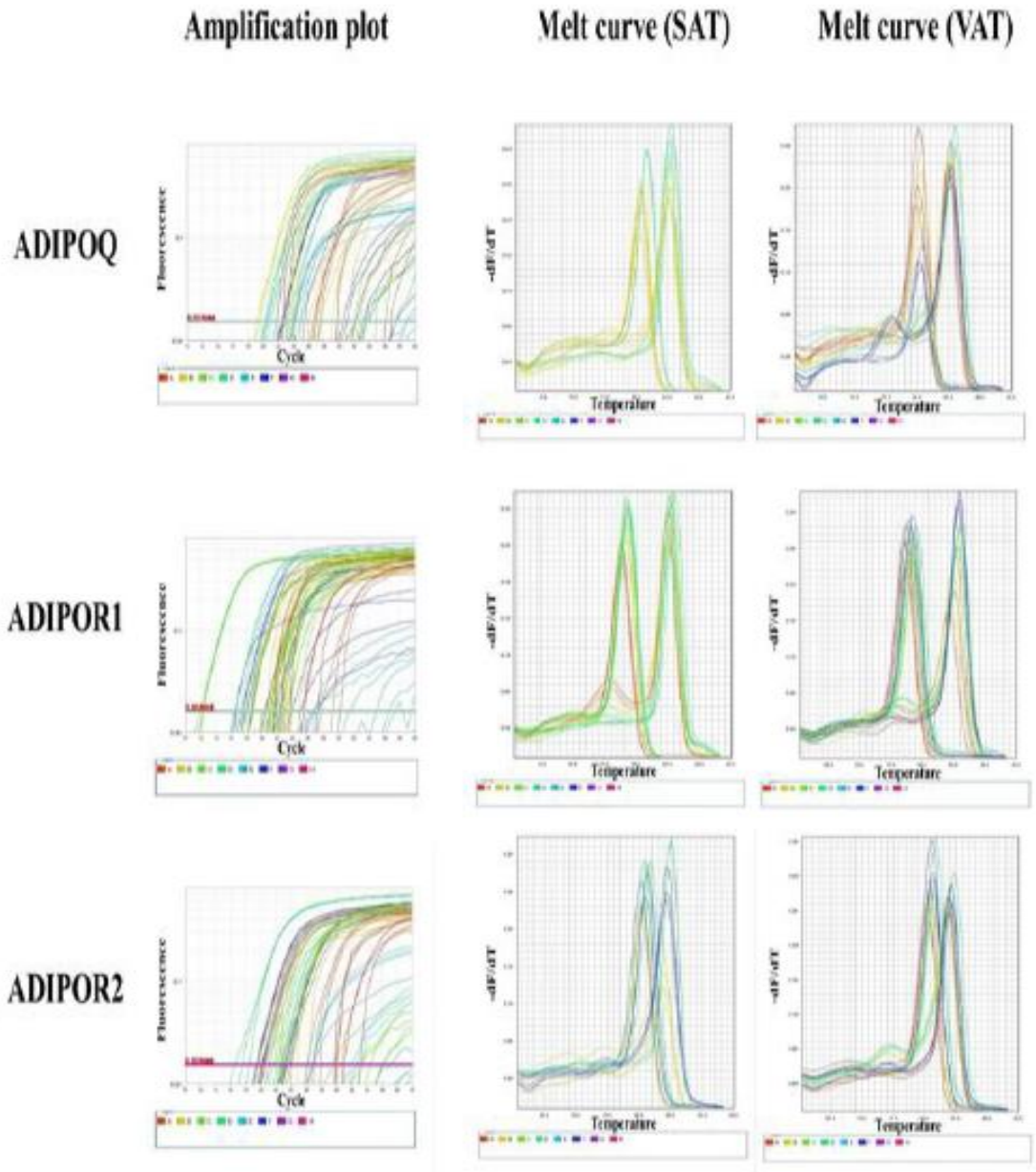


Figure 11. Amplification plots and melt curves for ADIPOQ (adiponectin), ADIPOR1 and ADIPOR2 expression in subcutaneous (SAT) and visceral (VAT) adipose tissue

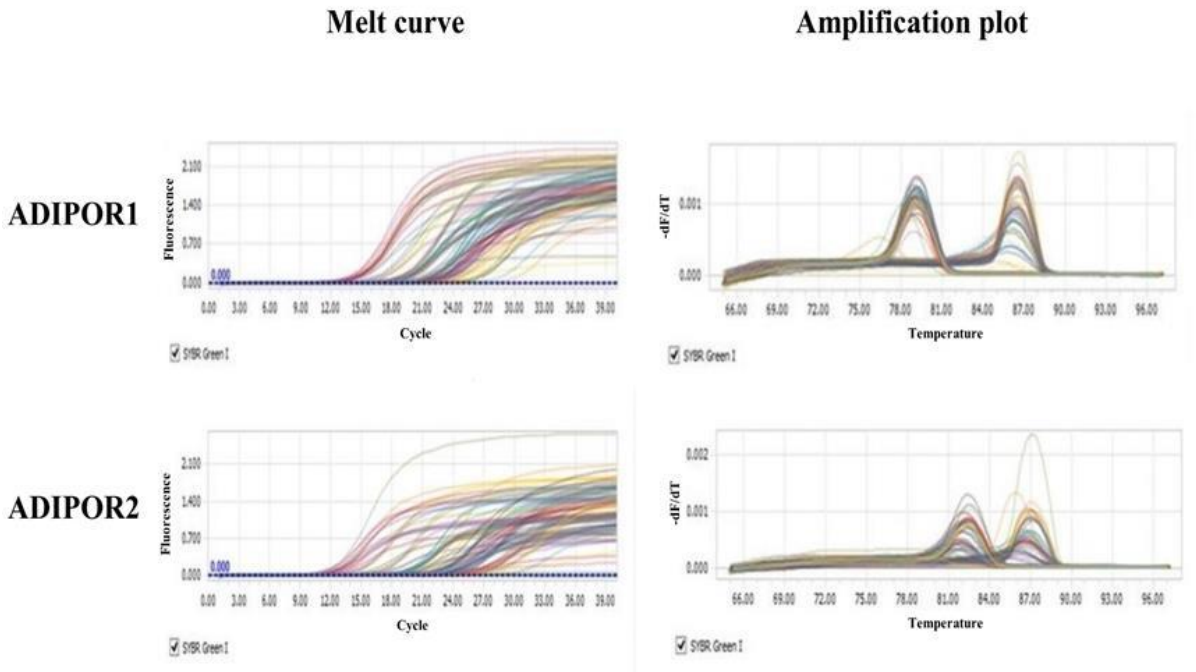


Figure12. Amplification plots and melt curves for ADIPOR1 and ADIPOR2 expression in PBMCs

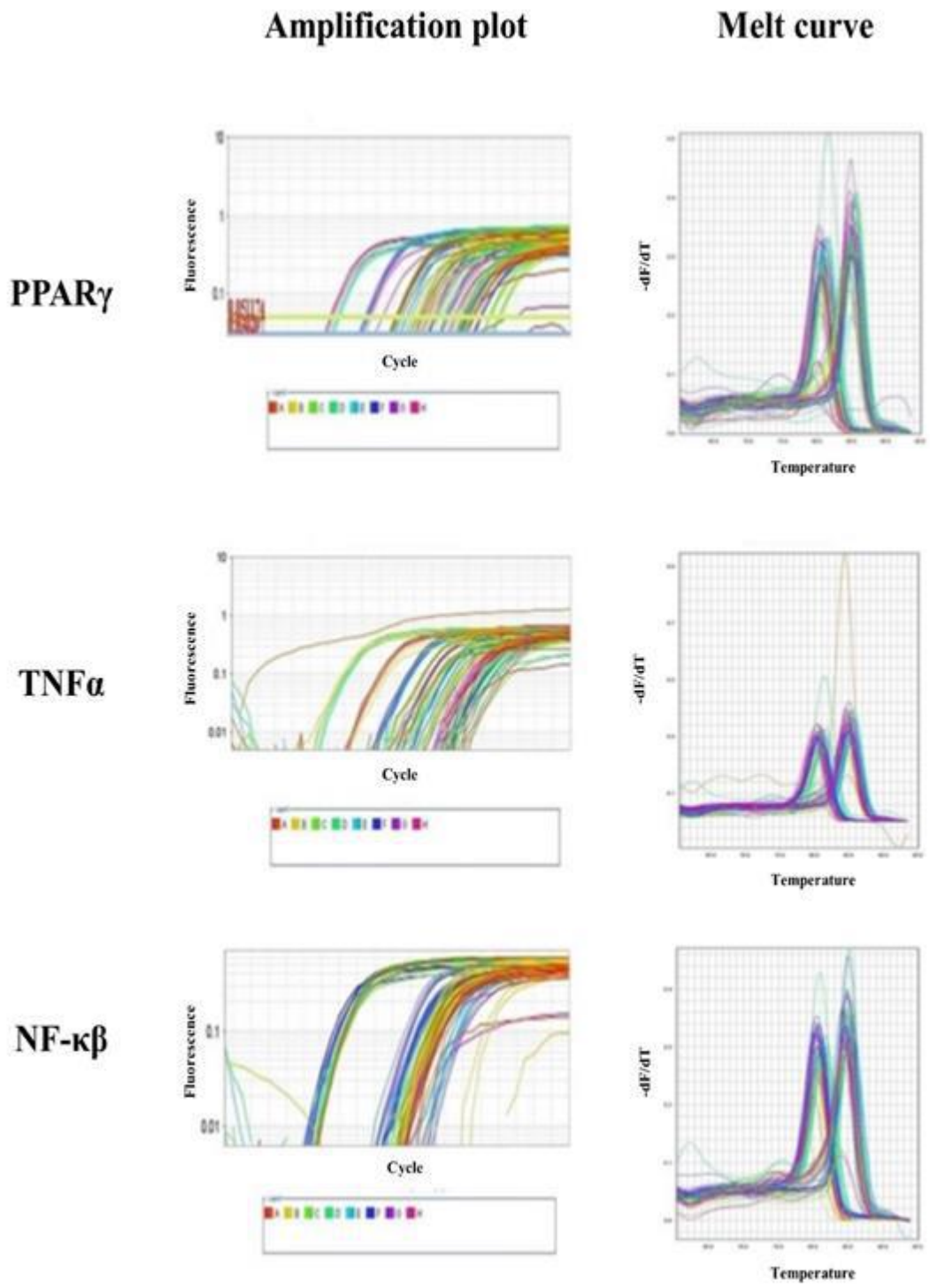


Figure: 13 Amplification plots and melt curves for PPARγ, TNFα, NF-κβ expression in PBMCs

Downregulation of PPARγ and upregulation NF-κβ and TNFα in PBMCs

PPARγ showed a 0.2124 fold decrease in diabetic dyslipidemic group (Figure 10 A) whereas diabetic subjects without dyslipidemia showed 0.4331 fold decrease ($P < 0.05$). Inflammatory biomarkers such as NF-κβ and TNFα showed significant upregulation in PBMCs of diabetic dyslipidemic group compared to diabetic subjects without dyslipidemia. A fold change of 5.61 and 2.395 for NF-κβ have been found to be associated with diabetic dyslipidemic and diabetic subjects without dyslipidemia respectively ($P < 0.05$). An upregulation of 6.852 for diabetic dyslipidemic and 3.313 for diabetic subjects without dyslipidemia for TNFα have been also observed ($P < 0.05$). The accuracy of the PCR is monitored by melt curve analysis (Figure 13).

Increase in lipid hydroperoxides (LOOH) in plasma

Lipid hydroperoxides (LOOH) is a biomarker to detect and quantify early stage lipid peroxidation. A significant increase (all, $P < 0.05$) in plasma LOOH (6.231 ± 0.719 μmol/l) were found to be associated with diabetic dyslipidemic condition compared to diabetic subjects without dyslipidemia (4.844 ± 0.425 μmol/l) and healthy controls (3.011 ± 0.307 μmol/l) . The data is represented in figure 11.

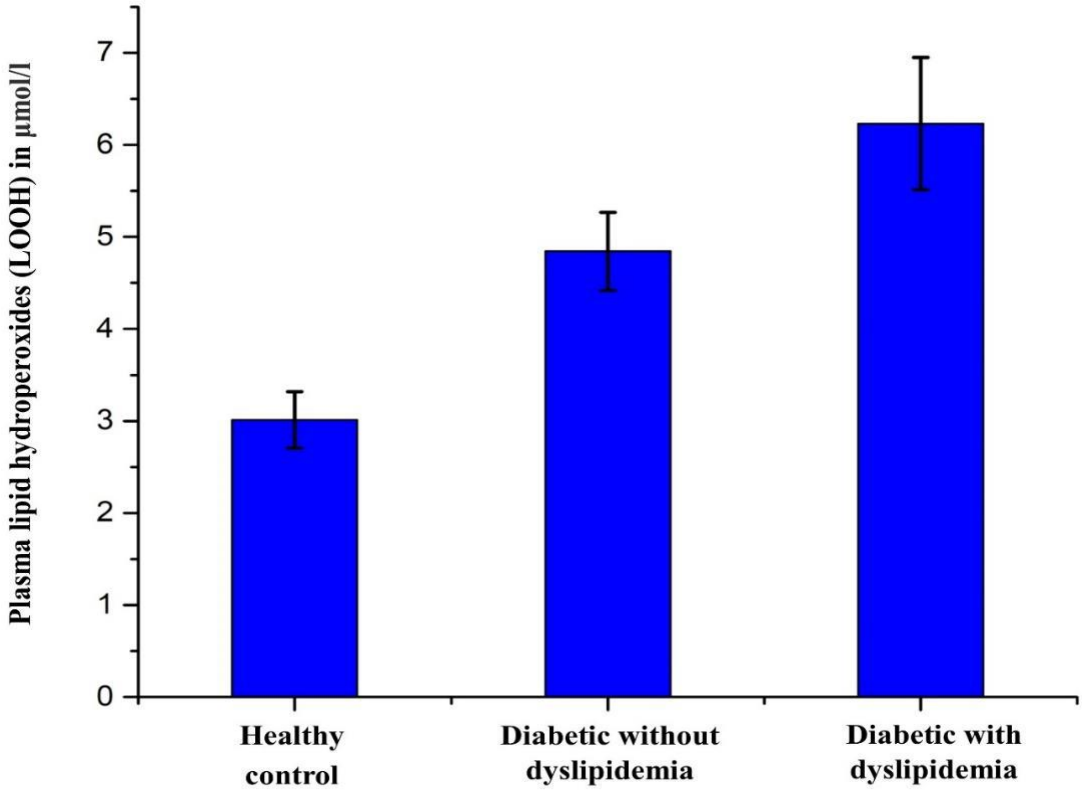


Fig 11.Comparison of plasma adiponectin, C reactive protein (CRP), 4 hydroxynonenal (4-HNE) adducts and malondialdehyde (MDA) levels

A significant increase in plasma level of CRP, 4-HNE adducts and MDA, and decrease in plasma adiponectin level have been noted in diabetic subjects with dyslipidemia compared to diabetic subjects without dyslipidemia and healthy controls ($p < 0.05$).

Genetic analysis: Comparisons of genotypes, alleles and haplotypes

To infer whether the genotype frequencies of four adiponectin SNPs differed significantly in two diabetic and healthy control groups, a global $\chi 2$ test was performed using a 3X3 format. Frequencies of rs2241766 ($\chi 2 = 20.885$, $p_{corrected} < 0.001$) and rs1501299 ($\chi 2 = 20.885$, $p_{corrected} < 0.001$) differed significantly among groups. Comparison of genotype frequencies was next conducted in four pairwise combinations namely diabetes without dyslipidemia vs healthy control, diabetes with dyslipidemia vs healthy control, diabetes with dyslipidemia vs diabetes without dyslipidemia and total diabetes patient vs healthy control following a recessive model. Genotype proportions of rs1501299 differed significantly between diabetes with dyslipidemia vs healthy control, diabetes with and without dyslipidemia and total diabetic subject vs healthy controls under recessive model following multiple correction (Table 6).

Genotype frequencies were significantly different for rs2241766 in diabetic with dyslipidemia and total diabetic subjects with respect to healthy control. Significant differences in the proportion of alleles were detected in all four pairwise comparisons for rs2241766 and rs1501299. None of the adiponectin receptor polymorphisms were associated with diabetes or diabetes induced dyslipidemia. Genetic association was further investigated at the level of haplotypes for the adiponectin SNPs, both of which were located in the promoter region of the gene

Table 6: Genetic association of adiponectin and its receptor polymorphisms with diabetes and dyslipidemia

	Diabetes without dyslipidemia and healthy control	**Diabetes with dyslipidemia and healthy control**	**Diabetic with dyslipidemia and diabetic without dyslipidemia**	**Total diabetic and healthy control**
rs2241766 (T>G) [Adiponectin]				
TT; TG; GG	57/72; 18/14; 11/4	44/72; 23/14; 21/4	44/57; 23/18; 21/11	101/72; 41/14; 32/4
OR, 95% CI,	3.1, 0.964-10.319,	6.7, 2.208-20.57,	2.1,0.96-4.758,	4.8,1.656-14.173,
p value	0.057	0.0008*	0.063	0.004*
T; G	132/158; 40/22	11/158; 65/22	111/132; 65/40	243/158; 105/22
OR, 95% CI,	2.2, 1.232-3.845,	4.2,2.448-7.223,	1.9,1.21-3.085,	3.1,1.88-5.123,
p value	0.007*	<0.0001*	0.006*	<0.0001*
rs1501299 (G>T) [Adiponectin]				
GG; GT; TT	37/61; 39/27; 10/2	26/61; 44/27; 18/2	26/37; 44/39; 18/10	63/61; 83/27; 28/2
OR, 95% CI,	5.8, 1.23-27.248,	11.3, 2.539-50.416, 0.0015*	2,0.845-4.52,	8.4,1.962-36.29,
p value	0.026*		0.1173	0.0042*
G, T	113/149; 59/31	96/149; 80/31	96/113, 80/59	209/149, 139/31
OR, 95% CI,	2.5, 1.524-4.133,	4.0, 2.46-6.522,	1.6, 1.035-2.46,	3.2, 2.054-4.976,
p value	0.0003*	<0.0001*	0.034*	<0.0001*
rs1342387 (G>A) [Adiponectin receptor 1]				
GG; GA; AA	33/44; 31/28; 22/18	28/44; 34/28; 26/18	28/33; 34/31; 26/22	61/44; 65/28; 48/18
OR, 95% CI,	1.4, 0.677-2.792,	1.7, 0.841-3.345,	1.2, 0.626-2.376,	1.5, 0.825-2.816,
p value	0.378	0.142	0.559	0.179
G; A	97/116; 75/64	90/116; 86/64	90/97; 86/75	187/116; 161/64
OR, 95% CI,	1.4, 0.913-2.152,	1.7, 1.132-2.65,	1.2, 0.8103-1.885,	1.5, 1.077-2.261,
p value	0.123	0.0113	0.325	0.0187

rs4766415 (A>T) [Adiponectin receptor 2]				
AA; AT; TT	21/23; 45/46; 20/21	17/23; 43/46; 28/21	17/21; 43/45; 28/20	38/23; 88/46; 48/21
OR, 95% CI,	0.9, 0.495-2.001,	1.5,0.79-2.976,	1.5, 0.786-3.015	1.2, 0.693-2.26,
p value	0.99	0.206	,0.208	0.456
A; T	87/92; 85/88	77/92; 99/88	77/87; 99/85	164/92; 184/88
OR, 95% CI,	1.0, 0.672-1.55,	1.3, 0.886-2.04,	1.3, 0.863-2.01,	1.2, 0.818-1.682,
p value	0.92	0.165	0.202	0.386

*Indicates P<0.05 and statistically significant

Interaction among multiple parameters

To predict the risk for diabetes induced dyslipidemia non-HDL-Cholesterol level was considered as a dependent variable in the linear regression analysis conducted between two groups of diabetic subjects. The variables that seemed to predict the risk for developing dyslipidemia in diabetes subjects included triglycerides, HDL, LDL, adiponectin, MDA, 4-HNE adducts and rs2241766 (T>G) were predicted to be the risk variables according to their respective *P* values (Table 7). MDR analysis was next carried out on these variables and three best interaction models each consisting of three independent factors with notable statistical significance (P <0.05) were identified (Table 8). Among the parameters studied, plasma MDA, adiponectin and rs2241766 yielded the best suited model indicating the intimate correlations they shared in diabetic dyslipidemia (χ^2=103.314, OR 89.687, 95% CI 28.65-280.75). This model was also selected on the basis of higher cross validation consistency coefficient (10/10) and testing balanced accuracy (0.872). A second model relating plasma adiponectin level with two risk associated SNPs (rs2241766 and rs1501299) also predicted the prevalence of dyslipidemia in diabetes (Table 9). Finally, a gene-gene interaction model which involved three SNPs (rs2241766, rs1501299 and rs1342387) was also associated with diabetic dyslipidemia (Table 5).

Table 7: Association of adiponectin promoter haplotypes with diabetes and dyslipidemia

Haplotypes	Reference (major) haplotype		Total diabetic vs healthy control	Diabetic with dyslipidemia vs diabetic without dyslipidemia	Diabetic without dyslipidemia vs healthy control	Diabetic with dyslipidemia vs healthy control
TG	-	Case[a]	147 (0.422)	91 (0.527)	94 (0.545)	52 (0.297)
		Control[b]	129 (0.716)	96 (0.545)	129 (0.716)	129 (0.716)
GT	TG	Case	43 (0.123)	62 (0.351)	21 (0.121)	21 (0.121)
		Control	2 (0.010)	21 (0.122)	2 (0.010)	2 (0.010)
		p value[c]	<0.01	<0.01	<0.01	<0.01
TT	TG	Case	95 (0.277)	18 (0.103)	38 (0.222)	59 (0.333)
		Control	30 (0.162)	38 (0.223)	29 (0.162)	29 (0.162)
		p value[c]	<0.01	0.091	0.111	<0.01
GG	TG	Case	62 (0.179)	3 (0.018)	19 (0.111)	44 (0.248)
		Control	19 (0.111)	19 (0.111)	20 (0.113)	20 (0.113)
		p value[c]	0.06	0.015	0.446	<0.01

[a]Haplotype frequency and number in case group

[b]Haplotype frequency and number in control group

[c]Between group Comparison was made between a haplotype and the reference haplotype

Table 8: Multiple linear regression analysis to identify the biochemical and genetic variables as predictors for diabetes associated dyslipidemia

Non-HDL-Cholesterol			
Independent variables	**B**	**Std. Error**	***P-value***
Fasting blood glucose	0.019	0.058	0.710
Postprandial blood glucose	0.029	0.053	0.615
HbA1c	0.020	0.104	0.829
Triglycerides	0.511	0.039	**<0.001**
HDL	-0.295	0.048	**<0.001**
LDL	0.408	0.024	**<0.001**
Adiponectin	-0.065	0.031	**0.046**
CRP	0.017	0.027	0.509
MDA	0.109	0.022	**<0.001**
4-HNE adducts	0.199	0.021	**<0.001**
rs2241766 (T>G)	0.009	0.003	**<0.001**
rs1501299 (G>T)	0.002	0.003	0.405
rs1342387 (G>A)	0.001	0.001	0.484
rs4766415 (A>T)	0.000	0.001	0.980

Table 9: Interaction models and performance evaluations

Interaction model	**Training sample accuracy**	**Checking sample accuracy**	**Cross validation consistency**	**Chi-square (*P value*)**	**OR (95% CI)**
Plasma adiponectin x Plasma MDA x rs2241766	0.884	0.872	10/10	103.314 ($P < 0.001$)	89.687 (28.65-280.75)
Plasma adiponectin x rs2241766 x rs1501299	0.778	0.697	10/10	56.989 ($P < 0.001$)	21.414 (8.393-54.632)
rs2241766 x rs1501299 x rs1342387	0.7377	0.6395	10/10	37.391 ($P< 0.001$)	7.615 (3.859-15.028)

DISCUSSION

The present observational study primarily focuses to identify a risk prediction model for dyslipidemia in type 2 diabetic patient. In this study markers of antioxidant defense in patients with type 2 diabetes were evaluated. The study included a group of type 2 diabetic patients with and without dyslipidemia. Results obtained in this study showed that patients with diabetes and dyslipidemia had significantly lower antioxidant defense activity reflected by decrease in plasma free thiols, glutathione (GSH), vitamin C, vitamin E and PBMCs catalase activity. From this study we may conclude that the presence of dyslipidemia among diabetic subjects degrades the antioxidant defense system, increases mitochondrial dysfunction and nitrosative stress. These factors together exert potential changes in platelet behavior increasing aggregation, further prompting metabolic condition. Therefore, these markers can be a good predictor of CVD among diabetic dyslipidemic subjects. Co-association of diabetes with other metabolic complications such as dyslipidemia increases the prevalence of hypertension, obesity and cardiovascular disorders (Figure 1.8).

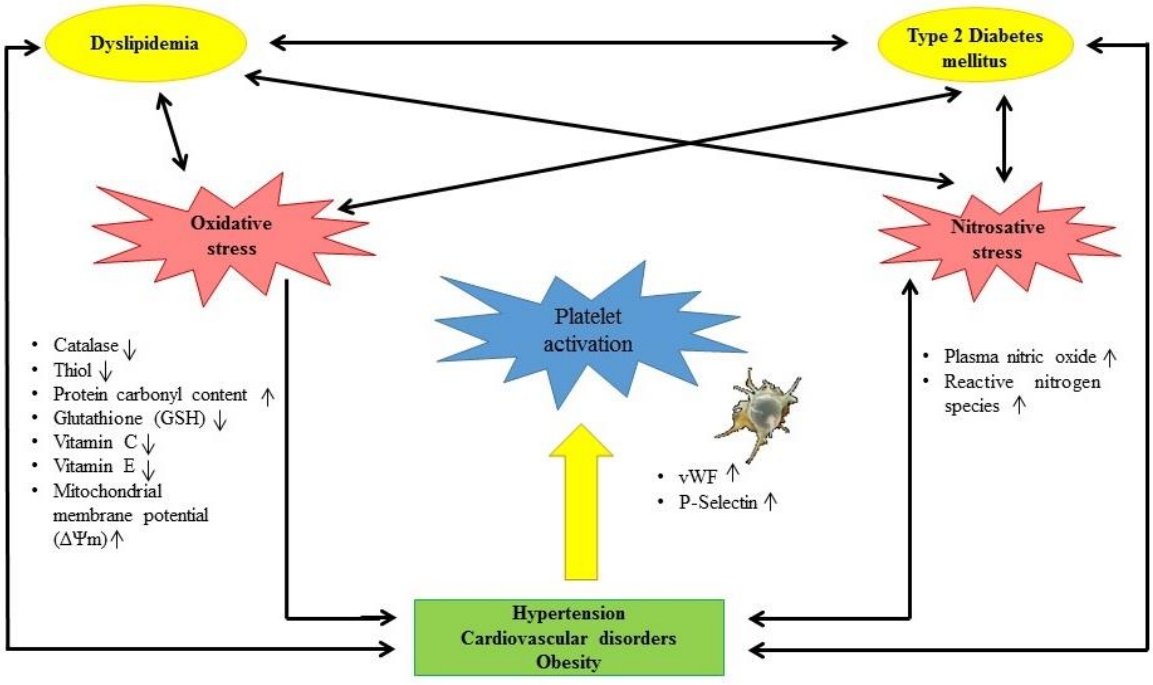


Figure 1.8: Hypothetical scheme representing the association between type 2 diabetes mellitus with dyslipidemia in term of oxidative, nitrosative stress and platelet hyperactivity, a precursor of CVD.

A battery of clinical, biochemical and genetic characteristics was analyzed to detect statistical association of a factor independently or in combination with others with development of dyslipidemia coupled with diabetes. Multiple linear regression analysis identifies correlation of triglycerides, HDL, LDL, adiponectin, MDA, 4-HNE adducts and rs2241766 (T>G) with non-HDL-Cholesterol in diabetic dyslipidemic condition. Of these variables, the association of plasma adiponectin, MDA, 4-HNE adducts and rs2241766 were new observations. Further MDR analysis identifies a three-factor model composed of plasma adiponectin and MDA levels along with ADIPOQ SNP rs2241766 to predict the risk of dyslipidemia in diabetes (Figure 2).

Adipocyte whose prime function remains to govern lipid metabolism is a storage site of a number of hormones viz. adiponectin, leptin, resistin and visfatin [38,39]. Expression of adiponectin and its plasma concentrations are inversely related with insulin resistance, fatty acid oxidation, lipid metabolism and obesity [15]. Since dyslipidemia is believed to be a pre-CVD metabolic state, lowering of adiponectin is a clear indication of worsening of diabetes related complications. Expressions of adiponectin as well as its receptors were detected in diabetic-dyslipidemic condition in both SAT and VAT in this study. The reason underlying the decreased expression of ADIPOQ in diabetes induced metabolic conditions remains elusive. And in absence of any further study, drawing any functional correlation of rs2241766 which results in a synonymous change will be speculative.

Increased plasma MDA level is another risk marker for dyslipidemia in diabetes. Generation of excess reactive oxygen species is a trademark of diabetic dyslipidemia [24]. Lipid peroxidation is a process under which free radicals attack lipids containing polyunsaturated fatty acids (PUFAs) resulting in lipid peroxyl radicals and hydroperoxides [39]. MDA and 4-HNE adducts are two classical examples of lipid peroxidation secondary products among which MDA reacts with thiobarbituric acid. MDA is believed to be the most mutagenic product of lipid peroxidation as it can be either enzymatically metabolized or can react on proteins or DNA to form adducts resulting in biomolecular damages [39,40].

The present study underscores the importance of assessing cumulative impact role of multiple factors in estimating disease risk. As per the genetic association conducted here the minor alleles of rs2241766 and rs1501299 are detected to be correlated with both diabetes and diabetic dyslipidemia. Towards finding additional factors associated with disease risk

multiple linear regression analysis is conducted which captures relatively weak genetic impact of rs2241766 in the increment of non-HDL-Cholesterol level in dyslipidemic individuals. This result reminds us about the utility of assessing quantitative contribution of study variables. Finally, application of multifactor dimensionality reduction captures the cumulative cue of rs2241766 in combination of plasma adiponectin and MDA. The cross-validation consistency score in MDR is a measure of the degree of consistency with which the selected interaction is identified as the best model among all possibilities considered. The testing balanced accuracy is a measure of the degree to which the interaction accurately predicts case–control status with scores between 0.50 (indicating that the model predicts no better than chance) and 1.00 (indicating perfect prediction). The outcome of this statistical analysis has an obvious biological underpinning as adipocyte dysfunction and oxidative stress have long been implicated in diabetes metabolic complications.

A careful attempt has been taken to minimize the effects of lifestyle and other confounding factors by following strict inclusion and exclusion criteria such as recruitment of age and gender-matched subjects from similar socioeconomic strata and dyslipidemic patients who are yet to start any anticholesterol medication which may influence inflammatory and oxidative markers. To capture the temporal pattern of biomarkers when dyslipidemia initially sets in T2DM, a longitudinal design would have been the most informative one. Nonetheless, the current study successfully addresses the direction of the association of biochemical and genetic signatures in dyslipidemia coupled with diabetes and the findings may potentially be incorporated in the future clinical practice as an indicator of poor prognosis of diabetes.

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SUMMARY OF THE FINDINGS:

Diabetes mellitus, a major multifactorial disorder, is closely associated with impaired lipid metabolism, dyslipidemia and obesity, emerging as a major health challenge of this millennium.. Rapid urbanization and changing lifestyle combined with genetic susceptibility results in metabolic syndrome like dyslipidemia and obesity. The characteristic features of dyslipidemia are increased plasma triglyceride, decreased HDL cholesterol and increased small dense LDL-cholesterol particles. Though, dyslipidemia in diabetes is an established risk factor for cardiovascular diseases, yet there is dearth of identifying appropriate signatures to address this issue. Adiponectin , is actively involved in lipid metabolism and plays crucial role in dyslipidemia and vascular complications.

To investigate the underlying genetic and molecular pattern of adiponectin metabolic pathway in diabetic dyslipidemia we performed a case-control study in a total 264 individuals belonging to 3 categories such as diabetes with dyslipidemia (n=88), diabetes without dyslipidemia (n=86) and normal healthy controls (n=90).The study was designed to correlate biochemical and oxidative stress parameters in T2DM and indices for antioxidant defence were evaluated. Results indicated that , patients with diabetes and dyslipidemia had significantly lower antioxidant defence status reflected by reduced plasma free thiols, glutathione (GSH), vitamin C, vitamin E and PBMCs catalase activity. Significantly low vitamin C level in diabetic dyslipidemic patients infers that oxidative stress in diabetes might have contributed to the depletion of such antioxidant levels. Hyperglycemic and

hyperlipidemic condition induce apoptosis through mitochondrial membrane depolarization by generating free radicals. Increased oxidative stress in diabetic patients leads to protein oxidation. The pro-thrombotic state in diabetic dyslipidemia reflects increased activity of platelets coagulation cascade and impaired fibrinolysis which in turn hyperactivates platelet von willebrand factor (vWF) and P-Selectin (CD62P) progressing atherosclerosis. Impaired calcium homeostasis has also been witnessed in diabetic dyslipidemia.

Adiponectin, an adipose tissue derived hormone plays protective roles against the development of T2DM, dyslipidemia and CVD. Expression of adiponectin (ADIPOQ) and its receptors (ADIPOR1 and ADIPOR2) were measured in visceral and subcutaneous adipose tissues. Significant downregulation of adiponectin receptors (ADIPOR1 and ADIPOR2) in diabetic-dyslipidemic condition was observed in peripheral blood mononuclear cells (PBMCs), visceral (VAT) and subcutaneous (SAT) adipose tissues. A decreased plasma adiponectin level in diabetic dyslipidemic conditions indicates impaired adiponectin metabolism. In this study an increase in the expression of inflammatory cytokine TNFα and NF-κβ were found to be associated with diabetic conditions with and without dyslipidemia. The expression of TNFα in PBMCs was even greater when diabetes is coupled with dyslipidaemia. Downregulation of PPARγ, increased plasma lipid peroxidation products (4-hydroxynonenal adducts, malonyldehyde and lipid hydroperoxides) and C-reactive protein (CRP) reveal greater risk in diabetic-dyslipidemic conditions culminating into CVD. Genetic polymorphisms in ADIPOQ gene and the genes of its receptors have been the major reasons for functional defect of novel adipokine, thus prompting the progression of insulin resistance, dyslipidemia and atherogenesis. A multiple linear regression followed by MDR analysis implicated elevated plasma malondialdehyde and decreased adiponectin level to be correlated with diabetic dyslipidemia. A strong correlation between ADIPOQ rs2241766 T>G and ADIPOQ rs1501299 G>T polymorphisms in diabetic and dyslipidemic condition was observed. SNP in ADIPOR1 (rs1342387 G>A) showed a correlation with diabetes but not with dyslipidemia and no association was found for rs4766415 A>T. Other biochemical factors including plasma C-reactive protein and 4-hydroxynonenal adducts found to be increased in diabetic dyslipidemic conditions. In summary, our results reveal interesting complex interplay of genetic and biochemical parameters in diabetic dyslipidemia which is significant from the perspective of risk stratification and therapeutic strategy development.

CONTRIBUTION TO THE SOCIETY

Type 2 Diabetes mellitus (T2DM) is growing worldwide with epidemic dimensions across all age groups. Diabetes mellitus affects large numbers of people from a wide range of ethnic groups and at all social and economic levels throughout the world. International Diabetes Federation predicts that, if the current rate of growth continues unchecked, the global burden of diabetics will exceed 642 million in 2040. According to the World Health Organization estimates, India had 32 million diabetic subjects in the year 2000 and this number would increase to 80 million by the year 2030.Prevalence of Type 2 Diabetes mellitus (T2DM) associated with dyslipidemia offers a greater threat than diabetes alone. Type 2 diabetes mellitus, is often accompanied by dyslipidemia which eventually lead to highly atherogenic profile responsible for developing various cardiovascular (CVD) complications. Dyslipidemia characterized by the elevation of plasma total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL) and reduced high density lipoprotein cholesterol (HDL).

The astounding upsurge of diabetes and dyslipidemia globally is believed to be due to rapid urbanization, diet/lifestyle transitions and obesity. Hyperglycemia and hyperlipidemia together confer an enhanced risk of CVD, which may be the results of insulin resistance and impaired lipid metabolism. Adiponectin, an adipose tissue derived hormone plays protective roles against the development of T2DM, dyslipidemia and CVD. Significant downregulation of adiponectin receptors (ADIPOR1 and ADIPOR2) in diabetic-dyslipidemic condition has been observed in peripheral blood mononuclear cells (PBMCs), visceral (VAT) and subcutaneous (SAT) adipose tissues. A decreased plasma adiponectin level and downregulation of adiponectin in PBMC, VAT and SAT has been also implicated in diabetic dyslipidemic conditions indicating impaired adiponectin metabolism. Therefore, this study concludes that plasma adiponectin level is a signature biomarker to predict the occurrence of dyslipidemia in diabetes. The underlying genetic background also plays a pivotal role in its expression. It is also explored that the prevalence of rs2241766 and rs1501299 risk alleles may decrease the expression of plasma adiponectin, therefore prompting dyslipidemia in diabetes. The present study underscores the importance of assessing cumulative impact role of multiple factors in estimating disease risk. The current study successfully addresses the direction of the association of biochemical and genetic signatures in dyslipidemia coupled with diabetes and the findings may potentially be incorporated in the future clinical practice opening a new avenue for therapeutic target development.

PUBLICATION OUT OF THE PROJECT:

1. Autophagy protects peripheral blood mononuclear cells against inflammation, oxidative and nitrosative stress in diabetic dyslipidemia. **Free Radic Biol Med**. 2019 Jul 29;143:309-323. doi: 10.1016/j.freeradbiomed.2019.07.034 Tanima Chatterjee, Rudradip Pattanayak, Anindita Ukila, Subhankar Chowdhury,Maitree Bhattacharyyaa
2. Plasma adiponectin and malondialdehyde levels together with adiponectin variant rs2241766 predict the risk of dyslipidemia in type 2 diabetes Kakali Ghoshal, Tanima Chatterjee, Subhankar Chowdhury, Sanghamitra Sengupta, Maitree Bhattacharyya (Manuscript communicated)
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WHETHER OBJECTIVES WERE ACHIEVED:

The project reached the target and the progress has been made according to original plan of work.

WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT:

The Project Fellow Tanima Chatterjee has been registered for her Ph. D. under the supervision of Prof. Maitree Bhattacharyya, Department of Biochemistry, University of Calcutta.

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11/9/19
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