2. Materials and Methods

2.1. Study design

This study was designed to investigate the association of apolipoprotein E and apolipoprotein A1 gene polymorphisms with diabetic nephropathy in Bangladeshi healthy individuals, type 2 diabetic patients without and with nephropathy. The study was carried out in the Department of Biochemistry and Molecular Biology at University of Dhaka and The Laboratory and Pathology department in Square Hospitals Limited from 1st December 2016 to 11th October 2018. The outline of this study has been shown in Figure 2.1

2.2. Study subject selection and diagnosis of the patients

Study subjects all were adult unrelated Bangladeshi individuals. Type 2 diabetic patients were confirmed using the levels of HbA1c according to the criteria set by the World Health Organization (HbA1c level: 6.5% or over). For comparison, healthy individuals were selected, who were appeared subjects were diabetic without nephropathy and with nephropathy therefore patients to be healthy and had no clinical features of type diabetes, kidney disease along with other complications i.e., viral disease, hypertension or liver diseases etc. pregnant women and children were excluded from the study.

Albuminuria is a well-known predictor of poor renal outcomes in patients with type 2 diabetes. Urine albumin (24 hour) and albumin creatinine ratio were used to diagnose nephropathy in the study participants. A microalbumin urine test determines the presence of the albumin in urine. The presence of a small amount of albumin in the urine may be an early indicator of kidney disease. However, the level of microalbumin <19 mg/L was considered as the normal value. Microalbuminuria is defined as levels of albumin ranging from 30 to 300 mg in a 24-h urine collection (ADA, 2005). Overt albuminuria, macroalbuminuria, or proteinuria is defined as a urinary albumin excretion of ≥300 mg/24 h. On the other hand, a ratio of albumin to creatinine (ACR) of less than 30 mg/L was considered as normal.

Other biochemical parameters like 2 hours postprandial blood glucose, HbA1c, urea, creatinine, total cholesterol, triglycerides, HDL, LDL, hemoglobin, insulin, urinary microalbumin, ACR were also measured.

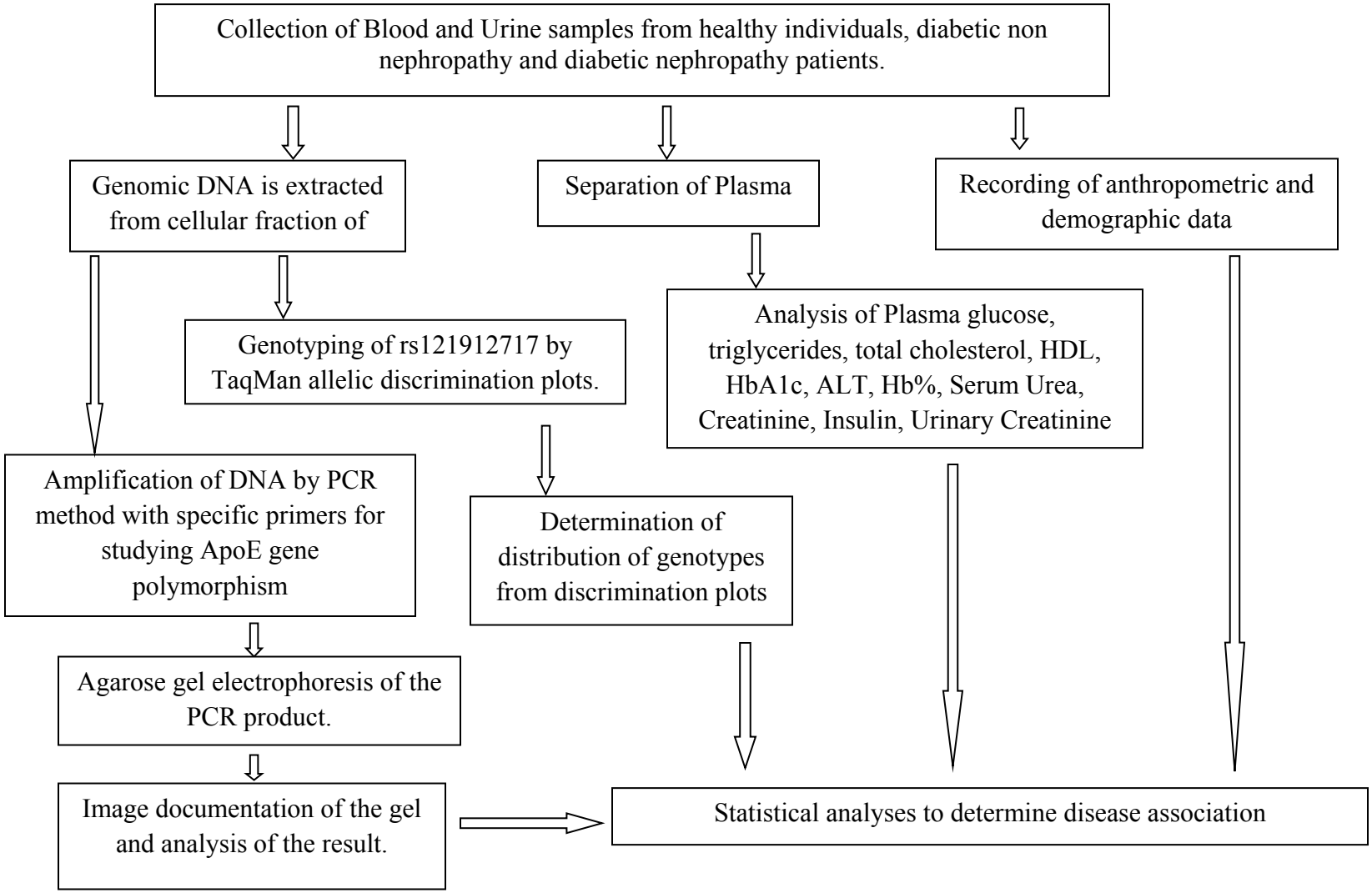

Figure 2.1: Schematic presentation of the overall activities to fulfill the research work.

2.2.1. Questionnaire analysis

A structured questionnaire to keep information of study subjects was prepared (Appendix) which are the associated clinical and physiological data such as age, sex, height, weight, BMI, Blood pressure etc of control subject, diabetic non nephropathy and diabetic nephropathy patients.

2.2.2. Sample collection

Blood and urine samples from diabetic without nephropathy, diabetic with nephropathy patients and control individuals were collected under protocols followed by participating institution. All the participants are Bangladeshi individuals and they were informed about the purpose of the study and gave their consent. With the assist of trained experts, 5 ml of venous blood were drawn with precautions using a disposable syringe. Blood was transferred to EDTA containing tube immediately and kept in an ice box for transportation. All participants were supplied a plain clean urine container to collect spot urine from them. When they had given the spot urine sample it was also kept in ice box to transport in the laboratory.

2.2.3. Separation of plasma from blood sample

Plasma was separated from each sample collected in EDTA tube. Collected blood samples were allowed to thaw and then centrifuged at 4000 rpm for 8 minutes. Plasma as supernatant was then separated in two 1.5 mL eppendorf tubes. After that, both plasma and plasma free blood cells were stored at -80°C until further use.

2.3. Genetic analysis

To determine polymorphisms in ApoE gene and ApoA1 gene, initially polymerase chain reaction (PCR) method was carried. Therefore genomic DNA was needed to be extracted. For this reason, blood cells that were previously separated from plasma, used for DNA extraction protocol.

2.3.1. Extraction of Genomic DNA from human blood sample

After collecting plasma, volume of each blood sample was adjusted with 0.9% NaCl to the respective volume of separated plasma. To extract genomic DNA from each sample following methods are used.

2.3.1.1. Reagents required for DNA extraction

2.3.1.1.1. EDTA (0.5 M), pH 8.0

Cells and nucleus are needed to be disrupted for DNA extraction. So cytoplasmic DNase can come into contact with DNA molecule and cut down DNA. Mg2+ is a cofactor for DNase. EDTA chelates divalent cations thus inactivates DNase. To prepare 0.5 M EDTA, 186.1 g of anhydrous EDTA was added to 800 mL of distilled water. The pH was adjusted to 8.0 with NaOH. The volume was then made up to 1 L with distilled water and autoclaved at 15 p.s.i. for 15 minutes.

2.3.1.1.2 Tris HCl (1M), pH 7.6

Cellular content and fragmented RNAs and proteins can change pH after lysing the cell. DNA is pH sensitive therefore, Tris HCl is used to maintain pH for DNA integrity. For preparing 1M tris HCl, 121.1 g of Tris base was dissolved in 800mL of distilled water then pH was adjusted to 7.6 with HCl. The solution was needed to be cool down to room temperature for final pH adjustment. The volume was made up to 1 L with distilled water. Then the solution was autoclaved at 15 p.s.i. for 15 minutes.

2.3.1.1.3. TE buffer

TE buffer or tris-EDTA buffer contains EDTA as a divalent cation chelating agent and tris HCl for maintaining pH. TE buffer dissolve nucleic acid protects it from degradation.

Here, TE buffer was prepared by mixing of 5mL of 1 M tris HCl (pH 7.6) with 2 mL of 0.5 M EDTA in distilled water. The volume was made up to 1L with distilled water. pH was adjusted to 8.0 and autoclaved for 15 minutes at 15 p.s.i.

2.3.1.1.4 Red blood cell (RBC) lysis buffer (pH 8.0)

Red blood cell lysis buffer is used to disrupt RBC leaving leukocytes intact. Red blood cell lysis buffer was prepared by mixing 10 mL of 1M Tris-HCl (pH 7.6), 109.54 g of pure sucrose, 1.01 g MgCl2 and 800 mL of distilled water. pH was adjusted to 8.0 with HCl. Then 10 mL of triton X-100 was added to the mixture carefully. Finally the volume was made upto 1 L with distilled water. Then the mixture was autoclaved for 10 minutes at 15 p.s.i.

2.3.1.1.5. Nucleic acid lysis buffer (pH 8.0)

Nucleic acid lysis buffer was prepared by mixing of 10 ml of 1 M tris –HCl (pH 7.6), 3.75 g of EDTA (pH 8.0), 10 g of SDS and 2.94 g of sodium citrate with 800 mL of distilled water. pH was adjusted to 8.0. The final volume was mad up to 1 L with distilled water. The solution was then autoclaved at 15 p.s.i. for 15 minutes.

2.3.1.1.6. Working solution of ethidium bromide

Ethidium bromide (0.5 µL) was mixed into 100 mL of TAE buffer to prepare working ethidium bromide solution. It is used to completely submerge the gel and kept in dark. It is useable for 1-2 months.

2.3.2. Procedure for DNA extraction

i. Frozen blood samples were brought to room temperature to make homogenous by brief vortexing .500 µL of blood from each sample tube was taken into a 1.5 mL eppendorf tube and 950 µL of RBC lysis buffer was added.

ii. The eppendorf tube was shaken gently to homogenize, then centrifuged for 2 minutes at 7000 rpm.

iii. Supernatant was carefully discarded.

iv. Steps i-iii was repeated for 2/3 times to increase pallet.

v. 1000 µL of RBC lysis buffer was added into the eppendorf tube. Pallet was disrupted by vortexing and eppendorf tube was centrifuged at 7000 rpm for 2 minutes.

vi. Supernatant was discarded carefully.

Steps v-vi was repeated 2-4 times to remove hemoglobin.

viii. When the pallet becomes white in color indicating clearance of hemoglobin, supernatant was discarded carefully and 400 µL of Nucleic acid lysis buffer was added. The pellet was pipette to be dissolved.

ix. Saturated 100 µL NaCl (5N) and of 600 µL of chloroform were added to eppendorf tube and mixed by gentle shaking and vortexing and then centrifuged for 2 minutes at 7000 rpm

x. Three phases were visible. Aqueous top phase contains DNA, interphase contains proteins, organic lower phase contains lipids and most of the RNAs. Nearly 400 µL supernatant from aqueous phase was transferred into another fresh eppendorf. 800 µL of cold (-20°C) absolute ethanol was added to the supernatant. The eppendorf tube was then shaken gently, white string of DNA was appeared (Figure 2.2).

xi. It was then centrifuged at 12000 rpm for 1 minute to precipitate DNA.

xii. The supernatant was discarded carefully. To make it dry eppendorf tube was placed downward on tissue paper overnight at room temperature.

xiii. To dissolve DNA 50 µL of TE buffer (pH 8.0) was added in the eppendorf . Dissolved DNA in eppendorf tube was kept in -20°C for later use.

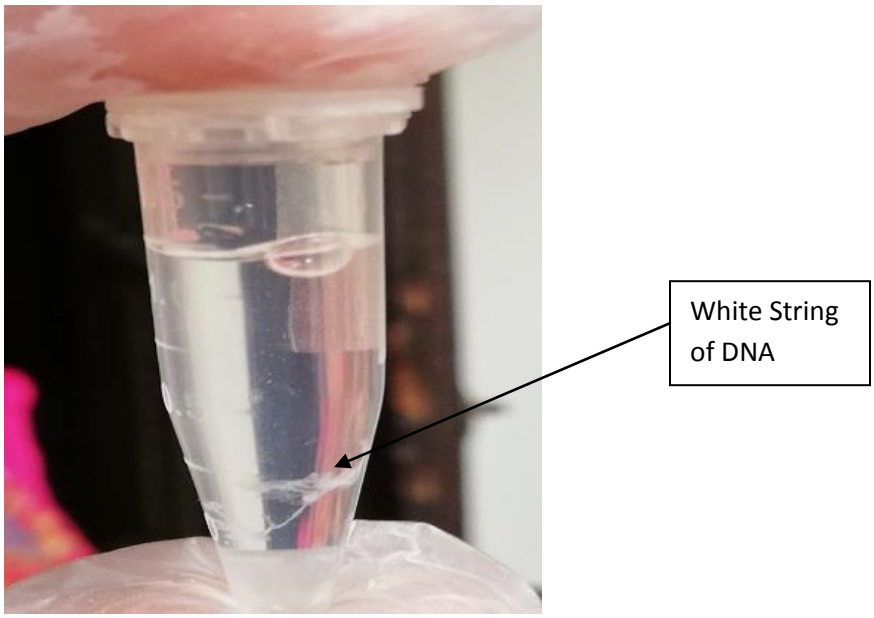

Figure 2.2: White string of DNA appeared after adding cold ethanol.

2.3.3. Purity check and quantification of extracted DNA:

It is very important to ensure the quality of extracted genomic DNA for Polymerase Chain Reaction (PCR). Chloroform, ethanol, EDTA, detergents, hemoglobin, heparin etc. can inhibit PCR (Holodniy *et al.*, 1991). Again, too much concentrated template can hampered PCR by

binding with primer nonspecifically as wellm as if template concentration is too low, amplification can be undetectable or will required many more cycles.

NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA) is a full spectrum (220nm-750nm) spectrophotometer. It was used to measure DNA concentration and purity. It uses patented sample withholding technology (patents US6628382 and US6809826) to hold the sample onto the pedestal. Before measuring concentration, both upper and lower measurement pedestal were washed multiple times with PCR grade water. 2 µL TE buffer was used to standardize before taking measurement as TE buffer was used to dissolve genomic DNA. After loading the sample and closing the arm, machine can analyze by taking command and showing result through NanoDrop software. It shows measurement results of absorbance at 260 nm, 280 nm, 260/280, 260/230, and concentration in ng/µL.

2.3.4. Evaluating the quality of extracted genomic DNA

Agarose gel electrophoresis was done to know the quality of extracted DNA, This method is based on DNA staining with ethidium bromide. Quality of extracted DNA, whether it is intact or fragmented, presence of RNA (smear) – can be visually identified on gel.

For this work, 0.8% agarose gel was used. To prepare the gel, 8g of agarose powder (Art. 3810.2, Agarose Standard, ROTI®GAROSE, CARL ROTH, Karlsruhe, Germany) was mixed with 100 ml of tris-acetate-EDTA (TAE) buffer, an electrophoresis buffer, heated inside a microwave oven for 2 minutes just till the solution became clear. Melted gel was then poured into a gel tray. A “comb” was placed on the tray at one end before the gel gets solidify, to make “wells” to load the samples. When the gel became cool and solidified, the comb was carefully removed. The gel was then placed on electrophoresis tank that was already poured with the same electrophoresis buffer, TAE, until the top surface of the gel was covered. In the tank, electricity was conducted by the buffer. 4 µL of DNA from each sample was mixed with 3µL of loading dye to make the sample to be loaded heavy and viscous so that it could not flooded out from the well. Also the brown colored loading dye helps to visually track the migration of sample during electrophoresis. Then the prepared DNA samples along with ladder were micropipetted to load on the gel. Positive and negative electrodes were checked. Electrophoresis was carried on for 1 hour at 90 volt. After that, the gel was fully submerged in ethidium bromide solution for 15 minutes. DNA

was visualized in AlphaImager® Mini (Protein simple, California, USA). Then under UV light, single pale orange colored thick band was observed for each sample under each well which migrated a small distance indicating that, extracted DNA was intact.

2.3.5. Polymerase chain reaction for ApoE genotyping

Amplification refractory mutation system PCR (ARMS-PCR) was employed for ApoE genotyping. Mismatches are included to maximize discrimination of the wild-type and mutant alleles. The ARMS-PCR is an amplification strategy in which a polymerase chain reaction primer is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue (Newton et al, 1989; Bottema et al, 1993). ARMS has also been termed allele-specific PCR (Wu et al, 1989).

Concentration of extracted DNA (which will be used as template DNA) was adjusted to 100 ng/µL. APOE genotyping was done by ARMS-PCR. APOE genotyping by ARMS-PCR was performed with specific Cys primers (Cys112 and Cys158) as well as Arg primers (Arg112 and Arg158). (Table 2.1).

Table 2.1. Primers for amplification refractory mutation system polymerase chain reaction.

Primer name	Primer Sequence	Product length
Arg112 (forward)	5'-CGCGGACATGGAGGACGTTC-3'	588
Arg158 (forward)	5'-ATGCCGATGACCTGCAGACGC-3'	451
Common primer (reverse)	5'-GTTCAGTGATTGTCGCTGGGCA-3'	--
Cys112 (forward)	5'- CGCGGACATGGAGGACGTTT-3'	588
Cys158 (forward)	5'-ATGCCGATGACCTGCAGACGT-3'	451

PCR was performed in a 20-µl reaction volume which consist the following components presented in Table 2.2

Table 2.2. Reagents required for genotyping of ApoE.

Name of the component	Volume	Concentration
Template DNA	2 µL	100 ng
GoTaq® Green Master Mix	10 µL	
Forward primer	0.8 µL	10 Molar
Reverse primer (Common)	0.8 µL	10 Molar
DMSO	1.6 µL	10%
Nuclease free H2O	4.8 µL	

PCR amplification was done by following the below condition;

- Initial denaturation and enzyme activation at 95°C for 5 minutes
- Then 35 cycles of denaturation at 95°C for 30 seconds, 63˚C for 30 sec and 72˚C for 30 seconds
- Final extension at 72˚C for 15 min
- After 35 cycles of amplification, amplicons were stored at 4°C.

2.3.6. Agarose gel electrophoresis

Amplified DNA samples were resolved by 2% agarose gel electrophoresis with a 100bp ladder as a marker. To prepare 2% agarose gel , 1.8 g agarose powder was mixed with 90 ml of tris-acetaed-EDTA(TAE) buffer, then heated inside the microwave oven for 2 minutes till the solution became clear.

Melted gel was then poured into two gel tray which contain 17 wells in each tray.. A “comb” was placed on the tray at one end before the gel gets solidify, to make “wells” to load the samples. When the gel became cool and solidified, the comb was carefully removed. The gel was then placed on electrophoresis tank that was already poured with TAE (1X), until the whole gel was submerged into buffer. 3 µL of amplified PCR product from each samples were loaded on each well. 1.5 µL of 100 bp ladder was loaded. Positive and negative electrodes were checked. Electrophoresis was carried on for 40 minutes at 90 volt. After that, the gel was fully submerged

in fresh ethidium bromide solution for 15 minutes. DNA was visualized in AlphaImager® Mini (Proteinsimple, California, USA). Then under UV light, sharp bands were observed as shown in Figure 2.3.

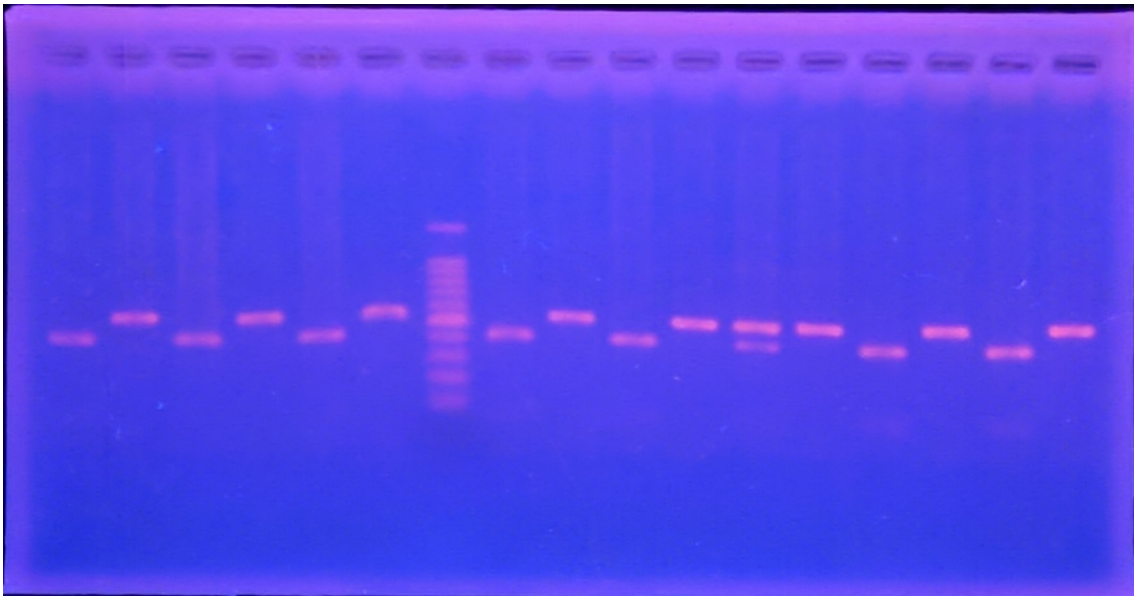

Figure 2.3: Image of agarose gel under uv light after electrophoresis of DNA samples.

2.3.7. Genotyping using Real Time Polymerase Chain Reaction

Probe-based real time PCR is relatively target specific. By adding primers, target specific probes, short oligonucleotides complementary to target DNA sequence, are designed commonly by incorporating a fluorophore at 5′ end and a quencher at 3′ end. During PCR, taq DNA polymerase hydrolizes the probe as primer. As the enzyme also has 5′exonuclease activity, during this event 5′ reporter fluorophore gets separated from quencher and fluorescence becomes detectable. Following reagents were used to perform the reaction:

2.3.7.1. TaqMan® SNP Genotyping Assay and TaqMan® Genotyping Master Mix

For amplification and detection of specific polymorphism, Taqman® SNP Genotyping assay follows Taqman® 5′ nuclease chemistry. TaqMan® SNP Genotyping Assay mix contains a pair of unlabeled forward and reverse primers for amplification of polymorphic sequence of interest and two TaqMan minor groove binder (MGB) probes with non-fluorescent quenchers . One

probe is labeled with VIC dye to detect allele 1, and the other is labeled with FAM dye to detect allele 2. Table 2.3 shows correlation between fluorescence signal and genotyping of samples.

Table 2.3. Correlation between fluorescence signal and sequences in the sample

Fluorescence signal	**Indication**
Only VIC dye fluorescence increase	Allele 1 homozygous
Only FAM dye fluorescence increase	Allele 2 homozygous
Both VIC and FAM dye signal increase	Allele 1/Allele 2 heterozygous

TaqMan® Genotyping Master Mix

TaqMan genotyping master mix contains AmpliTaq Gold® DNA polymerase, Ultra Pure (UP), deoxyribonucleotide triphosphates (dNTPs), ROX™ Passive Reference, optimized buffer components. AmpliTaq Gold® DNA polymerase is an ultrapure hot start enzyme, so in room temperature primer cannot be extended and there will be no enzymatic amplification even if any mispriming event occurs.

2.3.7.2. Protocol for performing genotyping study using TaqMan probe

For SNP rs121912717 genotyping real-time PCR was carried out using TaqMan® allelic discrimination assay in Applied biosystems 7500 fast real-time PCR instrument (Applied Biosystems, CA, USA). 10 µL volume of reaction mixture was prepared using TaqMan genotyping master mix, TaqMan genotyping assay mix, Nuclease free H_2O, and template DNA for each sample. As the reagents are light sensitive, the whole procedure was done avoiding exposure to light. The composition of reaction mixture for real-time PCR is given in Table 2.4.

Table 2.4. Composition of reaction mixture for real-time PCR.

Name of the component	Volume (μL)	Concentration
TaqMan genotyping master mix	2.5	2X
TaqMan genotyping assay mix	0.125	40X
PCR-H_2O	2.675	
Template DNA	4.7	10 ng/μL
Total	10	

The condition for amplification includes following steps:

I. Initial denaturation and enzyme activation at 95°C for 10 minutes,

II. Then 40 cycles of denaturation at 95°C for 15 seconds,

III. Annealing and elongation at 60°C for 60 seconds.

IV. After 40 cycles of amplification, amplicons were stored at 4°C covered in aluminium foil whenever required.

2.3.7.3. Analysis of genotyping

Genotype calls were made from allelic discrimination plot using Applied Biosystems 7500 software v2.3. The software requires windows XP or windows 7 operating system, 1 GB or more RAM, 20 GB or more of hard drive capacity. Comparing with reference control for each allele, the clusters were identified. 10% samples were analyzed randomly in duplicates to ensure quality of genotyping and the concordance was absolute.

2.4. Assay of biochemical parameters

2.4.1. Determination of the levels of glucose in plasma

Participants were requested to fast overnight to determine fasting plasma glucose level. Next morning blood was collected from where plasma was isolated. Glucose test was performed by automated analyzer V5600 which followed the dry chemistry method. The VITROS® 5600 is an

integrated system of Orthoclinical Diagnostics (OCD), USA which is the clinical diagnostic part of Johnson and Johnson Company, Norway.

2.4.1.1 Required reagents and materials

- GLU reagent Cartridge (Ready to use) supplied by Orthoclinical Diagnostics, USA

- The VITROS Chemistry Products Calibrator Kit 1 (Ready to use) supplied by Orthoclinical Diagnostics, USA.

Principle of assay procedure: The VITROS GLU Slide is a multilayered, analytical element coated on a polyester support. A drop of patient sample is deposited on the slide. The oxidation of sample glucose is catalyzed by glucose oxidase to form hydrogen peroxide and gluconate. This reaction is followed by an oxidative coupling catalyzed by peroxidase in the presence of dye precursors to produce a dye. The intensity of the dye is measured at 540 nm by reflected light.

Reaction scheme of Glucose

β-D-glucose + O_2 + H_2O $\xrightarrow{\text{glucose oxidase}}$ D-gluconic acid + H_2O

2H $_2O_2$ + 4-aminoantipyrine + 1,7-dihydroxynaphthalene $\xrightarrow{\text{peroxidase}}$ red dye

Calculation of result: This is a colorimetric type test. It's incubation time is 5 minutes. After the incubation it forms a colored dye which is measured and calculated by the software of Vitros system.

2.4.2. Determination of the level of Urea in the plasma of study participants:

The Urea level of plasma is determined by the automated Vitros 5600 Analyze. The origin of VITROS® 5600 integrated system is Orthoclinical Diagnostics (OCD), USA which is the clinical diagnostic part of Johnson and Johnson Company, Norway.

Required reagents and materials:

- BUN/UREA reagent Cartridge (Ready to use) supplied by Orthoclinical Diagnostics, USA

- The VITROS Chemistry Products Calibrator Kit 1 (Ready to use) supplied by Orthoclinical Diagnostics, USA.

Principle of assay procedure:

This is a colorimetric test. The VITROS UREA Slide is a multilayered, analytical element coated on a polyester support. A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. Water and non-proteinaceous components then travel to the underlying reagent layer, where the urease reaction generates ammonia. The semipermeable membrane allows only ammonia to pass through to the color-forming layer, where it reacts with the indicator to form a dye.

Reaction scheme of Urea:

$H_2NCONH_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$

NH_2 + ammonia indicator $\longrightarrow$ Dye

The reflection density of the dye is measured and is proportional to the concentration of urea in the sample.

Calculation of result: The incubation time for urea is 5 minutes. After the incubation it forms a colored dye which is measured by the reflectophotometre at 670 nm wavelength and calculated by the software of Vitros system .

2.4.3. Determination of the activity alanine aminotransferase (ALT) in the plasma of study participants

The ALT level of plasma is determined by the automated Vitros 5600 Analyze(VITROS® 5600 integrated system ; Orthoclinical Diagnostics,USA).

Required reagents and materials:

- ALT reagent Cartridge (Ready to use) supplied by Orthoclinical Diagnostics, USA

- The VITROS Chemistry Products Calibrator Kit 3 (Ready to use) supplied by Ortho Clinical Diagnostics, USA.

Principle of assay procedure:

The alanine aminotransferase test is a multi-point test. The slide is an analytical element coated on polyester support which multilayered. On the slide a drop of sample is deposited and is evenly distributed by the spreading layer to the underlying layers. Several sequential kinetic reactions between slide ingredients and sample eventually produce a colored dye that is monitored by reflectance spectrophotometry. The rate of change in reflectance density is proportional to ALT activity in the sample.

Reaction scheme of ALT:

Alanine + α-ketoglutarate $\xrightarrow{\text{ALT + pyridoxal-5-phosphate}}$ pyruvate + glutamate

Pyruvate + NADH + H+ $\xrightarrow{\text{LDH}}$ lactate + NAD+

Calculation of result: The approximate incubation time for ALT is 5 minutes. The rate of oxidation of NADH is monitored by reflectance spectrophotometry. The rate of change in reflection density is proportional to enzyme activity. The intensity is measured at 340 nm and value is calculated by the vitros software system.

2.4.4. Determination of the levels of creatinine in plasma

The creatinine level of plasma is determined by the automated Vitros 5600 Analyze(VITROS® 5600 integrated system ; Orthoclinical Diagnostics,USA).

Required reagents and materials:

- CREA reagent Cartridge (Ready to use) supplied by Orthoclinical Diagnostics, USA

- The VITROS Chemistry Products Calibrator Kit 1 (Ready to use) supplied by Orthoclinical Diagnostics, USA.

Principle of assay procedure:

The VITROS CREA Slide is a multilayered, analytical element coated on a polyester support which is used for plasma creatinine level. A drop of patient sample is deposited on the slide and is uniformly pass through the reagent layer, where it is hydrolyzed to creatine in the rate-determining step. The creatine is converted to sarcosine and urea by creatine amidinohydrolase. The sarcosine, in the presence of sarcosine oxidase, is oxidized to glycine, formaldehyde, and hydrogen peroxide. The final reaction involves the peroxidase-catalyzed oxidation of a leuco dye

to produce a colored product. Following addition of the sample, the slide is incubated. During the initial reaction phase, endogenous creatine in the sample is oxidized.

Reaction scheme of creatinine:

Creatinine + H_2O $\xrightarrow{\text{creatinine + amidohydrolase}}$ Creatine

Creatine + H_2O $\xrightarrow{\text{creatine + amidinohydrolase}}$ Sarcosine + urea

Sarcosine + O_2 + H_2O $\xrightarrow{\text{sarcosine + oxidase}}$ Glycine + Formaldehyde + H_2O_2

H_2O_2 + leuco dye $\xrightarrow{\text{peroxidase}}$ dye + $2H_2O$

Calculation of Result: This is a two point enzymatic test. The resulting change in reflection density is measured at 2 time points. The difference in reflection density is proportional to the concentration of creatinine present in the sample and the value is calculated by the software of Vitros system.

2.4.5. Determination of the levels of cholesterol in plasma

Cholesterol test is done by the VITROS CHOL Slide method on VITROS 5600 Integrated System (VITROS® 5600 integrated system; Orthoclinical Diagnostics,USA).

Required reagents and materials:

- CHOL reagent Cartridge (Ready to use) supplied by Ortho Clinical Diagnostics, USA

- The VITROS Chemistry Products Calibrator Kit 2 (Ready to use) supplied by Ortho Clinical Diagnostics, USA.

Principle of assay procedure:

The VITROS CHOL Slide is a multilayered, analytical element coated on a polyester support. The method is based on an enzymatic method. A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The Triton X-100

(TX100) surfactant in the spreading layer aids in dissociating the cholesterol and cholesterol esters from lipoprotein complexes present in the sample. Hydrolysis of the cholesterol esters to cholesterol is catalyzed by cholesterol ester hydrolase. Free cholesterol is then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Finally, hydrogen peroxide oxidizes a leuco dye in the presence of peroxidase to generate a colored dye.

Reaction scheme of Cholesterol:

Lipoprotein $\xrightarrow{\text{TX100}}$ cholesterol + cholesterol esters + proteins

Cholesterol esters + H_2O $\xrightarrow{\text{cholesterol ester hydrolase}}$ cholesterol + fatty acids

Cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ cholest-4-en-3-one + H_2O_2

H_2O_2 + leuco dye $\xrightarrow{\text{peroxidase}}$ dye + $2H_2O$

Calculation of result: The density of dye formed is proportional to the cholesterol concentration present in the sample and is measured by reflectance spectrophotometry and value is calculated by the vitros software.

2.4.6. Determination of the levels of triglyceride in plasma

Plasma triglyceride test was performed by automated analyzer Vitros 5600 (VITROS® 5600 integrated system; Ortho Clinical Diagnostics, USA) which followed the dry chemistry method.

Required reagents and materials:

- TRIG reagent Cartridge (Ready to use) supplied by Ortho Clinical Diagnostics, USA

- The VITROS Chemistry Products Calibrator Kit 2 (Ready to use) supplied by Ortho Clinical Diagnostics, USA.

Principle of assay procedure:

A drop of patient sample is deposited on the slide and is evenly distributed. The Triton X-100 dissociate the triglycerides from lipoprotein complexes present in the sample. The triglyceride molecules are then hydrolyzed by lipase to yield glycerol and fatty acids. Glycerol diffuses to the reagent layer, where it is phosphorylated by glycerol kinase in the presence of adenosine triphosphate (ATP). In the presence of L-α-glycerol-phosphate oxidase, L-α-glycerophosphate is oxidized to dihydroxyacetone phosphate and hydrogen peroxide. The final reaction involves the oxidation of a leuco dye by hydrogen peroxide, catalyzed by peroxidase, to produce a dye. The density of the dye formed is proportional to the triglyceride concentration present in the sample and is measured by reflectance spectrophotometry.

Reaction scheme of triglycerides:

lipoproteins $\xrightarrow{\text{surfactant}}$ triglycerides + proteins

triglycerides + H_2O $\xrightarrow{\text{lipase}}$ glycerol + fatty acids

glycerol + ATP $\xrightarrow{\text{glycerol kinase}}$ $MgCl_2$ L-α-glycerophosphate + ADP

L-α-glycerophosphate + O2 L-α-glycerol-phosphate $\xrightarrow{\text{oxidase}}$ dihydroxyacetone phosphate + H_2O_2

H_2O_2 + leuco dye $\xrightarrow{\text{peroxidase}}$ dye + $2H_2O$

Calculation of result: The density of the dye formed is proportional to the triglyceride concentration present in the sample and is measured by reflectance spectrophotometry at 540nm. Result is calculated by the machine's software.

2.4.7. Determination of the levels of HDL in plasma

The plasma dHDL test was done by Vitors 5600 (VITROS® 5600 integrated system; Orthoclinical Diagnostics, USA) automated analyzer.

Required reagents and materials:

- dHDL reagent Cartridge (Ready to use) supplied by Ortho Clinical Diagnostics, USA

- The VITROS Chemistry Products Calibrator Kit 25 (Ready to use) supplied by Ortho Clinical Diagnostics, USA.

Principle of the procedure: A drop of patient sample is deposited on the slide and is equally distributed to the underlying layers. HDL is separated by the precipitation of non-High Density

Lipoproteins (non-HDL) using phosphotungstic acid (PTA) and magnesium chloride ($MgCl_2$) in the spreading layer. The Emulgen B-66 surfactant in the spreading layer aids in the selective dissociation of the cholesterol and cholesterol esters from the HDL lipoprotein complexes present in the sample. Hydrolysis of the HDL-derived cholesterol ester to cholesterol is catalyzed by a selective cholesterol ester hydrolase. Free cholesterol is then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Finally, hydrogen peroxide oxidizes a leuco dye in the presence of peroxidase to generate a colored dye. The concentration of HDL is measured by reflectance spectrophotometry.

Reaction scheme of HDL:

HDL + non-HDL $\xrightarrow{PTA/MgCl_2}$ high density lipoproteins + non-HDL(↓)

high density lipoproteins $\xrightarrow{Emulgen\ B\text{-}66}$ cholesterol + HDL cholesterol esters + proteins

HDL cholesterol esters + H_2O $\xrightarrow{cholesterol\ ester\ hydrolase}$ cholesterol and fatty acids

Cholesterol + O_2 $\xrightarrow{cholesterol\ oxidase}$ cholest-4-en-3 + H_2O_2

H_2O_2 + leuco dye $\xrightarrow{peroxidase}$ dye + $2H_2O$

Calculation of result: At the final step of reaction hydrogen peroxide oxidizes a leuco dye and form a colored dye in the presence of peroxidase. It's incubation time is 5 minutes. The concentration of HDL is measured by reflectance spectrophotometry at 670 nm and result is carried out by using the vitros software system.

2.4.8. Determination of the levels of creatinine in urine

Urinary creatinine is determined from spot urine sample by using the automated Vitros 5600 and Vitros creatinine slide which is used in case of plasma creatinine determination therefore the test method is same as for plasma creatinine.

2.4.9. Determination of the levels of microalbumin in urine

Urine microalbumin test was done by The VITROS 5600 (VITROS® 5600 integrated system; Orthoclinical Diagnostics,USA) Chemistry Products mALB Reagent which consist a dual chambered package containing ready-to-use liquid reagents. When sample is added Reagent 1

containing a polymer and surfactant and antisera specific for human albumin (Reagent 2) produces antibody/antigen complexes by an immunochemical reaction. The light scattering properties of the antibody/antigen complexes increase solution turbidity proportional to albumin concentration in the sample. The turbidity is measured spectrophotometrically at 340 nm.

Reaction scheme of microalbumin:

Sample $\xrightarrow{\text{R1+Surfactant/Polymer}}$ Diluted Sample

Step 2: Formation of mALB/Antibody Complexes

Diluted Sample $\xrightarrow{\text{R2 + Polyclonal antibodies}}$ Antigen/Antibody Complexes

2.4.10. Determination of urine albumin creatinine ratio

Albumin-to-creatinine ratio (ACR) is the first method to determine the presence of albumin in the urine. Small amount of albumin excretion in the urine is sometimes referred to as urine microalbumin or microalbuminuria. "Microalbuminuria" is slowly being replaced with the term "albuminuria. To convert microalbumin and urine creatinine to its ratio (Albumin creatinine ratio, ACR) first make sure unit of both values. If the concentration of urine creatinine is in mg/Land microalbumin is in µg/L;

- Divide the microalbumin concentration by the creatinine concentration
- Multiply the resulting ratio by 1000 to get mg albumin over grams creatinine (mg/g).

The calculation is;

$$Albumin\ Creatinine\ Ratio\ (ACR) = \frac{Urinary\ Microalbumin\ (\mu g/L)}{Urinary\ Creatinine\ (mg/L)}\ \text{(mg/g)}$$

2.4.11. Determination of the levels of hemoglobin

The amount of hemoglobin from EDTA whole blood was estimated by automated Sysmex XT-4000i (Sysmex corporation Kobe, Japan Code No. AN 741742) hematology analyzer.

Required reagents:

- Cellpack
- Stromatolyzer-FB
- Stromatolyzer- 4DL
- Stromatolyzer-4DS
- Sulfolyzer
- Ret search (II) (diluents)
- Ret search (II) (dye solution)
- Cellclean

All reagents are ready to use and supplied by The Sysmex corporation Kobe, Japan Code No. AN 741742.

Principle of the procedure:

This analyzer performs the counting using the principle of the Hydro Dynamic Focusing (DC Detection), flow cytometry (using a semiconductor laser), and SLS hemoglobin method. Cytometry is used to analyze physiological and chemical characteristics of cells, thus used to analyze cells and particles. Aspirated blood sample is diluted to specified ratio, and stained and fed into the flow cell. A semiconductor laser beam is emitted to the flowing blood cells the emitting light scattered cells into forward and later direction (forward scattering and lateral scattering).This light is converted into electrical pulses for counting cells.

2.4.12. Determination of the levels of glycated hemoglobin

The glycated hemoglobin A1 c (HbA1c) is determined from EDTA whole blood by the auto machine 270-2455/270-2455X, VARIANT ™ II TURBO HbA1c Kit-2.0, (Bio-Rad Laboratories, United states) which uses the principle of HPLC method.

Required reagents:

- Elution Buffer A
- Elution Buffer B
- Wash Diluent

All reagents are ready to use and supplied by Bio-Rad Laboratories, United States.

Principle of the procedure:

There is a dual position pump in the VCS which deliver the required buffer to the analytical cartridge and detector. The sample is carried by the buffer through the analytical cartridge, where the sample is separated into its individual components. The separated components then pass through the dual-wavelength detector, where absorbance of the sample components is measured at 425 nm and background noise is minimized by the secondary wavelength at 690 nm. The reading is showed by using a real-time chromatograph (graph of absorbance vs time)

2.4.13. Determination of Plasma Insulin:

The quantitative determination of Insulin was carried out by the automated ARCHITECT *i* (Produced by DENKA SEIKEN CO., LTD. Tokyo, Japan for ABBOTT Diagnostics Division) machine which used the chemiluminescent microparticle immunoassay (CMIA) technology. This is also is a one-step immunoassay.

Reagents required for insulin:

- Reagent Kit 8K41. Antibody to human insulin (mouse, monoclonal) coated microparticles.
- Acridinium-labeled antibody to human insulin (mouse, monoclonal) conjugate
- ARCHITECT *i* Pre-Trigger Solution containing 1.32% (w/v) hydrogen peroxide.
- ARCHITECT *i* Trigger Solution which containing 0.35N sodium
- ARCHITECT *i* Wash Buffer containing phosphate buffered saline solution
- Preservative: antimicrobial agent.

All required reagents are ready to use and supplied by ABBOTT Diagnostics Division, Tokyo, Japan.

Assay procedure:

Before loading the ARCHITECT Insulin Reagent Kit on the system for the first time, the microparticle bottle requires mixing to resuspend microparticles. The ARCHITECT Insulin Reagent Kit was loaded on the ARCHITECT *i* System and sample was loaded on the machine at

sample load position and pressed RUN button. Test was completed by following the CIMA methodology.

2.4.14. Statistical analyses

Demographic data obtained from the structured questionnaire and clinical parameters were analyzed using Statistical Package for the Social Sciences (SPSS, v21.0) where the results were expressed as mean ± SD for continuous variables and % (no. of part) for categorical variables. To compare the differences between different variables from the control and patients, independent student's t-test was performed. Pearson's correlation test was performed to examine various correlations.

The chi-square test was used to compare the categorical variables. In case of genotypic frequencies, the odds ratio and 95% CI (confidence interval) were calculated using logistic regression to verify the association polymorphism with or without nephropathy under four inheritance or genetic models: codominant, dominant, recessive and over dominant. A p value </=0.05 was considered as statistically significant.

3. Results

3.1. Demographic and anthropometric characteristics of the study participants

Out of the total 349 study participants, 179 (51.29%) were male individuals and 170 (48.71%) were female individuals. Average age of healthy individuals, type 2 diabetic patients and diabetic patients with diabetic nephropathy were 40.48 ± 1.14, 53.28 ± 10.26 and 55.74 ± 12.03 years, respectively. Values of their respective BMI were 24.69 ± 2.76, 26.40 ± 3.49 and 26.67 ± 3.10. Mean values of their systolic blood pressure were 122.63 ± 7.68, 125.96 ± 7.06, 141.01 ± 8.54, respectively while these values for diastolic blood pressure were 82.36 ± 7.03, 84.47 ± 7.00 and 97.68 ± 7.82. Average duration of diabetes among the randomly selected patients was 8.59 ± 3.15 years (range: 5-15 years). To control blood sugar level, patients were under treatment using drugs like metformin, sulfonylureas etc. while few of the patients diagnosed with very high levels of blood sugar were advised to take insulin therapy for controlling glucose level. Data of demographic and anthropometric characteristics of the study participants have been presented in Table 3.1.

Table 3.1. Demographic and anthropometric characteristics of the study participants.

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Age (years)	40.48 ± 1.14	53.28 ± 10.26	55.74 ± 12.03	0.000	0.000	0.084
Height (cm)	162.50 ± 5.97	172.04 ± 89.63	163.47 ± 7.10	0.362	0.326	0.293
Weight (Kg)	65.59 ± 6.69	71.37 ± 6.46	71.01 ± 7.02	0.000	0.000	0.673
BMI	24.69 ± 2.76	26.40 ± 3.49	26.67 ± 3.10	0.001	0.000	0.523
SBP (mmHg)	122.63 ± 7.68	125.96 ± 7.06	141.01 ± 8.54	0.000	0.000	0.000
DBP (mmHg)	82.36 ± 7.03	84.47 ± 7.00	97.68 ± 7.82	0.000	0.000	0.000

CN: Healthy control individuals; T2D: Type 2 diabetic patients; T2D+DN: type 2 diabetic patients with nephropathy

3.2. Biochemical characteristics of the study participants

The mean fasting plasma glucose level of the healthy individuals, type 2 diabetic patients and diabetic patients with diabetic nephropathy were 5.07 ± 0.34, 9.49 ± 3.19 and 10.53 ± 3.62 mmol/L while after two hours of breakfast, these respective values were 6.15 ± 0.63, 13.97 ± 4.34 and 14.99 ± 4.40 mmol/L. The mean levels of fasting glucose of healthy individuals varied significantly with those of type 2 diabetic patients and diabetic patients with nephropathy ($p < 0.0001$). Also, the mean levels of HbA1c of healthy individuals (5.54 ± 0.32%) varied significantly with those of type 2 diabetic patients (8.73 ± 1.65%) and diabetic patients with nephropathy (9.36 ± 1.80%) ($p < 0.0001$). Insulin level was found to be the highest (39.68 ± 16.44 mlU/mL) among type 2 diabetic patients compared to that of healthy individuals (10.39 ± 3.53 mlU/mL) and diabetic patients with nephropathy (25.37 ± 21.63 mlU/mL). Mean level of insulin in healthy individuals was statistically significantly lower when compared with that of the levels of patients with type 2 diabetes and nephropathy. Mean levels of insulin in diabetic nephropathy was also significantly lower than that of patients with type 2 diabetes. Levels of serum creatinine in the study participants did not vary significantly between healthy and type 2 diabetic patients while the mean level of serum creatinine varied significantly between healthy individuals and patients with nephropathy as well as between type 2 diabetic patients with and without nephropathy. Urine creatinine level of healthy individuals was significantly lower compared to that of diabetic patients with and without nephropathy.

In this study, levels of microalbumin in healthy individuals, type 2 diabetic patients without and with nephropathy varied from 3.5-11.1 mg/L, 3.5-19 mg/L and 30.2-1140.0 mg/L, respectively. The mean value of microalbumin in diabetic nephrotic patients (238.58 ± 316.07 mg/L) was significantly higher than in healthy individuals (5.55 ± 2.32 mg/L) and diabetic patients without nephropathy (7.79 ± 4.68 mg/L). Albumin creatinine ratio (ACR) between healthy individuals (8.85 ± 4.07 mg/g; range: 3.24-20.2 mg/g) and type 2 diabetic patients (10.22 ± 6.07 mg/g; range: 1.4-25.5 mg/g) did not vary statistically while mean value of ACR of patients with diabetic nephropathy (313.66 ± 519.87 mg/g; range: 30.9-3442 mg/g) was significantly higher than healthy individuals and type 2 diabetic patients. Their respective ranges of urine creatinine were 41.2-140 mg/dL, 17.4-299 mg/dL and 16.3-345.0 mg/dL. According to ACR values, it was observed that out of total study participants with nephropathy 91 (74.6%) had microalbuminuria (range: 30.9-297.9 mg/g) while 31 (25.4%) had overt albuminuria or macroalbuminuria (range:

300.7-3442.0 mg/g). The mean levels of hemoglobin and activities of alanine transaminase did not vary significantly between the study participants. The data of the biochemical parameters of the study participants have been presented in Table 3.2.

Table 3.2. Biochemical characteristics of the study participants.

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Fasting serum (mmol/L)	5.07 ± 0.34	9.49 ± 3.19	10.53 ± 3.62	0.000	0.000	0.017
ABF (mmol/L)	6.15 ± 0.63	13.97 ± 4.34	14.99 ± 4.40	0.000	0.000	0.068
HbA1c (%)	5.54 ± 0.32	8.73 ± 1.65	9.36 ± 1.80	0.000	0.000	0.004
Insulin (mlU/mL)	10.39 ± 3.53	39.68 ± 16.44	25.37 ± 21.63	0.000	0.000	0.000
Urea (mg/dL)	22.33 ± 4.85	24.33 ± 6.21	29.45 ± 13.28	0.019	0.000	0.000
Serum Creatinine (mg/dL)	0.77 ± 0.15	0. .80 ± 0. .21	0.99 ± 0.49	0.334	0.001	0.000
Urine Creatinine (mg/dL)	66.36 ± 20.21	93.18 ± 60.22	96.26 ± 61.90	0.000	0.000	0.692
Microalbumin (mg/L)	5.55 ± 2.32	7.79 ± 4.68	238.58 ± 316.07	0.000	0.000	0.000
ACR (mg/g)	8.85 ± 4.07	10.22 ± 6.07	313.66 ± 519.87	0.087	0.000	0.000
HB (g/dL)	13.46 ± 1.53	12.67 ± 1.55	12.58 ± 1.76	0.121	0.130	0.675
ALT (U/L)	41.27 ± 24.24	45.4 ± 29.15	48.2 ± 28.87	0.305	0.085	0.276

CN: Healthy control individuals; T2D: Type 2 diabetic patients; T2D+DN: type 2 diabetic patients with nephropathy, ABF: Plasma glucose level 2 hours after breakfast; MALB: microalbuminuria; ACR: albumin creatinine ratio.

3.3. Determination of lipid profiles in the study participants

Serum lipid profile is measured for determining the possibility of diabetes associated macrovascular complications specially for cardiovascular risk prediction. Four basic parameters: total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were tested under this profile.

Table 3.3. Levels of lipid profile in the study participants.

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Cholesterol (mg/dL)	180.39 ± 33.73	166.58 ± 49.30	168.48 ± 44.15	0.016	0.048	0.750
Triglycerides (mg/dL)	173.98 ± 86.58	233.45 ± 235.84	244.56 ± 177.38	0.004	0.002	0.676
HDL-Cholesterol (mg/dL)	40.47 ± 10.04	39.20 ± 10.74	36.02 ± 7.54	0.413	0.001	0.008
LDL-Cholesterol (mg/dL)	105.12 ± 31.01	80.04 ±43.27	83.55 ± 42.06	0.000	0.000	0.126

CN: Healthy control individuals; T2D: Type 2 diabetic patients; T2D+DN: type 2 diabetic patients with nephropathy; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein

Total cholesterol less than 200 mg/dL is considered as the normal level in a healthy individual. It was observed that the levels of cholesterol in healthy individuals was significantly higher compared to that of the levels found in with ($p = 0.048$) and without ($p = 0.016$) nephropathy patients. Out of the total patients with diabetic nephropathy, 34 (27.9%) had higher cholesterol (range: 200-278 mg/dL) than normal level and rest of the 88 (72.1%) had cholesterol level within the normal range (69-194 mg/dL). In case of type 2 diabetic patients without nephropathy, 23 (18.7%) had higher level of cholesterol (202-444 mg/dL), 37 (30.1%) had cholesterol level more than 150 mg/dL (range: 151-199 mg/dL) and 13 (51.2%) had normal cholesterol level (range: 74-149 mg/dL). In case of healthy individuals, 21 (20.2%) had higher level of cholesterol (201-272 mg/dL), 35 (33.6%) had cholesterol level more than 150 mg/dL (range: 152-199 mg/dL) and 48 (46.2%) had normal cholesterol level (range: 111-149 mg/dL).

It was observed that the mean levels of triglyceride (TG) were higher than the normal level in all the study participants. Out of 122 diabetic nephropathy patients, 92 (75.4%) had higher levels of TG (range: 150-1362 mg/dL) while 30 (24.6%) had normal level of TG (range: 93-149 mg/dL).

In case of type 2 diabetic patients, 56 (45.5%) had higher and 67 (54.5%) had normal level (range: 62-148 mg/dL) of TG. On the other hand, more than 42% (n=44) and 57% (n=60) of the healthy individuals had >150 mg/dL and normal levels of TG, respectively. Further analyses revealed that the mean level of TG in healthy individuals was significantly lower compared to those of patients with and without nephropathy. The data have been presented in Table 3.3.

HDL cholesterol is considered as good cholesterol that acts as a scavenger, carrying LDL (harmful lipid) cholesterol away from the arteries and back to the liver. A range of 50-59 mg/dL of HDL-cholesterol is thought to be the normal level in human. Out of 122 diabetic nephropathy patients, 5 (4.1%) had normal levels of HDL-cholesterol (range: 50-59 mg/dL) while 117 (95.9%) lower level (range: 20-49 mg/dL). In case of type 2 diabetic patients, 18 (14.6%) had normal and 105 (85.4%) had lower levels (range: 18-48 mg/dL) of HDL-cholesterol. On the other hand, more than 9% (n=10) and 90% (n=94) of the healthy individuals had normal levels and lower of HDL-cholesterol, respectively. Further analyses revealed that the mean level of HDL-cholesterol was significantly higher in healthy individuals (p=0.001) and type 2 diabetic patients (p=0.008) compared to patients with diabetic nephropathy. However, levels of HDL-cholesterol did not vary between healthy individuals and type 2 diabetic patients. The data has been presented in Table 3.3. The mean level of LDL-cholesterol in healthy individuals was significantly higher compared to the mean values of patients with or without diabetic nephropathy. However, this level did not vary between diabetic patients with and without nephropathy.

3.4. Correlation of demographic and biochemical parameters of healthy individuals, type 2 diabetic patients with and without nephropathy

A direct relationship was observed between fasting plasma glucose and glycated hemoglobin in all the three groups of study participants, which is quite obvious that increasing levels of glucose will cause glycation of more hemoglobin. Correlation analyses demonstrated that there is a positive correlation between age and fasting plasma glucose as well as levels of glycated hemoglobin. Also, strong positive correlation exists between the age and BMI of the healthy study participants. The correlation data has been presented in Table 3.4, Table 3.5 and Table 3.6.

Table 3.4. Correlation between demographic and biochemical parameters of healthy control individuals.

Parameters		Age	Glc_C	HbA1C_C	ACR_C	Insulin_C	BMI_C
Age	Pearson Correlation	1	.242*	.215	-.206	-.086	.259*
	Sig. (2-tailed)		.038	.066	.078	.464	.016
Glc_C	Pearson Correlation		1	.256*	.111	.011	.007
	Sig. (2-tailed)			.028	.346	.926	.954
HbA1C_C	Pearson Correlation			1	-.074	.104	.237*
	Sig. (2-tailed)				.532	.380	.042
ACR_C	Pearson Correlation				1	.161	.130
	Sig. (2-tailed)					.170	.271
Insulin_C	Pearson Correlation					1	-.167
	Sig. (2-tailed)						.156
BMI_C	Pearson Correlation						1

*Age_C: Age of healthy individuals; Glc_C: Fasting plasma glucose level in healthy individuals; HbA1c_C: Glycated hemoglobin in healthy individuals; ACR_C: Albumin creatinine ratio in healthy individuals; Insulin_C: Levels of insulin in healthy individuals; BMI_C: Basal Metabolic Index of healthy individuals.**. Correlation is significant at the 0.05 level (2-tailed).

Table 3.5. Correlation between demographic and biochemical parameters of type 2 diabetic patients without nephropathy.

Parameters		Age_T2D	Glc_T2D	HbA1C_ T2D	ACR_ T2D	Insulin_ T2D	BMI_T2D
Age_T2D	Pearson Correlation	1	-.054	-.090	.116	-.075	.012
	Sig. (2-tailed)		.589	.371	.246	.466	.906
Glc_T2D	Pearson Correlation		1	.697**	.084	.158	.086
	Sig. (2-tailed)			.000	.403	.121	.391
HbA1C_T2D	Pearson Correlation			1	.122	.168	.020
	Sig. (2-tailed)				.222	.098	.845
ACR_T2D	Pearson Correlation				1	.099	-.098
	Sig. (2-tailed)					.332	.326
Insulin_T2D	Pearson Correlation					1	.041
	Sig. (2-tailed)						.688
BMI_T2D	Pearson Correlation						1

Age_T2D: Age of patients with type 2 diabetes without nephropathy; Glc_ T2D: Fasting plasma glucose level in patients with type 2 diabetes; HbA1c_T2D: Glycated hemoglobin in patients with type 2 diabetes without nephropathy; ACR_T2D: Albumin creatinine ratio of patients with type 2 diabetes without nephropathy; Insulin_ T2D: Levels of insulin in patients with type 2 diabetes without nephropathy; BMI_T2D: Basal Metabolic Index of patients with type 2 diabetes without nephropathy.

**. Correlation is significant at the 0.01 level (2-tailed).

Table 3.6. Correlation between demographic and biochemical parameters of type 2 diabetic patients with nephropathy.

Parameters		Age_DN	Glc_DN	HbA1C_ DN	ACR_ DN	Insulin_ DN	BMI_DN
Age_DN	Pearson Correlation	1	-.018	-.078	.058	-.023	-.001
	Sig. (2-tailed)		.840	.391	.523	.803	.995
Glc_DN	Pearson Correlation		1	.728**	.006	.154	-.048
	Sig. (2-tailed)			.000	.945	.095	.598
HbA1C_DN	Pearson Correlation			1	.065	.217*	-.107
	Sig. (2-tailed)				.473	.018	.238
ACR_DN	Pearson Correlation				1	-.014	-.025
	Sig. (2-tailed)					.876	.785
Insulin_DN	Pearson Correlation					1	-.040
	Sig. (2-tailed)						.668
BMI_DN	Pearson Correlation						1

Age_DN: Age of patients with diabetic nephropathy; Glc_DN: Fasting plasma glucose level of patients with diabetic nephropathy; HbA1C_DN Glycated hemoglobin of patients with diabetic nephropathy; ACR_DN: Albumin creatinine ratio of patients with diabetic nephropathy; Insulin_DN: Levels of insulin of patients with diabetic nephropathy; BMI_DN: Basal Metabolic Index of patients with diabetic nephropathy.

***. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed).*

In diabetic nephropathy, levels of insulin is also directly related to the levels of HbA1c (r^2=0.217, n=0.018). However, we did not find such association in healthy individuals and patients with type 2 diabetes.

3.5. Correlation analyses of the levels of HbA1c and insulin with lipid profiles among the study participants

In healthy individuals, levels of HbA1c showed positive relationship with that of plasma cholesterol (Table 3.7) while levels of plasma insulin showed a negative relation with the levels of plasma HDL cholesterol (Figure 3.1). Further, strong positive correlation was observed between plasma levels of cholesterol and triglycerides, between plasma cholesterol and LDL cholesterol while levels of plasma triglycerides and HDL cholesterol showed inverse relationship as presented in Table 3.7.

Table 3.7: Correlation analyses of the levels of HbA1c and insulin with lipid profiles of healthy control individuals.

Parameters		HbA1C_C	Insulin_C	Chol_C	TG_C	HDL_C	LDL_C
HbA1C_C	Pearson Correlation	1	.104	.238	.086	-.072	.230
	Sig. (2-tailed)		.380	.050	.500	.573	.068
Insulin_C	Pearson Correlation		1	.021	.200	-.298*	-.004
	Sig. (2-tailed)			.872	.112	.017	.976
Chol_C	Pearson Correlation			1	.377**	-.012	.881**
	Sig. (2-tailed)				.001	.917	.000
TG_C	Pearson Correlation				1	-.417**	-.013
	Sig. (2-tailed)					.000	.913
HDL_C	Pearson Correlation					1	-.104
	Sig. (2-tailed)						.376
LDL_C	Pearson Correlation						1

Chol_C: Levels of cholesterol in healthy individuals; TG_C: Levels of triglycerides in healthy individuals; HDL_C: Levels of High Density Lipoproteins in healthy individuals; LDL_C: Levels of Low Density Lipoproteins in healthy individuals; HbA1C_C Glycated hemoglobin of healthy individuals; Insulin_C: Levels of insulin in healthy individuals.

*. Correlation is significant at the 0.05 level (2-tailed); **. Correlation is significant at the 0.01 level (2-tailed).

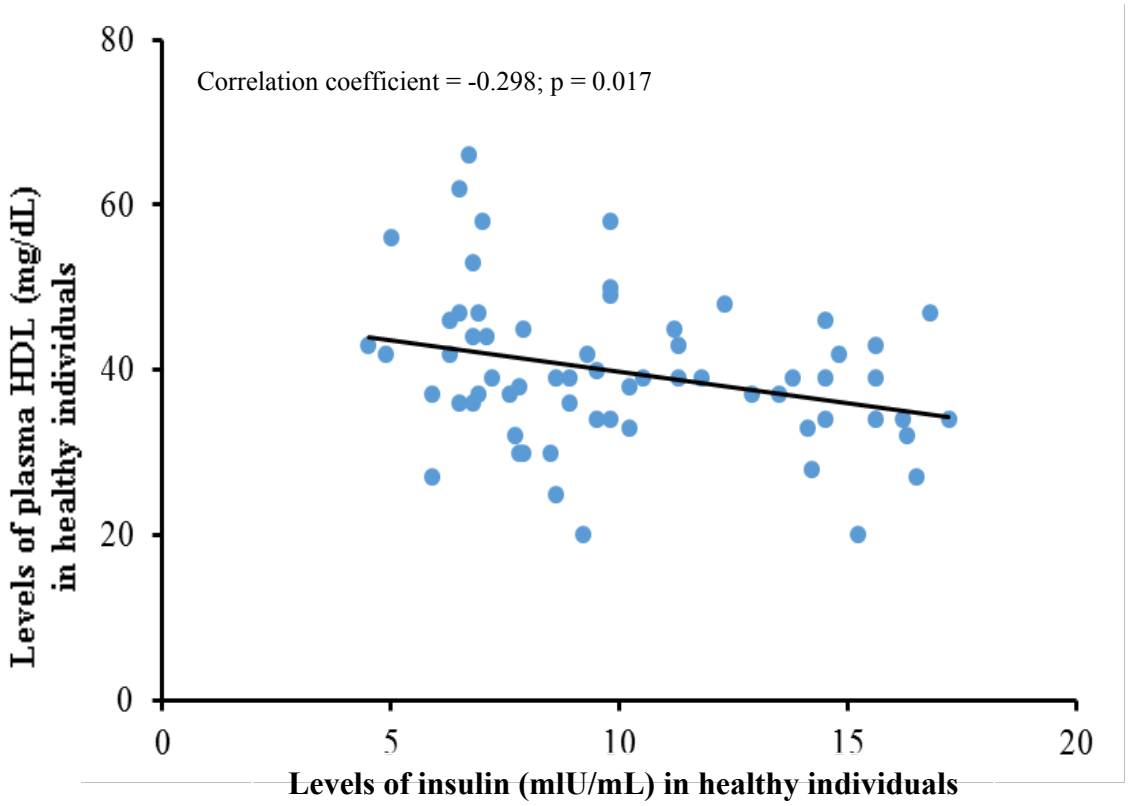

Figure 3.1: Correlation analyses between levels of insulin and plasma HDL cholesterol in healthy individuals. Statistically significant negative correlation was observed between the two parameters (Correlation coefficient = -0.298; p = 0.017) indicating increased level of insulin may reduce the levels of good HDL cholesterol.

Table 3.8: Correlation analyses of the levels of HbA1c and insulin with lipid profiles of type 2 diabetic patients without nephropathy.

Parameters		Chol_T2D	TG_T2D	HDL_T2D	LDL__T2D	HbA1C_T2D	Insulin_T2D
Chol_T2D	Pearson Correlation	1	.525**	.113	.537**	.085	.035
	Sig. (2-tailed)		.000	.257	.000	.397	.733
TG_T2D	Pearson Correlation		1	-.338**	-408**	.079	-.137
	Sig. (2-tailed)			.001	.000	.429	.179
HDL_T2D	Pearson Correlation			1	-.235*	.062	.195
	Sig. (2-tailed)				.017	.537	.055
LDL__T2D	Pearson Correlation				1	-.006	.142
	Sig. (2-tailed)					.949	.164
HbA1C_T2D	Pearson Correlation					1	.168
	Sig. (2-tailed)						.098
Insulin_T2D	Pearson Correlation						1

Chol_T2D: Levels of cholesterol in patients with type 2 diabetes; TG_T2D: Levels of triglycerides in patients with type 2 diabetes; HDL_T2D: Levels of High Density Lipoproteins in patients type 2 diabetes; LDL_T2D: Levels of Low Density Lipoproteins in patients with type 2 diabetes; HbA1C_T2D Glycated hemoglobin of patients with type 2 diabetes; Insulin_T2D: Levels of insulin in patients with type 2 diabetes.

*. Correlation is significant at the 0.05 level (2-tailed); **. Correlation is significant at the 0.01 level (2-tailed).

In type 2 diabetic patients triglycerides were directly related with the levels of cholesterol while inversely related with the levels of HDL cholesterol (as shown in Table 3.8). However, we did not find any relation of HbA1c and insulin with any of the parameters of lipid profile in these patients. Also, in these patients levels of triglycerides was inversely related to that of LDL cholesterol.

Table 3.9: Correlation analyses of the levels of HbA1c and insulin with lipid profiles of type 2 diabetic patients with nephropathy.

Parameters		Chol_DN	TG_DN	HDL_DN	LDL_DN	HbA1C_DN	Insulin_DN
Chol_DN	Pearson Correlation	1	.404**	.162	.680**	.253**	.059
	Sig. (2-tailed)		.000	.074	.000	.005	.526
TG_DN	Pearson Correlation		1	-.228*	-.378**	.044	.001
	Sig. (2-tailed)			.011	.000	.632	.992
HDL_DN	Pearson Correlation			1	.182*	-.094	.008
	Sig. (2-tailed)				.043	.300	.933
LDL_DN	Pearson Correlation				1	.245**	.059
	Sig. (2-tailed)					.006	.522
HbA1C_DN	Pearson Correlation					1	.217*
	Sig. (2-tailed)						.018
Insulin_DN	Pearson Correlation						1

*Chol_DN: Levels of cholesterol in patients with diabetic nephropathy; TG_DN: Levels of triglycerides in patients with diabetic nephropathy; HDL_DN: Levels of High Density Lipoproteins in patients with diabetic nephropathy; LDL_DN: Levels of Low Density Lipoproteins in patients with diabetic nephropathy; HbA1C_DN Glycated hemoglobin of patients with diabetic nephropathy; Insulin_DN: Levels of insulin in patients with diabetic nephropathy. **. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed).*

In case of patients with nephropathy, levels of cholesterol were found to be directly related to those of the levels of triglycerides and LDL cholesterol. On the other hand, both levels of HDL and LDL cholesterol are inversely related to that of triglycerides. Further analyses revealed that levels of HbA1c in these patients are positively correlated with those of the levels of plasma cholesterol and LDL cholesterol (Figures 3.2 A and B) indicating augmented probability of increased levels of bad cholesterol with increasing values of HbA1c. Though statistically not significant, levels of good cholesterol in these patients was inversely related to the levels of HbA1c. Data have been presented in Table 3.9.

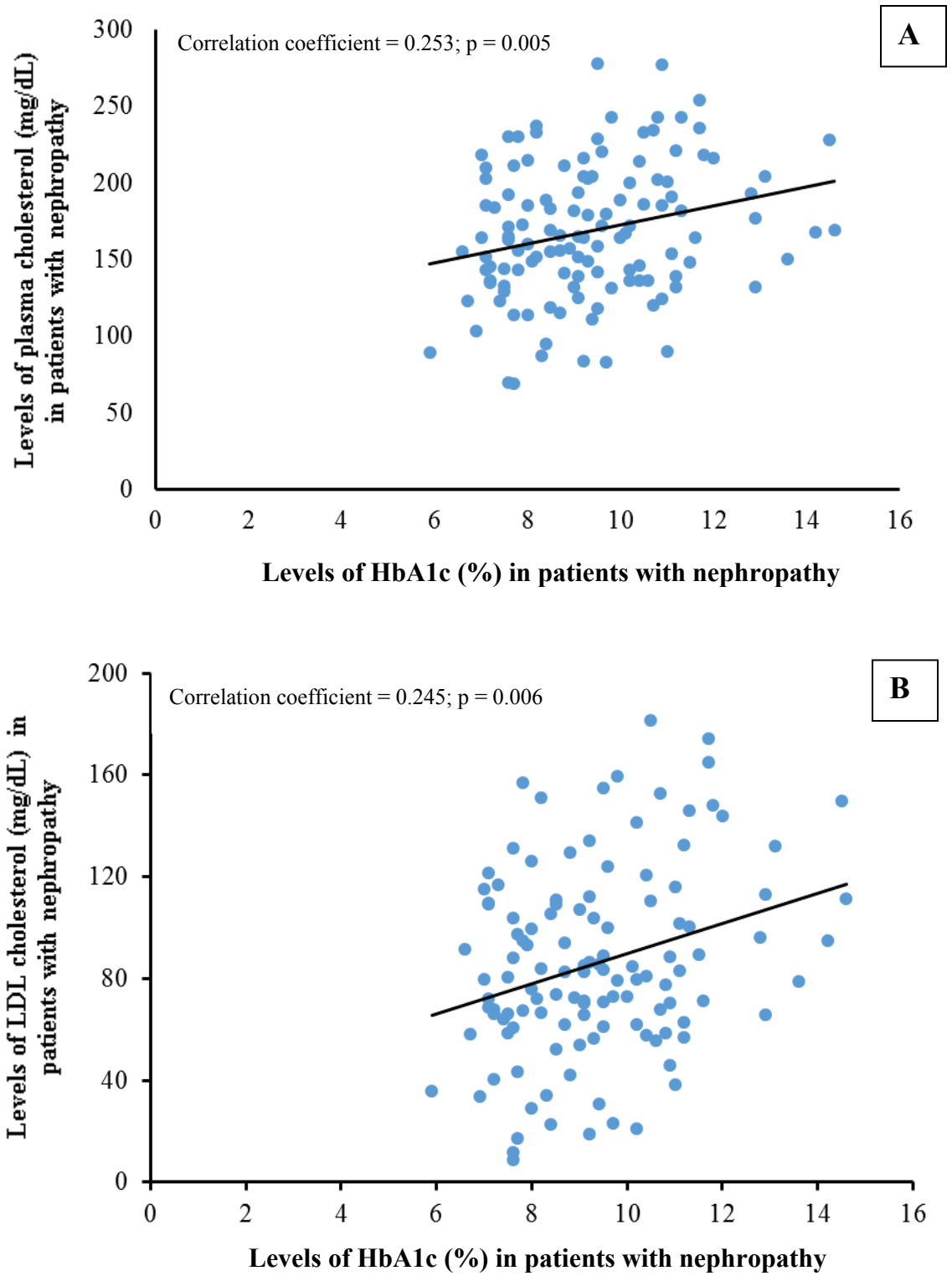

Figure 3.2: Correlation analyses between HbA1c, levels of plasma cholesterol (A) and LDL cholesterol (B) in type 2 diabetic patients with nephropathy. It was found that the levels of cholesterol and LDL cholesterol were significantly positively correlated with that of their

respective values of HbA1c (Correlation coefficients = 0.253 and 0.245; p = 0.005 and p = 0.006, respectively) indicating increased level of glycated hemoglobin may augment the levels of total cholesterol and bad LDL cholesterol.

Study participants were grouped into male and female individuals to compare their anthropometric, demographic and biochemical parameters which have been discussed below:

3.6. Demographic and anthropometric characteristics of the male study participants

Out of the total 179 male study participants, 56 (31.3%) were healthy individuals, 62 (34.6%) were type 2 diabetic patients and 61 (34.1%) were diabetic patients with nephropathy. Average age of male healthy individuals, type 2 diabetic patients and diabetic patients with nephropathy were 37.69 ± 12.36, 52.30 ± 9.83 and 55.72 ± 12.81 years, respectively. Values of their respective BMI were 24.84 ± 2.55, 26.78 ± 4.55 and 28.16 ± 2.76. Mean values of their systolic blood pressure were 123.61 ± 7.64, 126.57 ± 6.36, 141.02 ± 8.54 mmHg, respectively while these values for diastolic blood pressure were 83.29 ± 7.16, 85.69 ± 6.48 and 97.68 ± 7.82 mmHg, respectively. Average duration of diabetes among the randomly selected male patients was 8.95 ± 3.4 years (range: 6-18 years). To control blood sugar level, patients were under treatment using drugs like metformin, sulfonylureas etc. while few of the patients diagnosed with very high levels of blood sugar were advised to take insulin therapy for controlling glucose level. (Table 3.10).

Table 3.10. Demographic and anthropometric characteristics of the male study participants

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Age (years)	37.69 ± 12.36	52.30 ± 9.83	55.72 ± 12.81	0.000	0.000	0.070
Height (cm)	166.74 ± 4.67	169.27 ± 4.35	163.47 ± 7.11	0.022	0.062	0.001
Weight (Kg)	68.27 ± 8.13	72.82 ± 5.55	71.02 ± 7.02	0.009	0.007	0.403
BMI	24.84 ± 2.55	26.78 ± 4.55	28.16 ± 2.76	0.068	0.015	0.092
SBP (mmHg)	123.61 ± 7.64	126.57 ± 6.36	141.02 ± 8.54	0.049	0.000	0.022
DBP (mmHg)	83.29 ± 7.16	85.69 ± 6.48	97.68 ± 7.82	0.063	0.000	0.001

CN: Male healthy control individuals; T2D: Male type 2 diabetic patients; T2D+DN: Male type 2 diabetic patients with nephropathy.

3.7. Biochemical characteristics of the male study participants

The mean fasting plasma glucose level of the male healthy individuals, type 2 diabetic patients and diabetic patients with diabetic nephropathy were 5.08 ± 0.34, 13.54 ± 4.19 and 10.82 ± 3.60 mmol/L while after two hours of breakfast, these respective values were 6.13 ± 0.66, 8.96 ± 2.8 and 15.77 ± 4.30 mmol/L. The mean levels of fasting glucose varied significantly between all group of male participants ($p = 0.000$). Also, the mean levels of HbA1c of male healthy individuals (5.59 ± 0.24%) varied significantly with those of type 2 diabetic patients (8.51 ± 1.48%) and diabetic patients with nephropathy (9.60 ± 1.81%) ($p = 0.000$). Insulin level was found to be the highest (37.71 ± 14.2 mlU/mL) among female type 2 diabetic patients compared to that of healthy individuals (10.75 ± 3.52 mlU/mL) and diabetic patients with nephropathy (26.30 ± 28.88 mlU/mL). Mean level of insulin in male healthy individuals was statistically significantly lower when compared with that of the levels of male patients with type 2 diabetes and nephropathy. Mean levels of insulin in diabetic nephropathy was also significantly lower than that of patients with type 2 diabetes.

The level of serum creatinine did not vary significantly between male healthy and type 2 diabetic individuals while significant variation was observed when compared between healthy individuals and nephropathy female patients as well as between patients with or without nephropathy.

The mean value of microalbumin in male nephropathy patients (238.0 ± 316.08 mg/mL) was significantly higher than in healthy individuals (5.56 ± 2.32 mg/L) and diabetic patients without nephropathy (8.92 ± 4.97 mg/L). Albumin creatinine ratio (ACR) between healthy individuals (8.85 ± 4.07 mg/g; range: 3.2-22.2 mg/g) and type 2 diabetic patients (9.37 ± 5.91 mg/g; range: 1.6-25.5 mg/g) varied significantly and the mean value of ACR of patients with diabetic nephropathy (313.67 ± 519.87 mg/g; range: 30.9-1143.4 mg/g) was significantly higher than healthy individuals and type 2 diabetic patients. The mean levels of hemoglobin and activities of alanine transaminase did not vary significantly between the female study participants. The data of the biochemical parameters of the study participants have been presented in Table 3.11.

In the present study, levels of microalbumin in male healthy individuals, type 2 diabetic patients without and with nephropathy varied from 3.5-11.1 mg/L, 3.5-19.0 mg/L and 30.2-1140.0 mg/L, respectively. Their respective ranges of urine creatinine levels were 41.2-140 mg/dL, 21.7-299.0 mg/dL and 17.3-344.0 mg/dL. According to ACR values, it was observed that out of total male

study participants with nephropathy 16 (73.8%) had microalbuminuria (range: 30.9-297.5 mg/g) while 45 (26.2%) had overt albuminuria or macroalbuminuria (range: 300.7-1143.4 mg/g).

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Table 3.11. Biochemical characteristics of the male study participants.

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Fasting serum (mmol/L)	5.08 ± 0.34	13.54 ± 4.19	10.82 ± 3.60	0.000	0.000	0.022
ABF (mmol/L)	6.13 ± 0.66	8.96 ± 2.8	15.77 ± 4.30	0.000	0.000	0.000
HbA1c (%)	5.59 ± 0.24	8.51 ± 1.48	9.60 ± 1.81	0.000	0.000	0.253
Insulin (mlU/mL)	10.75 ± 3.52	37.71 ± 14.2	26.30 ± 28.88	0.000	0.000	0.005
Urea (mg/dL)	22.46 ± 5.03	24.4 ± 6.5	30.24 ± 16.58	0.069	0.010	0.032
Serum Creatinine (mg/dL)	0.88 ± 0.12	0.9 ± 0.2	1.01 ± 0.41	0.801	0.003	0.062
Urine Creatinine (mg/dL)	66.37 ± 20.22	114.84±64.16	106.42 ± 57.73	0.000	0.000	0.054
Microalbumin (mg/L)	5.56 ± 2.32	8.92 ± 4.97	238.0 ± 316.08	0.043	0.000	0.000
ACR (mg/g)	8.85 ± 4.07	9.37 ± 5.91	313.67 ± 519.87	0.075	0.000	0.000
HB (g/dL)	13.47 ± 1.53	13.29 ± 1.43	13.44 ± 1.57	0.875	0.890	0.925
ALT (U/L)	45.59 ± 25.32	47.6 ± 33.2	54.40 ± 31.67	0.200	0.058	0.051

CN: Male healthy control individuals; T2D: Male type 2 diabetic patients; T2D+DN: Male type 2 diabetic patients with nephropathy, ABF: Plasma glucose level 2 hours after breakfast; MALB: microalbuminuria; ACR: Albumin Creatinine Ratio.

3.8. Levels of plasma cholesterol, triglycerides, HDL cholesterol and LDL cholesterol in the female study participants

Total cholesterol less than 200 mg/dL is considered as the normal level in a healthy individual. From Table 3.12, it can be observed that the levels of cholesterol of male healthy individuals varied significantly with that of patients with and without type 2 diabetes. Out of the total male patients with diabetic nephropathy, 18 (29.51%) had higher cholesterol (range: 213-278 mg/dL) than normal level, 30 (49.18%) had >150 mg/dL of cholesterol and rest of the 13 (21.31%) had cholesterol level within the normal range (82-149 mg/dL). In case of type 2 diabetic patients without nephropathy, 20 (32.26%) had higher level of cholesterol (200-263 mg/dL), 21 (33.87%) had cholesterol level >/= 150 mg/dL (range: 150-198 mg/dL) and 21 (33.87%) had normal cholesterol level (range: 70-148 mg/dL). In case of healthy individuals, 30 (53.57%) had higher level of cholesterol (201-238 mg/dL), 18 (32.14%) had cholesterol level more than 150 mg/dL and 8 (14.29%) had normal cholesterol level (range: 124-148 mg/dL).

Table 3.12: Levels of plasma cholesterol, triglycerides, HDL cholesterol and LDL cholesterol in the female study participants

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Cholesterol (mg/dL)	190.3 ± 35.6	159.9 ± 56.58	160.87 ± 43.8	0.010	0.021	0.875
Triglycerides (mg/dL)	194.5 ± 54.9	254.6 ± 78.21	248.39 ± 75.6	0.040	0.021	0.105
HDL-Cholesterol (mg/dL)	36.48 ± 6.61	36.59 ± 10.99	34.15 ± 7.10	0.652	0.531	0.545
LDL-Cholesterol (mg/dL)	114.9 ± 32.8	72.37 ± 38.33	82.02 ± 36.14	0.015	0.033	0.062

CN: Male healthy control individuals; T2D: Male Type 2 diabetic patients; T2D+DN: Male type 2 diabetic patients with nephropathy; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein.

The mean levels of triglyceride (TG) were higher than the normal level in all the male study participants. Out of 61 male diabetic nephropathy patients, 44 (72.13%) had higher levels of TG while 17 (27.87%) had normal level of TG (range: 72-148 mg/dL). In case of type 2 diabetic patients, 23 (37.10%) had higher and 39 (62.9%) had normal level (range: 65-142 mg/dL) of TG. On the other hand, almost 35.7% (n=20) and 64.3% (n=36) of the healthy individuals had >/=150 mg/dL and normal levels of TG, respectively. Further analyses revealed that the mean level of TG between healthy individuals and patients with or without nephropathy varied significantly. However, while this level did not vary significantly between the two groups of patients. The data has been presented in Table 3.15.

Out of 61 diabetic nephropathy male patients, 5 (8.2%) had normal levels of HDL-cholesterol (range: 50-59 mg/dL) while 56 (91.8%) lower level (range: 20-49 mg/dL). In case of type 2 diabetic patients, 17 (27.42%) had normal (range: 50-78 mg/dL) and 45 (72.58%) had lower levels (range: 18-48 mg/dL) of HDL-cholesterol. On the other hand, 32.14% (n=18) and 67.86% (n=38) of the healthy individuals had normal levels and lower of HDL-cholesterol, respectively. Further analyses revealed that the mean level of HDL-cholesterol was significantly higher in healthy individuals (p=0.001) and type 2 diabetic patients (p=0.015) compared to patients with diabetic nephropathy. However, levels of HDL-cholesterol did not vary between healthy individuals and type 2 diabetic patients. The data has been presented in Table 3.15. With regard to mean LDL-cholesterol, mean level of healthy individuals varied significantly with the mean values of male patients with or without diabetic nephropathy. However, this level did not vary between male diabetic patients with and without nephropathy.

3.9. Demographic and anthropometric characteristics of the female study participants

Out of the total 170 female study participants, 48 (28.2%) were healthy individuals, 61 (35.9%) were type 2 diabetic patients and 61 (35.9%) were diabetic patients with nephropathy. Average age of female healthy individuals, type 2 diabetic patients and diabetic patients with nephropathy were 39.26 ± 12.59, 51.70 ± 10.03 and 57.72 ± 11.01 years, respectively. Values of their respective BMI were 24.97 ± 2.56, 26.78 ± 4.55 and 28.16 ± 2.76. Mean values of their systolic blood pressure were 118.38 ± 8.35, 125.10 ± 7.18, 141.05 ± 7.53, respectively while these values for diastolic blood pressure were 78.25 ± 8.05, 83.70 ± 6.84 and 97.66 ± 7.72. Average duration of diabetes among the randomly selected female patients was 7.35 ± 3.5 years (range: 5.5-15

years). To control blood sugar level, patients were under treatment using drugs like metformin, sulfonylureas etc. while few of the patients diagnosed with very high levels of blood sugar were advised to take insulin therapy for controlling glucose level.

Table 3.13. Demographic and anthropometric characteristics of the female study participants.

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Age (years)	39.26 ± 12.59	51.70 ± 10.03	57.72 ± 11.01	0.000	0.000	0.027
Height (cm)	157.42 ± 2.36	178.38 ± 141.55	157.82 ± 2.79	0.432	0.000	0.315
Weight (Kg)	61.57 ± 6.55	68.36 ± 5.55	70.10 ± 6.62	0.000	0.000	0.353
BMI	24.84 ± 2.55	26.78 ± 4.55	28.16 ± 2.76	0.004	0.655	0.142
SBP (mmHg)	118.38 ± 8.35	125.10 ± 7.18	141.05 ± 7.53	0.001	0.000	0.000
DBP (mmHg)	78.25 ± 8.05	83.70 ± 6.84	97.66 ± 7.72	0.023	0.000	0.000

CN: Female healthy control individuals; T2D: Female type 2 diabetic patients without nephropathy; T2D+DN: Female type 2 diabetic patients with nephropathy.

3.10. Biochemical characteristics of the female study participants.

The mean fasting plasma glucose level of the female healthy individuals, type 2 diabetic patients and diabetic patients with diabetic nephropathy were 5.08 ± 0.34, 9.71 ± 3.52 and 10.26 ± 3.66 mmol/L while after two hours of breakfast, these respective values were 6.17 ± 0.61, 13.49 ± 4.00 and 14.23 ± 4.41 mmol/L. The mean levels of fasting glucose of female healthy individuals varied significantly with those of type 2 diabetic patients and diabetic patients with nephropathy ($p = 0.000$). However, this level did not vary between female diabetic patients with and without nephropathy. Also, the mean levels of HbA1c of female healthy individuals (5.50 ± 0.38%) varied significantly with those of type 2 diabetic patients (8.76 ± 1.78%) and diabetic patients with nephropathy (9.14 ± 1.77%) ($p < 0.000$). Insulin level was found to be the highest (41.63 ± 19.99 mlU/mL) among female type 2 diabetic patients compared to that of healthy individuals

(10.03 ± 3.52 mlU/mL) and diabetic patients with nephropathy (24.46 ± 10.68 mlU/mL). Mean level of insulin in female healthy individuals was statistically significantly lower when compared with that of the levels of female patients with type 2 diabetes and nephropathy. Mean levels of insulin in diabetic nephropathy was also significantly lower than that of patients with type 2 diabetes. Mean level of serum creatinine did not vary significantly between female healthy and type 2 diabetic individuals while significant variation was observed when compared between healthy individuals and nephropathy female patients as well as compared between patients with and without nephropathy. Urine creatinine level of female participants did not vary statistically.

The mean value of microalbumin in female nephropathy patients (239.39 ± 328.00 mg/mL; range: 20.8-1140.43 mg/mL) was significantly higher than in healthy individuals (5.41 ± 2.13 mg/L; range: 2.14-9.60 mg/mL) and diabetic patients without nephropathy (6.81 ± 4.14 mg/L; range: 3.5-16.1 mg/mL). Albumin creatinine ratio (ACR) between healthy individuals (8.16 ± 3.73 mg/g; range: 3.33-17.1 mg/g) and type 2 diabetic patients (10.92 ± 5.90 mg/g; range: 1.4-22.6 mg/g) varied significantly and mean value of ACR of patients with diabetic nephropathy (385.10 ± 670.03 mg/g; range: 34.2-3442 mg/g) was significantly higher than healthy individuals and type 2 diabetic patients. The mean levels of hemoglobin and activities of alanine transaminase did not vary significantly between the female study participants. The data of the biochemical parameters of the study participants have been presented in Table 3.14

In the present study, levels of microalbumin in female healthy individuals, type 2 diabetic patients without and with nephropathy varied from 2.14-9.6 mg/L, 3.5-16.1 mg/L and 20.8-1140.0 mg/L, respectively. Their respective ranges of urine creatinine were 45.3-140 mg/dL, 17.4-251 mg/dL and 16.3-345.0 mg/dL. According to ACR values, it was observed that out of total study participants with nephropathy 46 (87.7%) had microalbuminuria (range: 34.2-289.8 mg/g) while 15 (12.3%) had overt albuminuria or macroalbuminuria (range: 313.14-3442.0 mg/g).

Table 3.14 .Biochemical characteristics of the female study participants.

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Fasting serum (mmol/L)	5.08 ± 0.34	9.71 ± 3.52	10.26 ± 3.66	0.000	0.000	0.682
ABF (mmol/L)	6.17 ± 0.61	13.49 ± 4.00	14.23 ± 4.41	0.000	0.000	0.856
HbA1c (%)	5.50 ± 0.38	8.76 ± 1.78	9.14 ± 1.77	0.000	0.000	0.553
Insulin (mlU/mL)	10.03 ± 3.52	41.63 ± 19.99	24.46 ± 10.68	0.000	0.000	0.000
Urea (mg/dL)	22.22 ± 4.67	24.60 ±6.09	28.71 ± 9.17	0.079	0.000	0.002
Serum Creatinine (mg/dL)	0.68 ± 0.12	0.70 ± 0.18	0.96 ± 0.56	0.553	0.003	0.000
Urine Creatinine (mg/dL)	70.72 ± 23.70	76.71 ± 54.54	86.28 ± 64.67	0.648	0.163	0.262
Microalbumin (mg/L)	5.41 ± 2.13	6.81 ± 4.14	239.39 ± 328.00	0.103	0.000	0.000
ACR (mg/g)	8.16 ± 3.73	10.92 ± 5.90	385.10 ± 670.03	0.005	0.001	0.000
HB (g/dL)	12.30 ± 1.08	12.07 ± 1.43	11.74 ± 1.54	0.336	0.060	0.265
ALT (U/L)	36.95 ± 22.24	43.08 ± 19.86	42.21 ± 24.69	0.200	0.298	0.800

CN: Female healthy control individuals; T2D: Female type 2 diabetic patients; T2D+DN: Female type 2 diabetic patients with nephropathy, ABF: Plasma glucose level 2 hours after breakfast; MALB: microalbuminuria; ACR: Albumin Creatinine Ratio.

3.11. Determination of lipid profiles in the female study participants

Total cholesterol less than 200 mg/dL is considered as the normal level in a healthy individual. It was observed that the levels of cholesterol among the female study participants were not statistically significant. Out of the total female patients with diabetic nephropathy, 20 (32.8%) had higher cholesterol (range: 203-277 mg/dL) than normal level, 24 (39.3%) had 150-200 mg/dL of cholesterol and rest of the 17 (27.9%) had cholesterol level within the normal range

(84-149 mg/dL). In case of type 2 diabetic patients without nephropathy, 15 (24.6%) had higher level of cholesterol (202-253 mg/dL), 25 (41.0%) had cholesterol level >/= 150 mg/dL (range: 150-196 mg/dL) and 21 (34.4%) had normal cholesterol level (range: 74-149 mg/dL). In case of healthy individuals, 7 (14.6%) had higher level of cholesterol (201-228 mg/dL), 18 (37.5%) had cholesterol level more than 150 mg/dL (range: 156-196 mg/dL) and 23 (47.9%) had normal cholesterol level (range: 124-148 mg/dL).

Table 3.15. Determination of lipid profiles in the female study participants

Parameters	CN	T2D	T2D+DN	p - values		
				CN vs T2D	CN vs T2D+DN	T2D vs T2D+DN
Cholesterol (mg/dL)	170.46 ± 28.86	167.68 ± 41.30	175.98 ± 43.54	0.998	0.494	0.475
Triglycerides (mg/dL)	153.46 ± 79.33	191.84 ± 156.35	240.81 ± 143.74	0.070	0.001	0.305
HDL-Cholesterol (mg/dL)	44.46 ± 11.32	42.36 ± 11.41	37.87 ± 7.57	0.252	0.001	0.015
LDL-Cholesterol (mg/dL)	95.31 ± 26.27	86.95 ± 39.51	89.95 ± 35.82	0.005	0.003	0.102

CN: Female healthy control individuals; T2D: Female type 2 diabetic patients; T2D+DN: Female type 2 diabetic patients with nephropathy; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein.

It was observed that the mean levels of triglyceride (TG) were higher than the normal level in all the female study participants. Out of 61 female diabetic nephropathy patients, 47 (77.05%) had higher levels of TG (range: 150-792 mg/dL) while 14 (22.95%) had normal level of TG (range: 80-142 mg/dL). In case of type 2 diabetic patients, 25 (41.5%) had higher and 36 (59.0%) had normal level (range: 62-148 mg/dL) of TG. On the other hand, almost 44% (n=21) and 56% (n=27) of the healthy individuals had >/=150 mg/dL and normal levels of TG, respectively. Further analyses revealed that the mean level of TG between healthy individuals and T2D patients without nephropathy as well as between patients with or without nephropathy did not

vary significantly. However, while this level varied significantly between healthy individuals and patients with nephropathy. The data has been presented in Table 3.15.

Out of 61 diabetic nephropathy female patients, 3 (4.92%) had normal levels of HDL-cholesterol (range: 50-59 mg/dL) while 58 (95.08%) lower level (range: 21-49 mg/dL). In case of type 2 diabetic patients, 13 (21.3%) had normal (range: 50-87 mg/dL) and 48 (78.7%) had lower levels (range: 24-48 mg/dL) of HDL-cholesterol. On the other hand, 20.8% (n=10) and 79.2% (n=34) of the healthy individuals had normal levels and lower of HDL-cholesterol, respectively. Further analyses revealed that the mean level of HDL-cholesterol was significantly higher in healthy individuals ($p=0.001$) and type 2 diabetic patients ($p=0.015$) compared to patients with diabetic nephropathy. However, levels of HDL-cholesterol did not vary between healthy individuals and type 2 diabetic patients. The data has been presented in Table 3.15. With regard to mean LDL-cholesterol, mean level of healthy individuals varied significantly with the mean values of female patients with or without diabetic nephropathy. However, this level did not vary between female diabetic patients with and without nephropathy.

Genotype analyses, disease association study and distribution of clinical parameters according to different genotypes with regard to ApoE and ApoA1 gene polymorphisms

Each participant was genotyped with regard to their ApoE and ApoA1 gene polymorphisms. Frequency distribution of respective genotypes and alleles were determined to determine their association with the diseases and clinical parameters.

3.12. Genotype analyses and frequency distribution of genotypes and alleles with regard to ApoE gene polymorphism in healthy individuals, type 2 diabetic patients with and without nephropathy

Considering all the individuals participated in this study, data analyses of genotypic frequency distribution with regard to ApoE gene polymorphism revealed that E3/E3 genotype was distributed about among 70% while E3/E4 among 26%, E4/E4 among 2.01%, E2/E3 among 1.43% and E2/E2 among 0.57% of the total population. However in the present study, we did not find individuals with E2/E4 genotype.

Out of total healthy individuals, almost 56% had E3/E3 which is 1.3-fold (~74%) and 1.4-fold (~78%) lower than those found in type 2 diabetes without and with diabetes, respectively. On the other hand, E3/E4 genotype frequency was found to be 2-fold and 2.5-fold higher in healthy individuals when compared with those of type 2 diabetes without (42.31% vs 21.13%) and with (42.31%

individuals when compared with those of type 2 diabetes without (42.31% vs 21.13%) and with (42.31% vs 17.21%) nephropathy, respectively. Distribution of genotypic frequencies E2/E2, E2/E3 and E4/E4 have been presented in Table 3.16. Also, it has been noticed that E2/E2 genotype was missing in healthy individuals with diabetic nephropathy patients (Table 3.16).

In case of distribution of allelic frequencies, E3 allele was found in 77.4%, 85.37% and 87.29% of healthy individuals, type 2 diabetic patients without and with nephropathy, respectively. Frequency of E4 allele in healthy individuals was found 1.8-fold higher compared to that of type 2 diabetic patients without (12.19%) and with (11.89%) nephropathy. Their respective E2 allele was found to be distributed in 0.48%, 2.44% and 0.82% of the individuals (Table 3.16).

This is to mention here that for further association analyses, E3/E3 genotype and E3 allele were considered to be the reference or wild type for our population as most of the study participants represent this genotype and allele. Different ApoE genotypes have been identified from the gel images represented as an example in Figure 3.2.

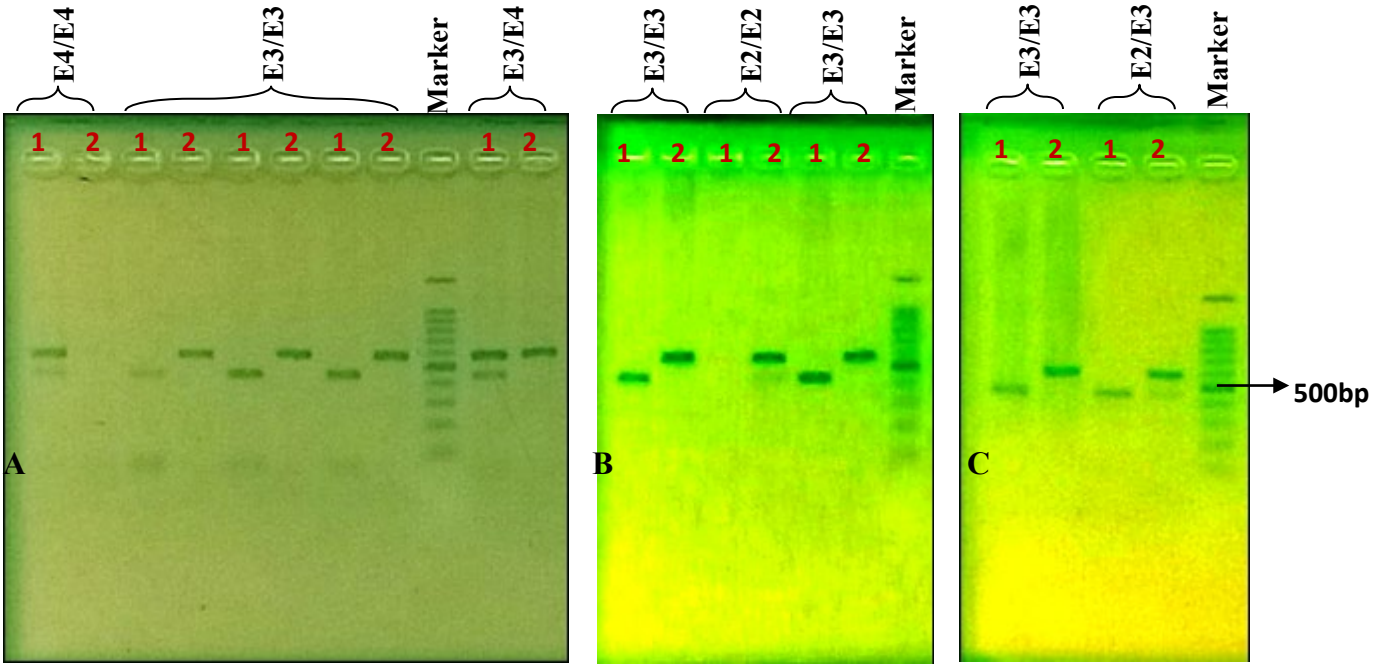

Figure 3.2: Different ApoE genotypes E2/E2, E2/E3, E3/E3, E3/E4 and E4/E4 have been identified in the study participants using ARMS PCR. PCR was performed using arginine primers (lanes 1) containing arginine 112 (588bp) and arginine 158 (451bp), and cysteine primers (lanes 2) containing cysteine 112 (588bp) and cysteine 158 (451bp). E4/E4 genotype was identified when bands for arg112/arg158 were found but not for cys112/cys158 (A); E3/E3 genotype was indentified when bands for arg158/cys112 were found (A); E3/E4 genotype was indentified when bands for arg158/arg112 and cys112 were found (A); E2/E2 genotype was identified when bands for cys112/cys158 were found but not for arg112/arg158 (B); E2/E3 genotype was indentified when bands for cys158/cys112 and arg158 were found (C). For marker DNA, 100 bp ladder was used. In this study, E2/E4 genotype was not recognized.

Table 3.16. Frequency distribution of genotypes and alleles with regard to ApoE gene polymorphism in healthy individuals, type 2 diabetic patients without and with nephropathy.

Genotypes	Healthy individuals (%), n=104	Type 2 diabetes without nephropathy (%), n=123	Type 2 diabetes with nephropathy (%), n=122	Total (%), n=349
E2/E2	0	2 (1.63)	0	2 (0.57)
E2/E3	1 (0.96)	2 (1.63)	2 (1.64)	5 (1.43)
E3/E3	58 (55.77)	91 (73.98)	95 (77.87)	244 (69.91)
E2/E4	0	0	0	0
E3/E4	44 (42.31)	26 (21.13)	21 (17.21)	91 (26.08)
E4/E4	1 (0.96)	2 (1.63)	4 (3.28)	7 (2.01)
Alleles				
E2	1 (0.48)	6 (2.44)	2 (0.82)	9 (1.29)
E3	161 (77.40)	210 (85.37)	213 (87.29)	584 (83.67)
E4	46 (22.12)	30 (12.19)	29 (11.89)	105 (15.04)
Total	208	246	244	698

3.13. Association of genotypic and allelic frequencies in regard to ApoE gene polymorphism with type 2 diabetes without nephropathy among Bangladeshi population

Multiple regression analyses revealed that heterozygous E3/E4 genotype played a statistically significant role in developing resistance against type 2 diabetes in Bangladeshi population [Odds ratio: 0.38 [95% CI: {0.21-0.68)}, chi square: 10.96, p <0.00]. Also, E4 allele showed resistance against developing type 2 diabetes [odds ratio: 4.6 {0.55-38.6}, chi square: 7.44, p = 0.006]. Association of any other genotypes and alleles with respect to ApoE gene polymorphism with type 2 diabetes were not observed in the present study. Data have been presented in Table 3.17.

Table 3.17. Association analyses of genotypic and allelic frequencies with type 2 diabetes without nephropathy in regard to ApoE gene polymorphism among Bangladeshi population.

Genotypes	Healthy individuals	Type 2 Diabetes without nephropathy	OR	X^2	p-value
E3/E3 (ref)	58	91			
E2/E2	0	2			
E2/E3	1	2	1.27 (0.11-14.37)	0.04	0.84
E2/E4	0	0			
E3/E4	44	26	0.38 (0.21-0.68)	10.96	<0.00
E4/E4	1	2	1.27 (0.11-14.37)	0.04	0.84
Total	**104**	**123**			
Alleles					
E3 (ref)	161	210			
E2	1	6	4.6 (0.55-38.6)	2.38	0.12
E4	46	30	0.5 (0.30-0.83)	7.44	0.006
Total	**208**	**246**			

3.14. Association of genotypic and allelic frequencies in regard to ApoE gene polymorphism with type 2 diabetes with nephropathy among Bangladeshi population

Multiple regression analyses revealed that heterozygous E3/E4 genotype played a statistically significant role in developing resistance against developing nephropathy in type 2 diabetic patients [odds ratio: 0.29 [95% CI: {0.16-0.58)}, chi square: 16.25, p <0.00]. Also, E4 allele showed resistance against developing nephropathy in type 2 diabetic patients [odds ratio: 0.31 {0.18-0.55}, chi square: 17.08, p < 0.00]. Association of any other genotypes and alleles with respect to ApoE gene polymorphism with nephropathy in type 2 diabetic patients were not observed in the present study. Data have been presented in Table 3.18.

Table 3.18. Association analyses of genotypic and allelic frequencies with type 2 diabetic nephropathy in regard to ApoE gene polymorphism among Bangladeshi population.

Genotypes	Healthy individuals	Type 2 Diabetes with nephropathy	OR	X^2	p-value
E3/E3 (ref)	58	95			
E2/E2	0	0			
E2/E3	1	2	1.22 (0.11-13.77)	0.03	0.87
E2/E4	0	0			
E3/E4	44	21	0.29 (0.16-0.58)	16.25	<0.00
E4/E4	1	4	2.44 (0.27-22.38)	0.66	0.42
Total	**104**	**122**			
Alleles					
E3 (ref)	161	213			
E2	1	2	1.51 (0.14-16.82)	0.11	0.73
E4	46	29	0.31 (0.18-0.55)	17.08	<0.00
Total	**208**	**244**			

Table 3.19 Association analyses of genotypic and allelic frequencies with type 2 diabetes without and with nephropathy in regard to ApoE gene polymorphism among Bangladeshi population.

Genotypes	Type 2 Diabetes without nephropathy	Type 2 Diabetes with nephropathy	OR	X^2	p-value
E3/E3 (ref)	91	95			
E2/E2	2	0			
E2/E3	2	2	0.95 (0.13-6.95)	0.002	0.96
E2/E4	0	0			
E3/E4	26	21	1.19 (0.62-2.26)	0.27	0.60
E4/E4	2	4	1.92 (0.34-10.72)	0.57	0.45
Total	**123**	**122**			
Alleles					
E3 (ref)	210	213			
E2	6	2	0.33 (0.07-1.65)	2.02	0.16
E4	30	29	1.02 (0.59-1.76)	0.005	0.94
Total	**246**	**244**			

We also wanted to analyze the fact that whether the pattern of genotypic and allelic frequencies with regard to ApoE gene polymorphism among type 2 diabetes had any association with the risk of developing nephropathy. We found that neither distribution patterns of genotypic nor allelic frequencies of type 2 diabetes were associated with the risk of nephropathy. The data have been presented in Table 3.19.

3.15. Genotype distribution of ApoE according to clinical parameters among healthy participants

No E2/E2, E2/E4 and E4/E4 genotypes were found with respect to ApoE gene polymorphism in healthy study participants. So, we did not consider distribution pattern of clinical parameters for these genotypes. E2/E3 genotype was recognized only in one sample. So we could not calculate standard deviation (SD) for this genotype.

3.15.1. Albumin creatinine ratio

Average albumin creatinine ratio (ACR) of healthy study participants carrying E3/E3 and E3/E4 genotypes were 8.75 ± 4.04 mg/g And 8.53 ± 3.44 mg/g, respectively. Only one healthy study participant was found to have E2/E3 genotype with ACR value of 20.2 mg/g (so standard deviation could not be calculated). All healthy study participants had normal ACR value (<30mg/g). Data is shown in Figure 3.3.

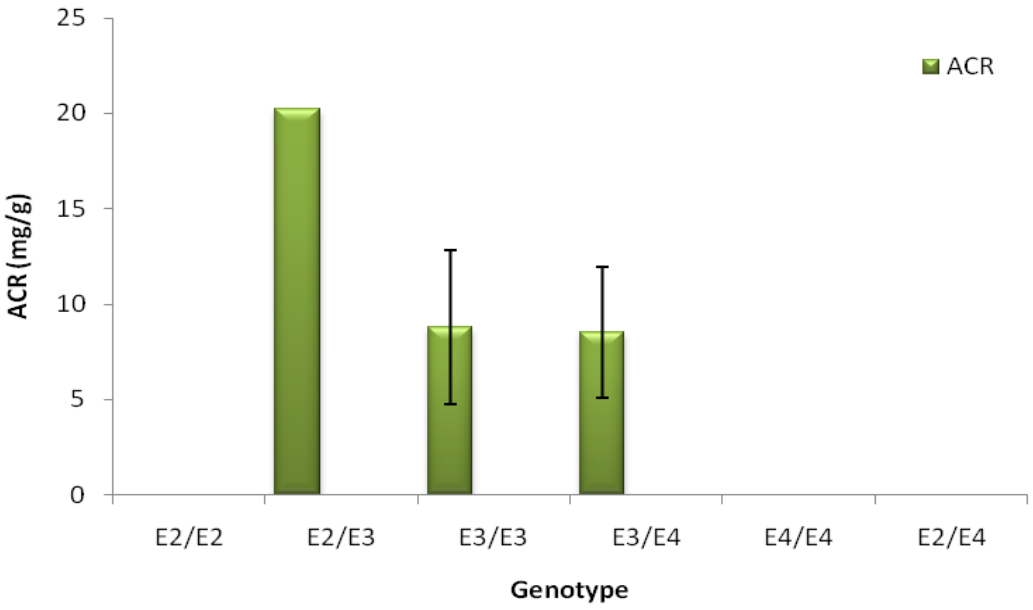

Figure 3.3: Graphical representation of distribution of ACR with genotypes regarding ApoE among healthy study participants. (Normal: < 30 mg/g; microalbuminurea 30-300 mg/g, macroalbuminurea > 300 mg/g).

3.15.2. Fasting plasma glucose and HbA1c

Average fasting plasma glucose of E3/E3 and E3/E4 genotypes were 5.08± 0.36 mmol/L and 5.07 ± 0.29 mmol/L, respectively. Also HbA1c of healthy participants with E3/E3 and E3/E4 genotypes were 5.54 ± 0.34 % and 5.4 ± 0.29%, respectively. Only participant with E2/E3 genotype had fasting plasma glucose level 5.20 mmol/L and HbA1c 5.70%. All of them had fasting plasma glucose and HbA1c level within the normal range (fasting blood glucose 3.9-6.1 mmol/L and HbA1c level 4.3-6.0%). Data is shown in Figure 3.4.

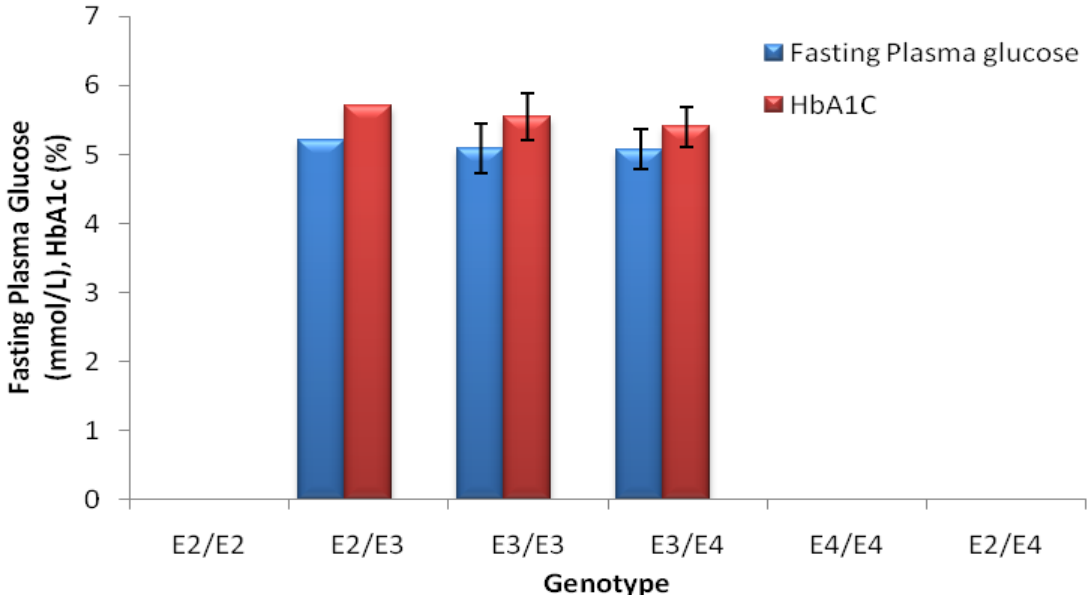

Figure 3.4: Graphical representation of distribution of Fasting plasma glucose and HbA1c with genotypes regarding ApoE among healthy study participants.

3.15.3. Lipid profiles

Healthy participants with E3/E3 and E3/E4 genotypes had average cholesterol levels of 181.5 ± 33.5 mg/dL and 177.0 ± 35.9 mg/dL, respectively which is normal (<200 mg/dL) to upper limit of border line (200-240 mg/dL).

Their mean level of triglyceride was 169.0 ± 70.2 mg/dL and 189.0 ± 61.9 mg/dL respectively. E3/E3 genotype had average triglyceride level (169.0 ± 70.2 mg/dL) normal to upper limit of border line whereas E3/E4 had average triglyceride level (189.0 ± 61.9 mg/dL) normal to high risk level (>240mg/dL).

E3/E3 and E3/E4 participants also had average HDL level (41.3 ± 9.26 mg/dL and 37.6 ± 11.9 mg/dL) normal (35-60 mg/dL) to border line risk (<35 mg/dL). Although, their average LDL level was normal (106.3 ± 30.3 and 101.6 ± 34.3 respectively).

Only participant with E2/E3 genotype had normal levels of cholesterol (183 mg/dL), triglyceride (157 mg/dL), HDL (49 mg/dL) and LDL (102 mg/dL) as shown in Figure 3.3.

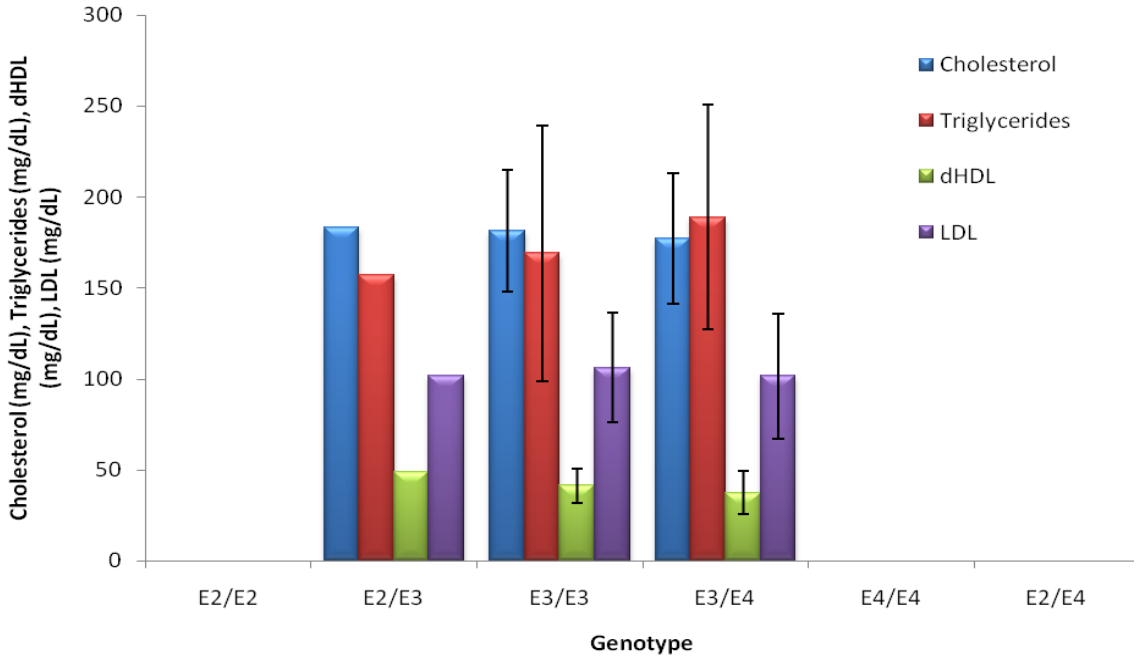

Figure 3.5: Graphical representation of distribution of cholesterol, triglycerides, HDL and LDL among various genotypes of ApoE in healthy participants.

3.16. Genotype distribution of ApoE according to clinical parameters among diabetic patients without nephropathy

No E2/E4 genotype regarding ApoE was found among diabetic non-nephropathy study participants. So distribution pattern was not generated for this particular genotype. Also only one participant was recognized with E2/E3 genotype, so standard deviation could not be calculated for E2/E3 genotype with diabetic non-nephropathy.

3.16.1. Albumin creatinine ratio

No ApoE E2/E4 genotype was found in type 2 diabetic patients without nephropathy and E2/E3 was recognized in only one individual who had ACR value of 6.6 mg/g. E2/E2, E3/E3, E3/E4 and E4/E4 genotype carrying participants had average ACR value of 12.1 ± 0.75 mg/g, 10.24 ± 5.90 mg/g, 9.11 ± 1.09 mg/g, 11.1 ± 1.09 mg/g (Figure 3.6). All of them had ACR value within normal range.

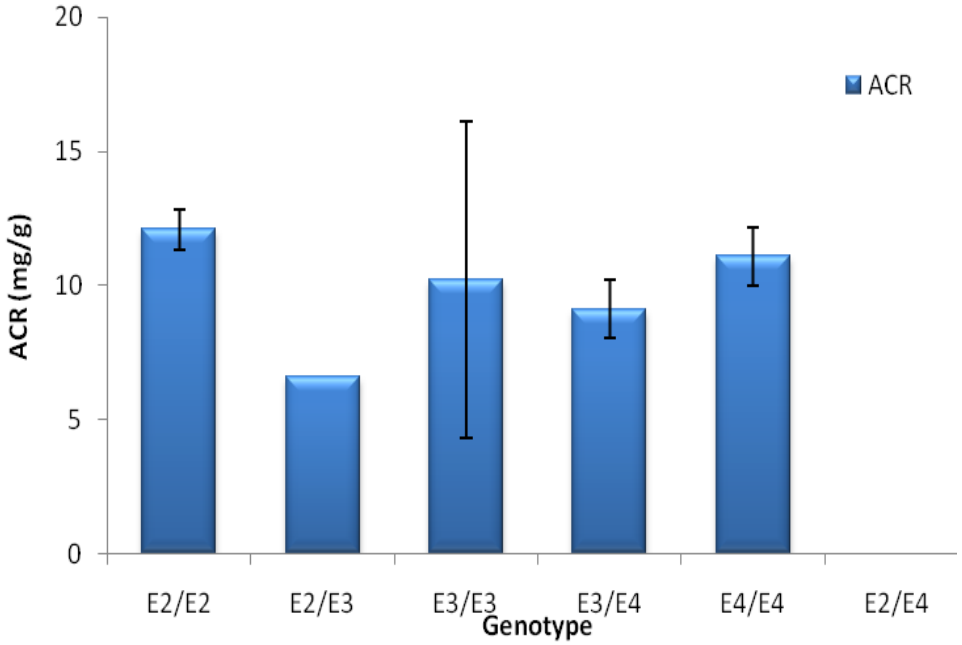

Figure 3.6: Graphical representation of distribution of ACR among different ApoE genotypes in type 2 diabetic patients without nephropathy.

3.16.2. Fasting plasma glucose and HbA1c

E2/E2, E3/E3, E3/E4 and E4/E4 genotype in type 2 diabetic patients without nephropathy had average fasting plasma glucose levels of 10.4 ± 3.67 mmol/L, 9.4 ± 3.18, 8.45 ± 1.86 and 9.40 ± 3.39 mmol/L, respectively which were higher than normal. Also, their HbA1c levels were higher than normal (8.85 ± 0.35, 8.59 ± 1.57, 8.10 ± 1.25 and 9.65 ± 1.62 respectively). E2/E3 genotype in this group of patients also had higher fasting blood glucose (7.8 mmol/L) and HbA1c (8.2%), levels than normal (Figure 3.7).

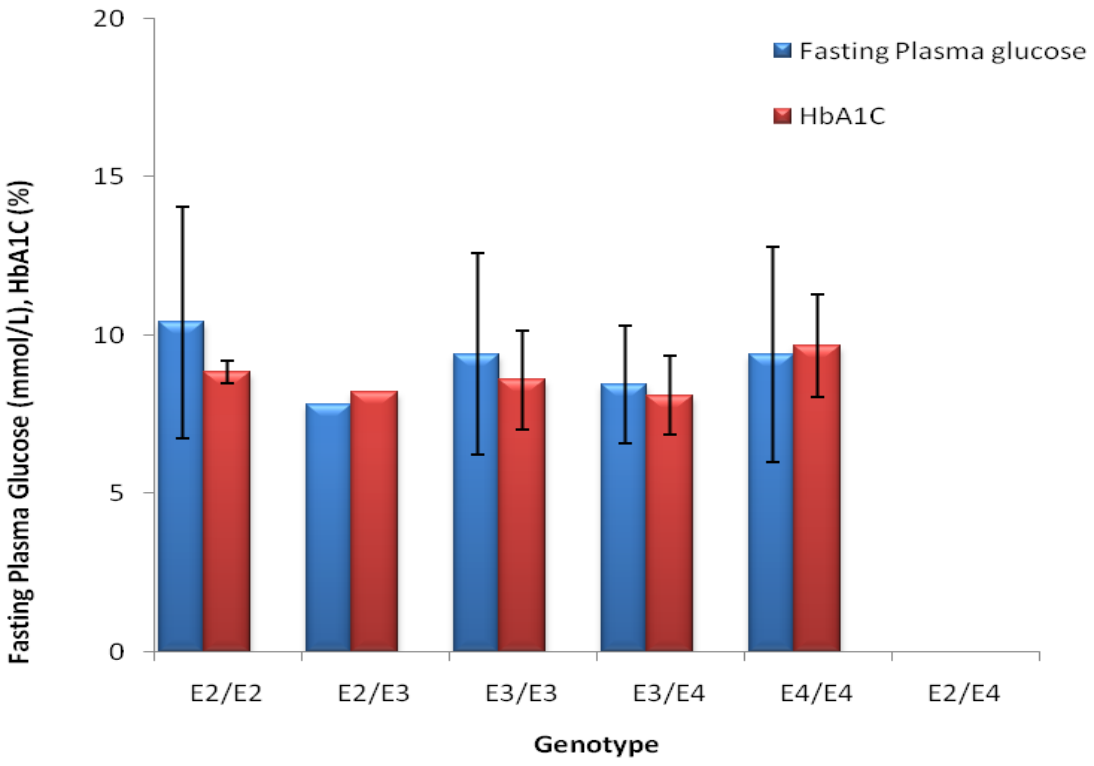

Figure 3.7: Graphical representation of distribution of genotypes regarding ApoE with fasting plasma glucose and HbA1c in type 2 diabetic patients without nephropathy.

3.16.3. Lipid profile

Figure 3.8 indicates that in type 2 diabetic patients without nephropathy, E2/E2 and E3/E4 genotype participants had normal to upper limit of border line cholesterol and TG level while they had normal levels of HDL and LDL. E3/E3 and E4/E4 had normal cholesterol and LDL level but their levels of triglycerides and HDL varied from normal to upper limit of border line. Only participant with E2/E3 genotype had higher level of cholesterol, very high level of TG and very low level of HDL.

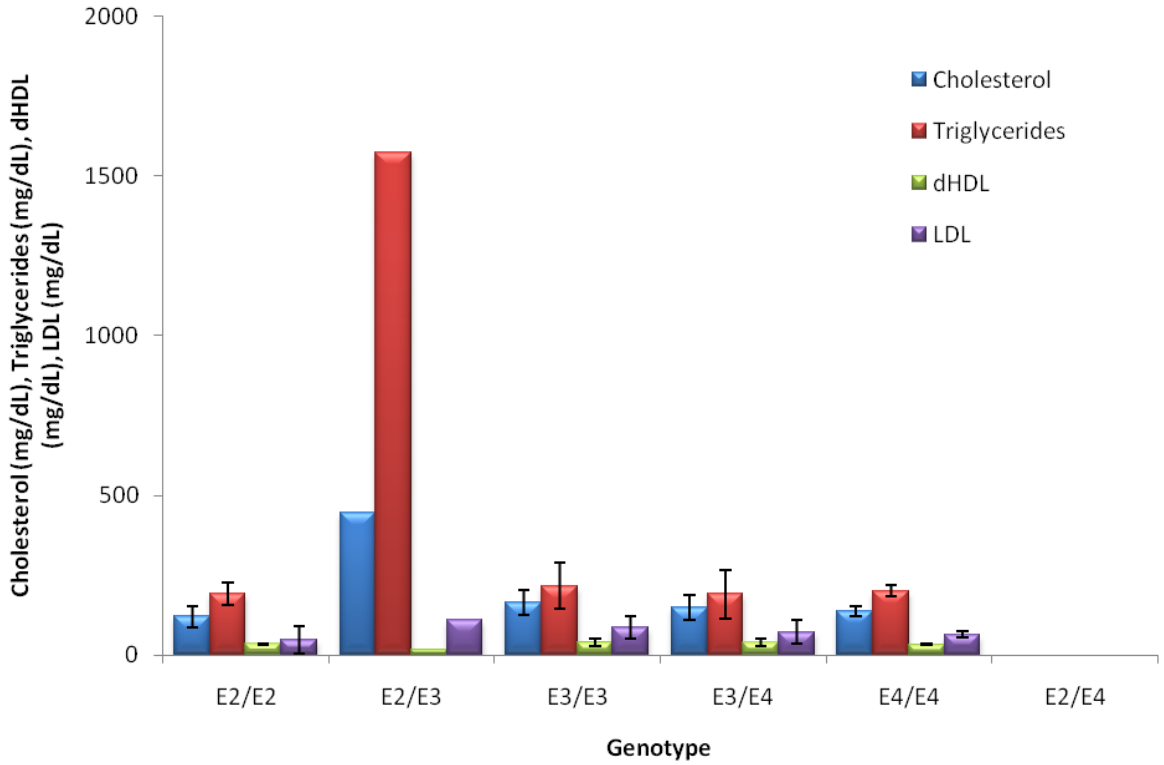

Figure 3.8: Graphical representation of various Apo E genotypes with cholesterol, triglycerides, HDL and LDL in type 2 diabetic patients without nephropathy.

3.17. Genotype distribution with clinical data among diabetic patients with nephropathy

In this study, no E2/E2 and E2/E4 genotypes were found with respect ApoE gene.

3.17.1. Albumin creatinine ratio

Among the diabetic nephropathy patients, no E2/E2 and E2/E4 genotypes were found. ACR of E2/E3, E3/E3, E3/E4 and E4/E4 were 771.1 ±10.2 mg/g, 322.03 ± 92.17 mg/g, 251.30 ± 86.63 mg/g and 429.4 ± 14.7 mg/g respectively. These four genotypes showed significantly high value of ACR. Among them, E2/E3, E3/E3 and E4/E4 genotypes had mocroalbuminurea or overt proteinurea, whereas E3/E4 genotype had microalbuminurea. Figure 3.9 is showing the respective data.

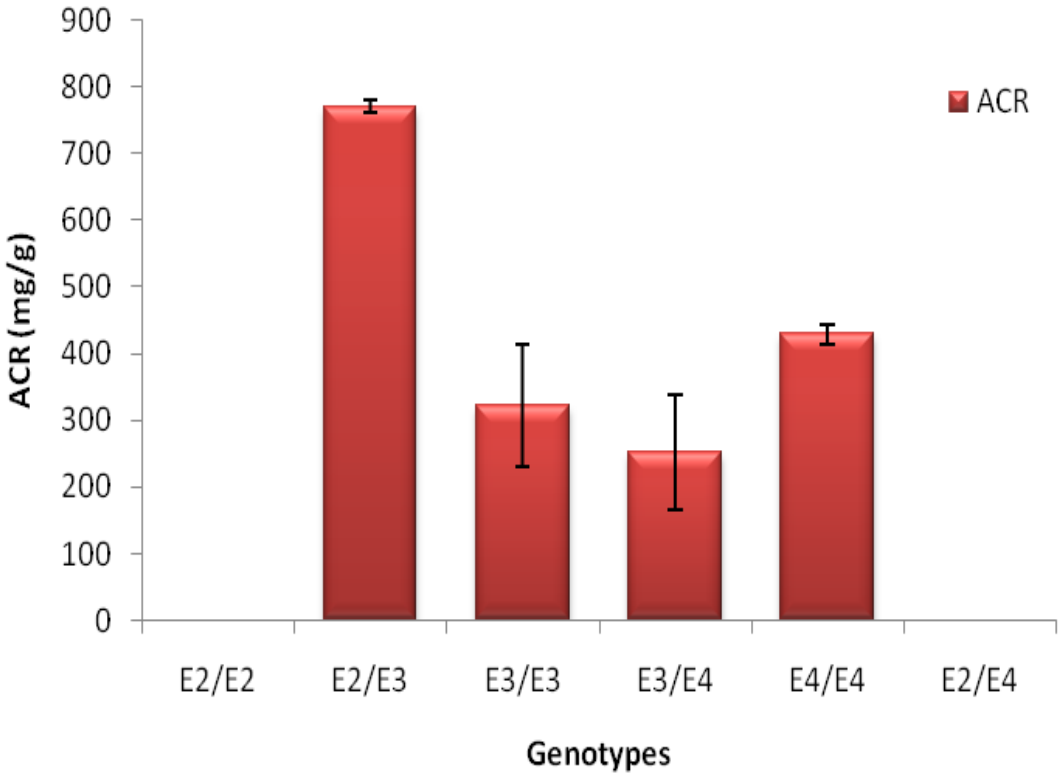

Figure 3.9: Distribution of albumin creatinine ratio among different genotypes in diabetic nephropathy patients.

3.17.2. Fasting plasma glucose and HbA1c

Fasting plasma glucose level of E2/E3, E3/E3, E3/E4 and E4/E4 diabetic nephropathy participants were 10.2 ± 4.66 mg/g, 10.26 ± 3.32 mg/g, 10.58 ± 3.61 mg/g and 16.5 ± 5.03 mg/g. All of them had plasma glucose level higher than normal, among them E4/E4 genotype individuals have notably higher fasting plasma glucose than other three genotypes (Figure 3.10). HbA1c of E2/E3, E3/E3, E3/E4 and E4/E4 9.8 ± 4.38 %, 9.34 ± 1.85 %, 9.28 ± 1.20% and 10.7 ± 0.91% which is also higher than normal. Figure 3.10 demonstrates the distribution fasting plasma glucose as well as HbA1c in diabetic nephropathy patients.

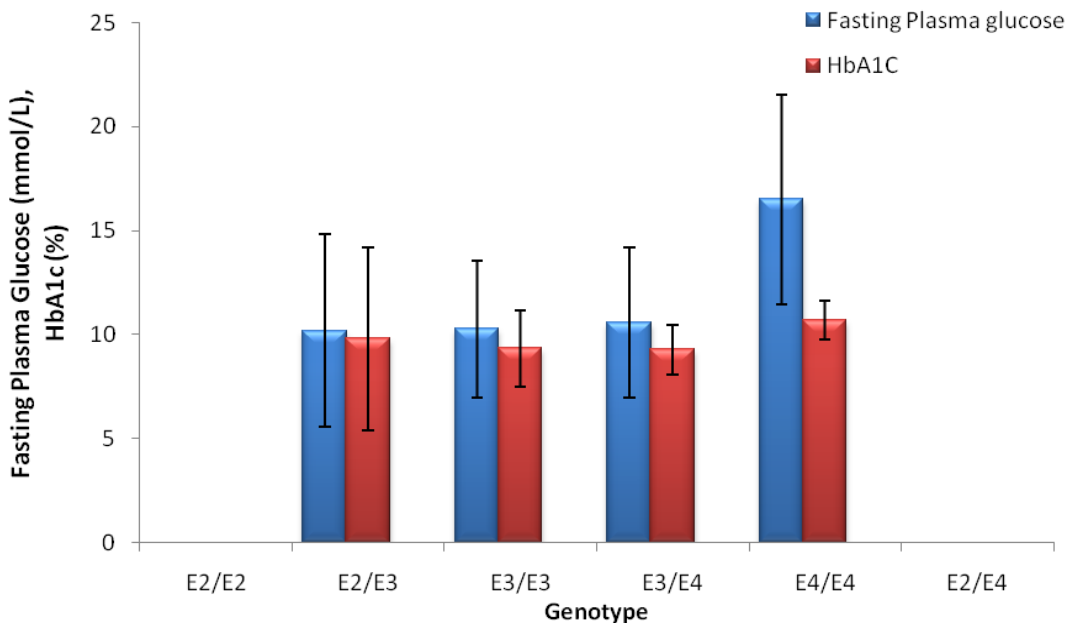

Figure 3.10: Representation of Association of genotype with fasting plasma glucose and HbA1c among diabetic nephropathy patients.

3.17.3. Lipid profile

From the Figure 3.11 it is found that E2/E3 diabetic nephropathy participants had normal levels of cholesterol (150 ± 38.2 mg/dL), triglycerides (127 ± 18.3 mg/dL), HDL (39.0 ± 4.24 mg/dL) and LDL (85.6 ± 38.0 mg/dL). Both E3/E3 and E3/E4 genotypes had normal Cholesterol and LDL level. Their levels of triglycerides and HDL is normal to upper limit of border line. Level of cholesterol in E4/E4 genotypes varied from normal to upper limit of border line (210 ± 56.9 mg/dL), while triglyceride level was significantly high (642 ± 57.2 mg/dL) but had normal HDL (36.5 ± 3.0 mg/dL) and LDL levels (97.7 ± 49 mg/dL).

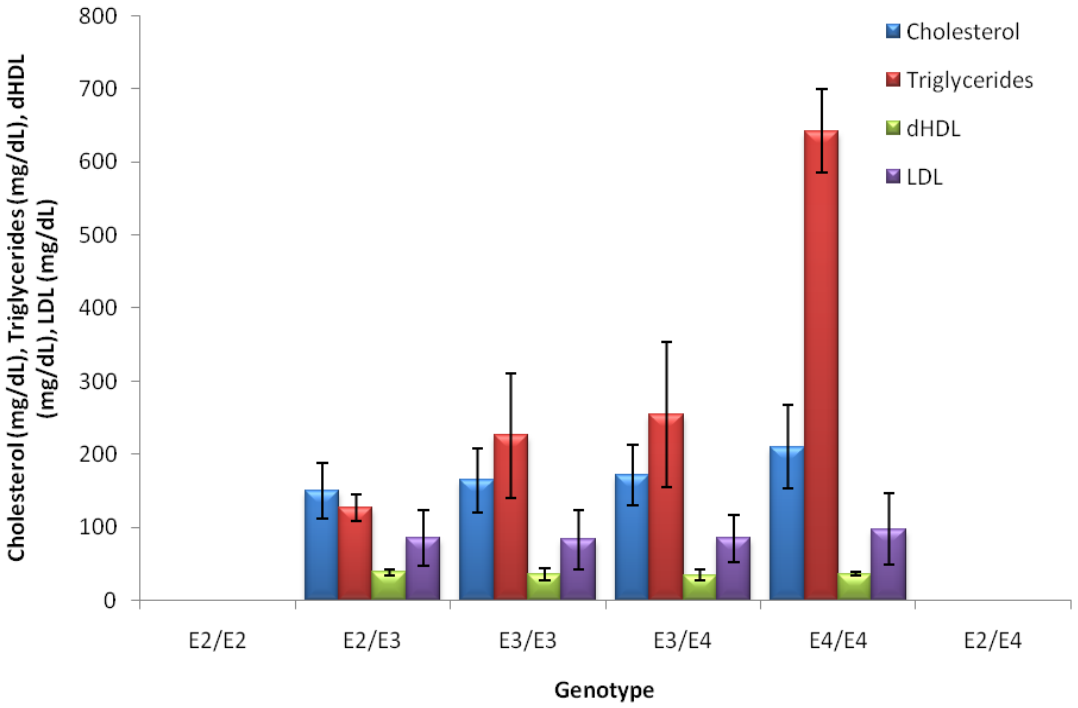

Figure 3.11: Graphical representation of distribution of the levels of cholesterol, triglycerides, HDL and LDL among diabetic nephropathy participants according to different genotypes of ApoE gene.

3.18. Evaluation of allele discrimination plot for genotyping with respect to rs121912717 in ApoA1 gene

An allelic discrimination plot, also known as a "cluster plot" or an "AD plot" shows three clusters, and near the origin, the No Template Control (NTC) (figure 3.14). Lower right corner of the plot shows allele 1 homozygous or wild homozygous (red) labeled with VIC® dye, upper left corner of the plot shows allele 2 homozygous or mutant homozygous (blue) labeled with FAM™ approximately midway between the homozygous allele 1 and homozygous allele 2 clusters, heterozygous clusters (green) are plotted. The points in each cluster are grouped closely together,

and each cluster is well separated from the other clusters. Bottom left corner of the plot has negative control (without template, black cross). Figure 3.12 represents the allelic discrimination plot.

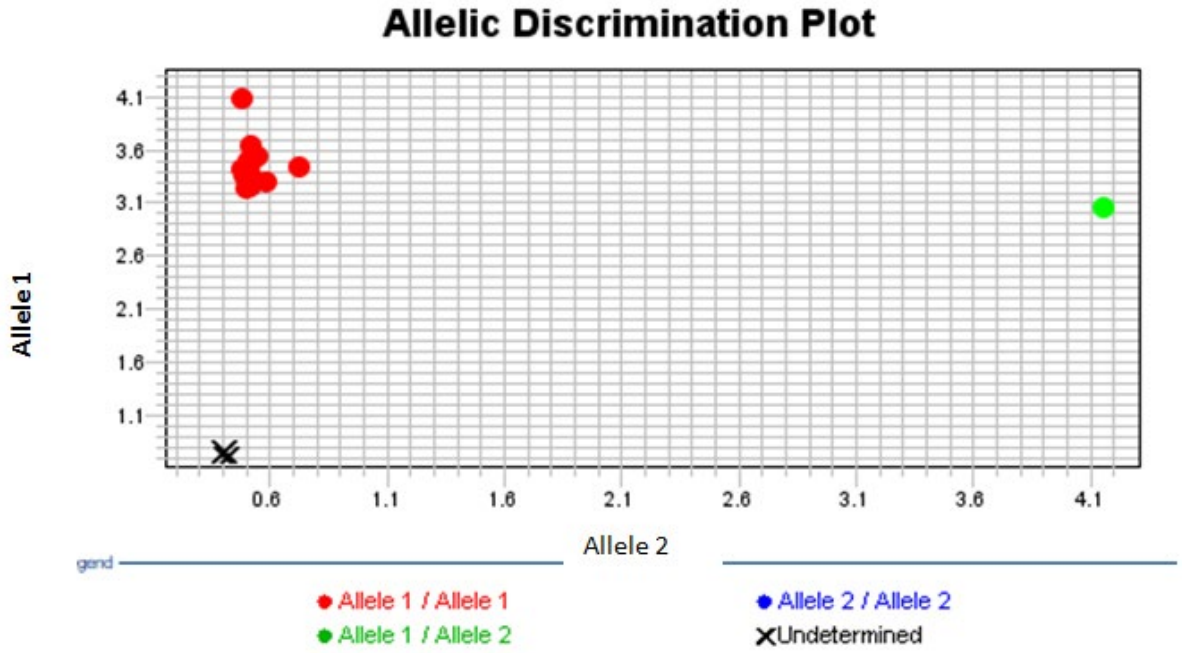

Figure 3.12: Representation of the allelic discrimination plot for the determination of homozygous, heterozygous and mutant homozygous genotypes in the study participants with regard to rs121912717 polymorphism of ApoA1 gene.

3.19. Determination of genotype frequency distribution of ApoA1 with respect to rs121912717 in healthy participants, diabetic patients and diabetic patients with nephropathy

From the allele discrimination plot, genotype distribution of rs121912717 of ApoA1 gene was determined in study participants. There were two type of genotype found; wild type homozygous GG genotype and heterozygous GT genotype in the study participants (104 healthy individuals, 123 type 2 diabetic patients without nephropathy and 122 type 2 diabetic with nephropathy). However, no mutant TT homozygous genotype was identified in total 349 study participants.

Table 3.20. Genotype distribution with respect to rs121912717 of ApoA1 gene.

rs121912717	GG	GT	TT
Healthy individuals (104)	99 (95.2%)	5 (4.8%)	0
Type 2 diabetes without nephropathy (123)	119 (96.7%)	4 (4.3%)	0
Type 2 diabetes with nephropathy (122)	117 (95.9%)	5 (4.1%)	0

Among healthy individuals, type 2 diabetic patients without nephropathy and type 2 diabetic patients with nephropathy, GG genotype frequencies was 95.2%, 96.7% and 95.9% respectively as well as GT genotype frequencies were 4.8%, 4.3% and 4.1% respectively. From Table 3.20 it can be shown that both genotypes were evenly distributed among all three categories of study participants.

3.20. Determination of association of genotype frequencies with respect to rs121912717 within ApoA1 gene with type 2 diabetes without and with nephropathy.

Logistic regression analyses were performed to determine the association of genotypic and allelic frequencies with type 2 diabetes without and with nephropathy with respect to rs121912717 within ApoA1 gene

3.20.1. Association of genotype frequencies with respect to rs121912717 with type 2 diabetes without nephropathy

In this case control study, 104 healthy individuals and 123 patients with diabetes non-nephropathy participated. Among the case group, 4 individuals had heterozygous GT genotype and 119 had homozygous GG genotype.

In control group, among 104 healthy individuals, 5 individuals were identified with heterozygous GT genotype regarding rs121912717, the rest carried wild type GG genotype. No mutant homozygous TT genotype was found in case or control group.

Table 21. Association of genotype frequencies with regard to rs121912717 of ApoA1 gene with the probable risk of diabetes in Bangladeshi population.

Parameters		T2D, n (%)	Control, n (%)	OR (95% CI Lowest-Highest)	χ^2	p
Genotype	GG/GT/TT	119/4/0	99/5/0	-	-	-
Alleles	G/T	242/4 (98.37/1.63)	203/5 (97.60/2.40)	0.33 (0.09-1.24)	2.95	0.085
Co-dominant model	GG	119 (96.7)	99 (95.2)	1	-	-
	GT	4 (4.3)	5 (4.8)	1.02 (0.39-5.74)	0.36	0.55
	TT	0	0	--	--	--
Dominant model	TT	0	0	1	-	-
	GT+GG	123 (100)	104 (100)	--	--	--
Recessive	TT+GT	4	5	1	-	-
	GG	119	99	0.66 (0.17-2.54)	0.35	0.55
Over dominant	GG+TT	119	99	1	-	-
	GT	4	5	1.02 (0.39-5.74)	0.36	0.55

Wild type G allele frequencies among diabetic individuals and healthy individuals were 98.37% and 97.60%. Also mutant T allele frequencies were 1.63% and 2.40%, respectively. Overall, wild type G allele and mutant T allele frequencies were evenly distributed among case and control group.

From the Table 3.21, no significant allelic association was found with the disease. Odds ratio for both co-dominant and over dominant model was 1.02 which indicates association of the model with the risk of disease. But it was not insignificant ($p>0.005$). Recessive model showed disease protective association with odds ratio of 0.66 but it is also not significant ($p>0.005$). Dominant model could not be generated as there was no mutant TT genotype found.

3.20.2. Association of genotype frequencies with respect to rs121912717 with type 2 diabetes with nephropathy

Among the total of 104 of healthy individuals 99 had wild type GG and 5 had heterozygous GT genotypes while 122 diabetic patients with nephropathy, 117 patients had wild type GG genotype and 5 had GT genotype. This study identified no TT genotype among case and control group.

Table 3.22. Association of genotype frequencies with regard to rs121912717 of ApoA1 gene with the probable risk of diabetic nephropathy in Bangladeshi population

Parameters		T2D+DN, n (%)	Control, n (%)	OR (95% CI Lowest-Highest)	χ^2	p
Genotype	GG/GT/TT	117/5/0	99/5/0	-	-	-
Alleles	G/T	239/5 (97.95/2.05)	203/5 (97.60/2.40)	0.84 (0.24-2.97)	0.06	0.79
Co-dominant model	GG	117 (95.9)	99 (95.2)	1	-	-
	GT	5 (4.1)	5 (4.8)	0.84 (0.24-3.01)	0.07	0.79
	TT	0	0	--	--	--
Dominant model	TT	0	0	1	-	-
	GT+GG	122 (100)	104 (100)	--	--	--
Recessive	TT+GT	5	5	1	-	-
	GG	117	99	1.18 (0.33-4.20)	0.06	0.79
Over dominant	GG+TT	117	99	1	-	-
	GT	5	5	0.84 (0.24-3.01)	0.07	0.79

Wild type G allele frequency among healthy individuals and diabetic nephropathy patients were 97.95% and 97.60%, mutant T allele frequency were 2.05% and 2.40% respectively. So both allele were evenly distributed. From the Table 3.22, no significant allelic association was found regarding diabetic nephropathy.

Recessive model has odds ratio of 1.18 which indicates that rs121912717 may increase the risk of diabetic nephropathy, but this finding is not significant ($p>0.005$). Though co-dominant and over- dominant model showed protective association but it is also insignificant. Association study could not be conducted for dominant model as no mutant TT genotype was found.

3.21. Genotype distribution of ApoA1 according to clinical parameters among healthy participants

No mutant TT homozygous genotype regarding apoA1 among healthy individuals was found in this study. Heterozygous GT genotype was recognized in only one sample, so standard deviation could not be calculated.

3.21.1. Albumin creatinine ratio

Figure 3.13 shows that the average ACR of healthy study participants carrying GG (Wild type homozygous) genotype was 8.87 ± 4.12 mg/g. Only one healthy participant was found to have GT (Mutant heterozygous) genotype with ACR value of 6.83 mg/g (so standard deviation could not be calculated). All healthy study participants had normal ACR value (<30mg/g). No healthy individuals were found to have TT (Mutant homozygous) genotype.

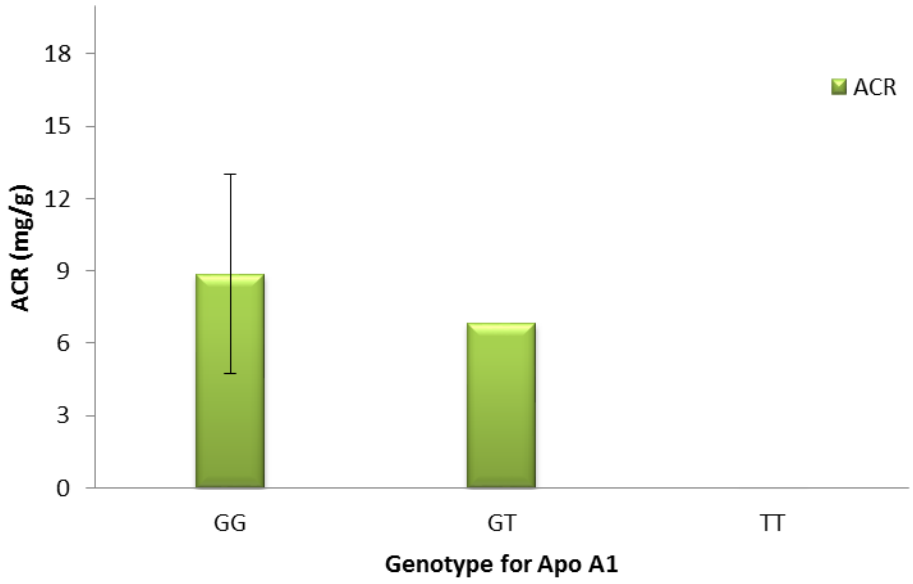

Figure 3.13: Distribution of the levels of ACR in various genotypes with regard to rs121912717 within ApoA1 gene in among healthy study participants.

3.21.2. Fasting plasma glucose and HbA1c

Average fasting plasma glucose for GG genotype was 5.07 ± 0.34 mmol/L and level of HbA1c of this group was 5.54 ± 0.33 %. Only one participant with GT genotype had fasting plasma glucose level 5.40 mmol/L and HbA1c 5.9%. All of them had fasting plasma glucose and HbA1c level within the normal range. The data is shown in Figure 3.14.

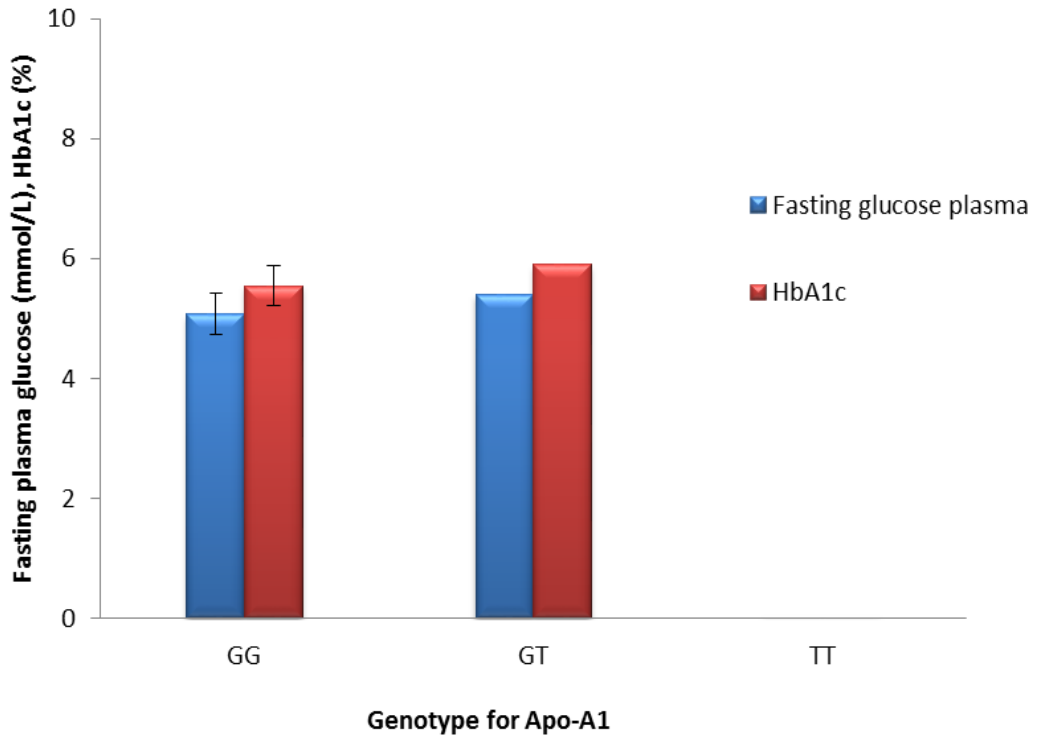

Figure 3.14: Distribution of various genotypes of ApoA1 gene with fasting Glucose and HbA1c among healthy study participants.

3.21.3. Lipid profile

Healthy participants with GG genotype had average cholesterol 180 ± 33.9 mg/dL which was close to upper limit of the normal value. The average triglyceride level of this genotype was 170.1 ± 63.2 mg/dL that is higher than the normal level. Average HDL value was 40.5 ± 10.1 40 mg/dL). Also, the average LDL level of GG genotype was normal (105.4 ± 31.3 mg/dL). The genotype GT had only in one subject and it's cholesterol, triglycerides, HDL and LDL value

were 170 mg/dL, 195 mg/dL, 34 mg/dL and 97 mg/dL, respectively. The data are presented in Figure 3.15.

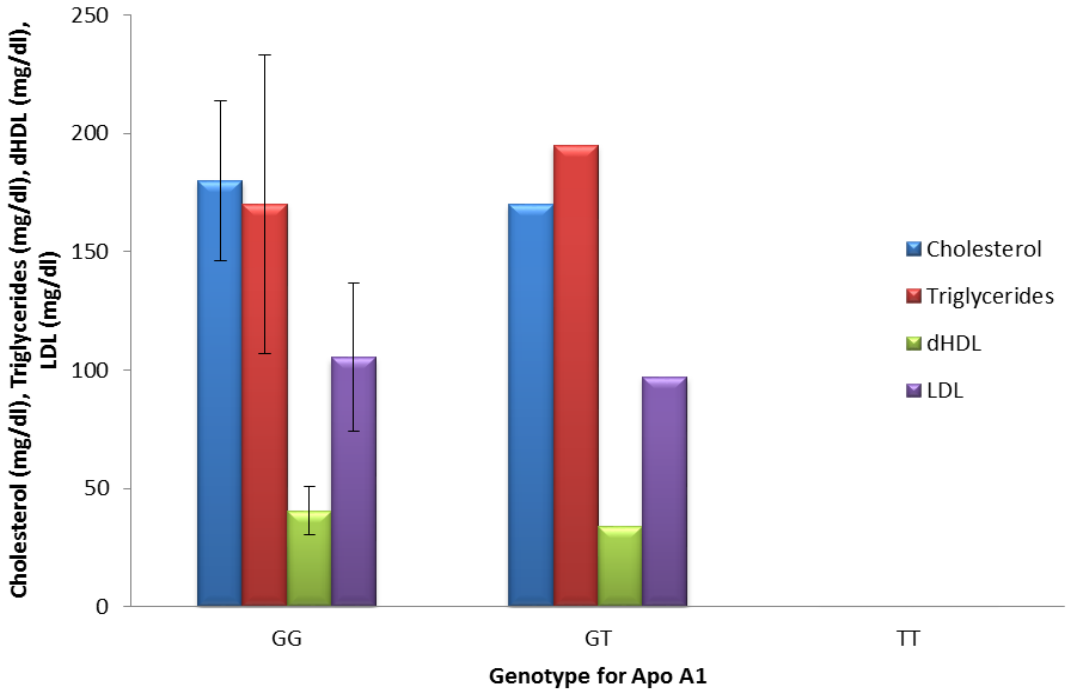

Figure 3.15: Association of various genotypes of ApoA1 gene with Cholesterol, Triglycerides, HDL and LDL among healthy participants.

3.22. Genotype distribution of ApoA1 according to clinical parameters among diabetic non-nephropathy patients

No mutant TT homozygous genotype regarding apoA1 among diabetic patients without nephropathy was found in this study. Genotype distribution with ACR, fasting plasma glucose, HbA1c and lipid profile was calculated for wild type homozygous GG genotype and heterozygous GT genotype.

3.22.1. Albumin creatinine ratio

The mean level of ACR in GG (wild type homozygous) genotype of type 2 diabetic patients was 9.86 ± 5.82 mg/g. In addition, the GT genotype carrying participants had average ACR value of 18.75 ± 4.15 mg/g (Figure 3.16). All of them had ACR within the normal range.

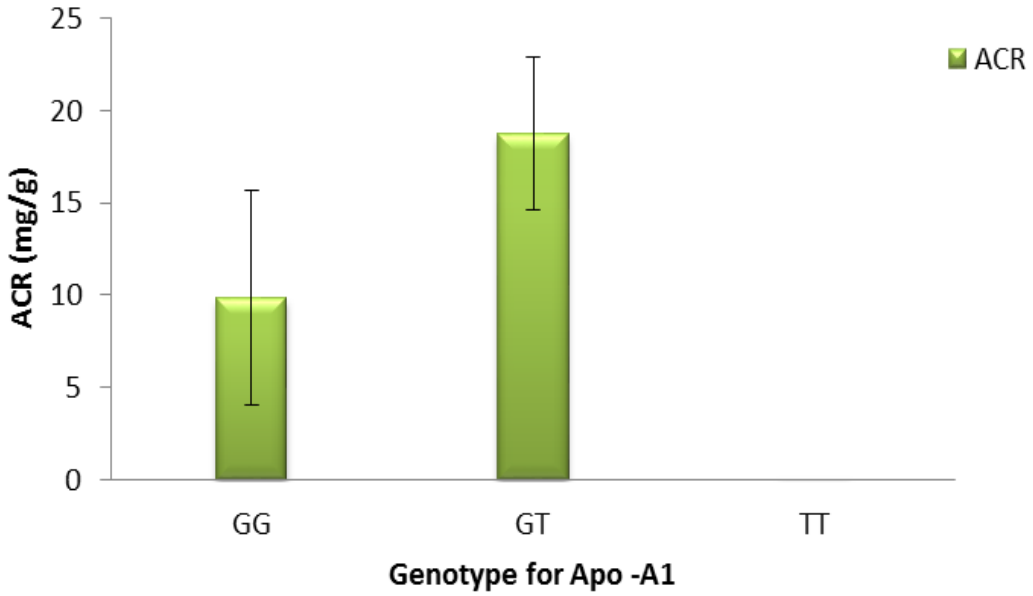

Figure 3.16: Distribution of the levels of ACR in various genotypes with regard to rs121912717 within ApoA1 gene in type 2 diabetic patients without nephropathy.

3.22.2. Fasting plasma glucose and HbA1c

The GG (wild type homozygous) and GT (mutant heterozygous) genotypes carrying participants had average fasting plasma glucose level of 9.24 ± 2.97 mmol/L, and 8.0 ± 2.4 mmol/L, respectively which were higher than normal. Also, their HbA1c level was high (8.52 ± 1.51%and 9.55 ± 3.46%, respectively) presented in Figure 3.17.

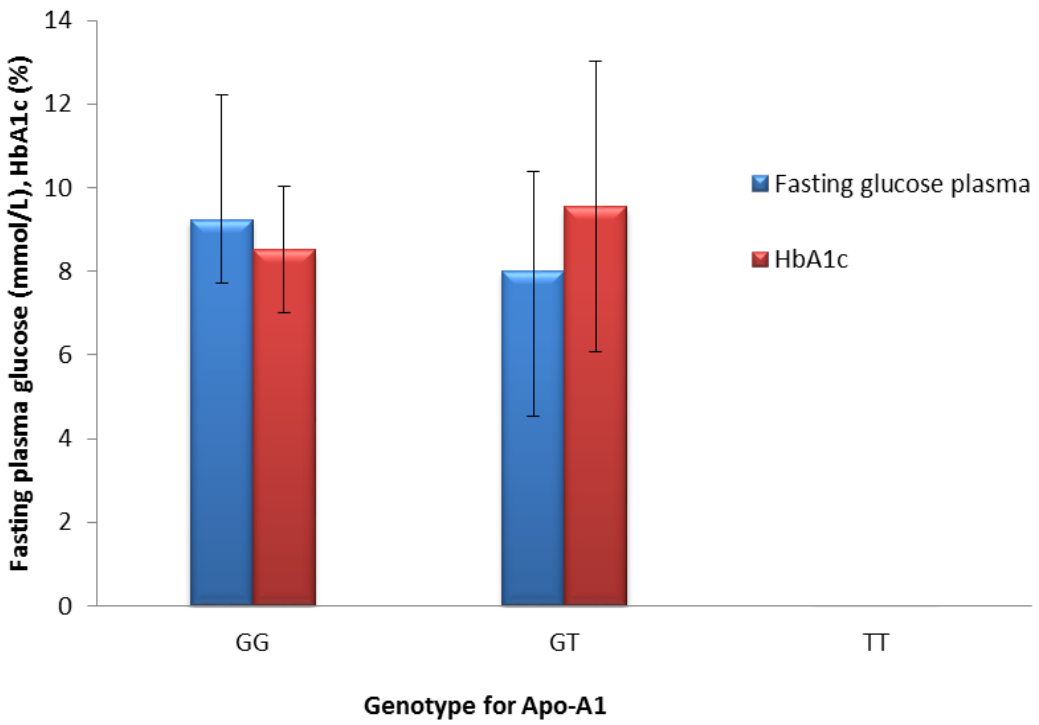

Figure 3.17: Distribution of fasting Glucose and HbA1c in various genotypes with regard to rs121912717 within ApoA1 gene in type 2 diabetic patients without nephropathy.

3.22.3. Lipid profile

Figure 3.18 stated that, among the diabetic non-nephropathy participants, GG and GT genotype participants had normal to border line high cholesterol and Triglycerides level with normal to borderline high HDL and LDL. In case of GG genotype the average value of cholesterol, triglycerides, HDL, LDL are 164.4 ± 49.1, 224.7 ± 88.8, 39.5 ± 11.2, 79.9 ± 36.1 respectively. (Figure 3.18). As well as in case of GT genotype the average level of cholesterol, triglycerides, HDL, LDL are 203 ± 59.4, 251.5 ± 91.2, 33.5 ± 2.12, 119.4 ± 39.0, respectively.

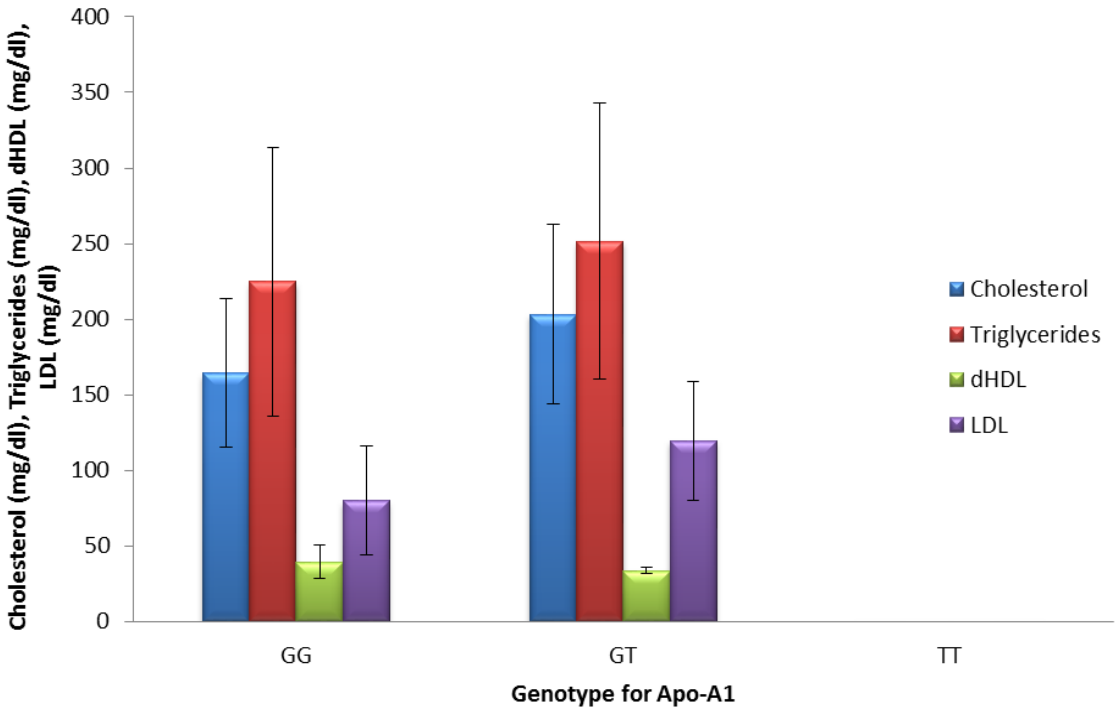

Figure 3.18: Distribution pattern of cholesterol, triglycerides, HDL and LDL in various genotypes with regard to rs121912717 within ApoA1 gene in type 2 diabetic patients without nephropathy.

3.23. Genotype distribution of ApoA1 with clinical data among diabetic nephropathy patients

No mutant TT homozygous genotype regarding ApoA1 among diabetic patients with nephropathy was found in this study. Genotype distribution with ACR, fasting plasma glucose, HbA1c and lipid profile was calculated for wild type homozygous GG genotype and heterozygous GT genotype.

3.23.1. Albumin creatinine ratio

Among the diabetic nephropathy patients, the GG (wild type homozygous) and GT (Mutant heterozygous) genotypes were found though there is no TT (mutant homozygous) genotype. The mean value of ACR for GG and GT genotypes were 319 ± 99.9 mg/g and 83.1± 45.3 mg/g,

respectively. These two genotypes showed significantly high value of ACR which is the indication of nephropathy. Figure 3.19 is showing the data.

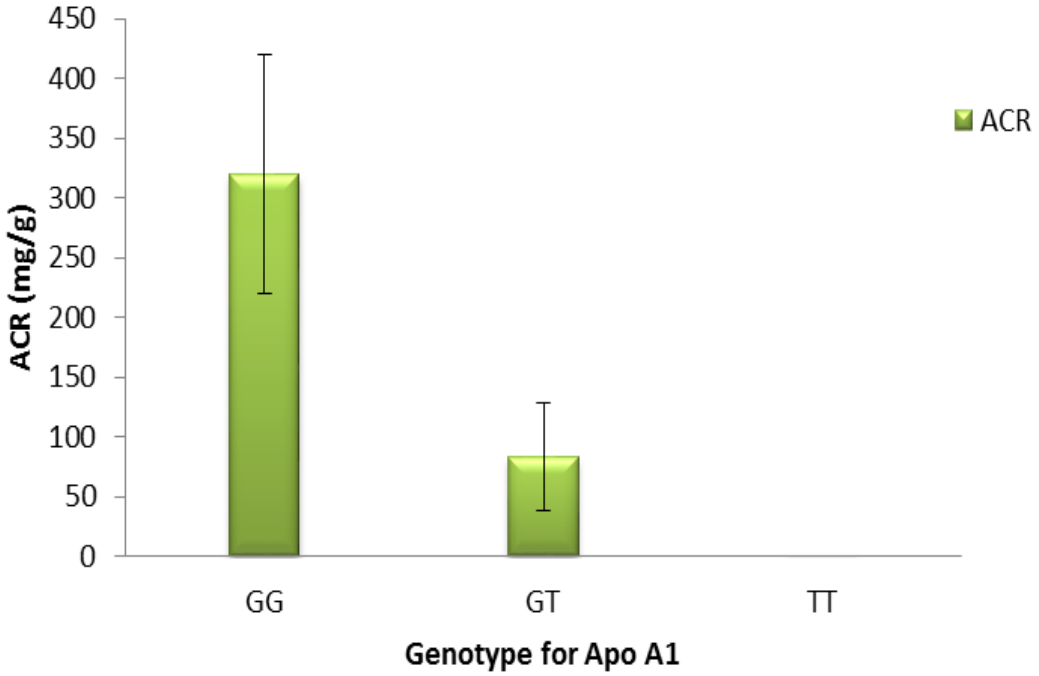

Figure 3.19: Distribution of ACR level in various genotypes with regard to rs121912717 within ApoA1 gene in type 2 diabetic patients with nephropathy.

3.23.2. Fasting plasma glucose and HbA1c

The mean level of fasting plasma glucose of GG and GT genotype with regard to rs121912717 within ApoA1 gene in type 2 diabetic patients with nephropathy were 10.7 ± 3.69 mmol/L and 9.45 ± 1.82 mmol/L respectively. All of them had plasma glucose level higher than normal. The GG genotype individuals had notably higher fasting plasma glucose. The mean level of HbA1c in GG and GT genotypes were 9.45 ± 1.82% and 8.83 ± 1.11%, respectively which is also higher than normal. Figure 3.20 represents the distribution of fasting plasma glucose and HbA1c level in various genotypes with regard to rs121912717 within ApoA1 gene in type 2 diabetic patients with nephropathy.

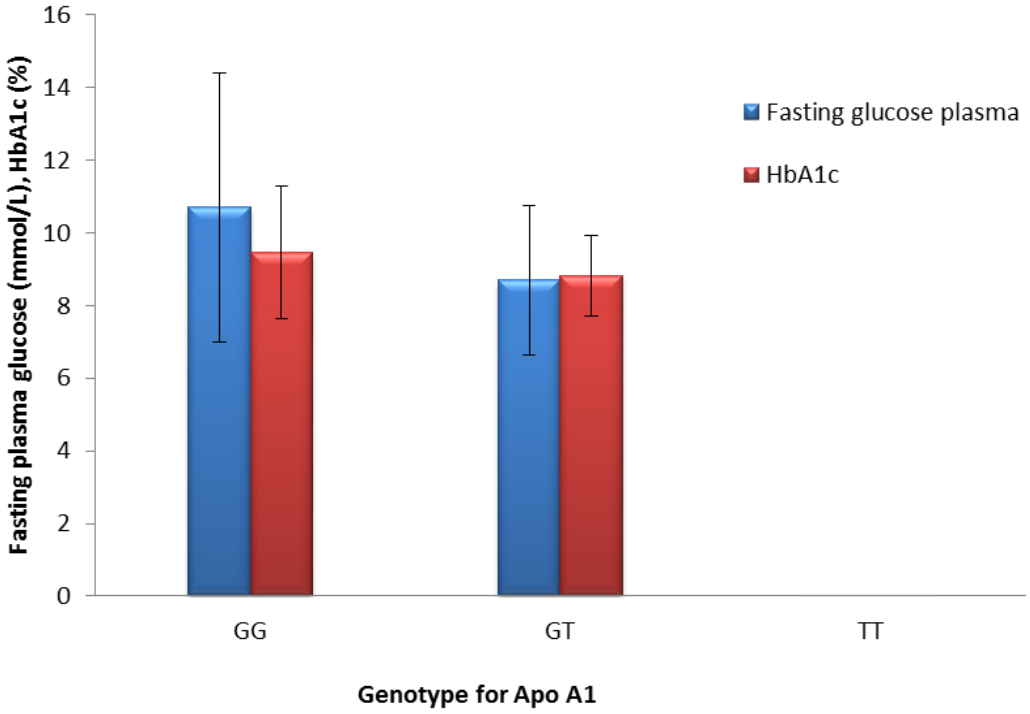

Figure 3.20: Distribution pattern fasting plasma glucose and HbA1c level in various genotypes with regard to rs121912717 within ApoA1 gene in type 2 diabetic patients with nephropathy.

3.23.3. Lipid profile

It can be concluded from the Figure 3.21 that GG (wild type homozygous) type 2 diabetic patients with nephropathy had higher levels of cholesterol (168 ± 44.6 mg/dL), TG (240.6 ± 84.02 mg/dL) than normal. Their HDL level was lower (36.04 ± 7.58 mg/dL) than expected value while level of LDL was within the normal range (84.11 ± 36.7 mg/dL). Also, GT genotypes had higher levels of cholesterol (190.3 ± 2.0 mg/dL) and triglycerides (237.3 ± 48.8 mg/dL). Their HDL level was lower than normal (37.3 ± 12.5 mg/dL) though slightly higher than GG genotypes and the mean LDL level was higher (105± 20.9 mg/dL) than normal level.

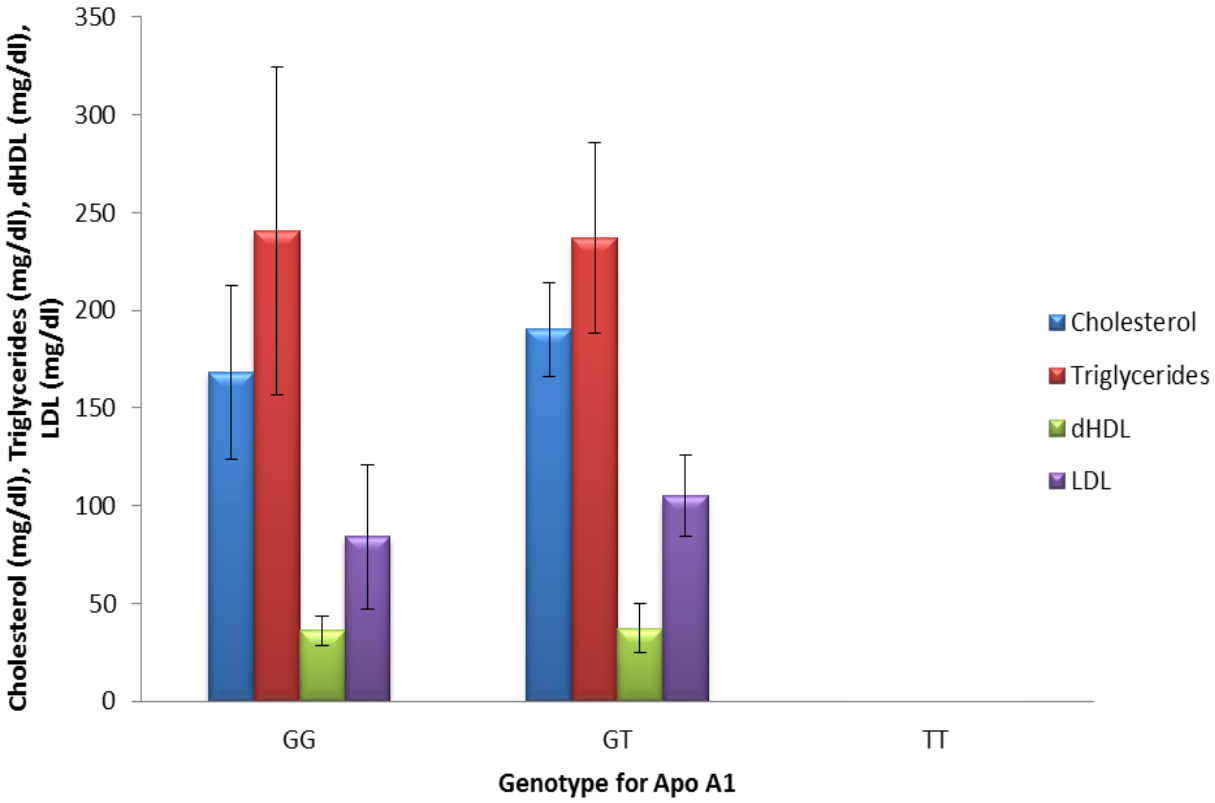

Figure 3.21: Distribution of cholesterol, triglycerides, HDL, and LDL levels in various genotypes with regard to rs121912717 within ApoA1 gene in type 2 diabetic patients with nephropathy.

4. Discussion

Diabetes is a chronic metabolic disorder which is characterized by polyphagia, polydipsia and polyurea. The complications of diabetes can damage different organs including renal lesions which is the major cause of end stage renal disease. Among microvascular complications in case of diabetic patients, nephropathy is one of the major challenges for the health care system as it is as yet poorly understood in many aspects. It is the leading cause of premature death in young diabetic patients (between 50 and 70 years old) (Rizvi et al., 2014). It is a heterogeneous and a multifactorial disease with several genes, proteins and environmental factors contributing to its risk. It is important to identify diabetic nephropathy predictors for the proper management of this disease. Thus, genetic susceptibility has been proposed as an important factor for diabetic nephropathy. Several allelic polymorphisms as well having demonstrable effects in the development and progression of the disease thus contributing to the overall risk. Genetic factors and abnormal lipid metabolism are assumed to develop and progression of diabetic nephropathy. Polymorphisms of apolipoprotein E (ApoE) and lipoprotein (a) (Apoliproprotein A1 or ApoA1) are important dyslipidemia genetic markers and the polymorphisms of these two genes are associated with the nephropathy in diabetes mellitus patients. Thus, this study aims to find out the genotypic and functional association of apolipoprotein E and lipoprotein(a) gene polymorphisms with diabetic nephropathy in Bangladeshi population and thus, to evaluate the possibility of these genes for their involvement as the independent risk factor for the development of diabetic nephropathy.

The anthropometric and biochemical data were recorded. Height, weight, BMI, sex, age and blood pressure of all study participants were recorded using a structured questionnaire. The averages of BMI of control individual, type 2 diabetic patients without and with nephropathy were 24.69 ± 2.76, 26.40 ± 3.49 and 26.67 ± 3.10, respectively. The average age, systolic blood pressure and diastolic blood pressure has been shown in Table 3.1 of three types of study participant. In case of biochemical analysis, fasting blood glucose and HbA1c was measured that facilitated the diagnosis of diabetic and non-diabetic individuals. According to the guideline of WHO, individuals having fasting glucose level >7.0 mmol/L and HbA1c level >6.5% were considered as diabetic patients while levels of fasting glucose <5.7 mmol/L and HbA1c < 6.0%

were considered as healthy individuals and the average data of all study subjects have been shown in Table 3. To define the diabetic nephropathy the urinary microalbumin was considered as > 30 mg/L with diabetic. According to ACR values, it was observed that out of total study participants with nephropathy 74.6% had microalbuminuria (range: 30.9-297.9 mg/g) while 25.4% had overt albuminuria or macroalbuminuria (range: 300.7-3442.0 mg/g).

High blood glucose level is related to the development of insulin resistance (Borai et.al, 2010). Study showed the evidence of the direct relationship between hyperglycemia and diabetic nephropathy and about 35% patients with diabetes develop nephropathy separately of hyperglycemia (Feng *et al.*, 2010). In this study, it was found that HbA1c level is directly related to the levels of insulin in plasma (Table 3.6) of patients with diabetic nephropathy i.e. with increasing concentration of HbA1c (in other words glucose), levels of insulin will be increased in plasma which is a probable indication of less utilization of insulin by the cells or development of insulin resistance in these particular group of patients rather than effects on insulin secretion. Studies also demonstrated the fact that hyperglycemia exerts an inhibitory effect on the first phase insulin secretory response (Toschi et.al., 2002; Ferner et.al., 1986). Hyperglycemia also cause impairment of lipid metabolism mainly in diabetic dyslipidemia individuals which is characterized by elevated triglycerides followed by low HDL (Parhofer, 2015). It was found that levels of HbA1c were positively correlated with the levels of cholesterol and LDL cholesterol in type 2 diabetic patients with nephropathy (as shown in Figures 3.2A and 3.2B, respectively) i.e. persistent high glucose levels in blood may increase the levels of cholesterol and LDL cholesterol, which may increase the probability of cardiovascular diseases in these patients. Moreover, in case of healthy individuals, it was observed that levels of HDL cholesterol is inversely related to that of plasma insulin (Figure 3.1) indicating increased insulin level i.e., unused insulin due to insulin resistance may hamper metabolism of good HDL cholesterol.

Apolipoprotein E, a 299-amino acid glycoprotein, is involved in the metabolism of lipoproteins. ApoE exists in three common isoforms i.e., E2, E3, and E4, which are encoded by three alleles in exon 4 of the ApoE (Mahley, 1988). The E2 allele was shown to be associated with higher triglyceride levels, while the others have been associated with elevated plasma cholesterol levels and a higher frequency of cardiovascular and Alzheimer's disease (Reilly et al, 1991;

Bickeboller et al, 1997). Out of all genotypes identified in type 2 diabetic patients with nephropathy, E2/E3 genotype had higher level of albumin creatinine ratio compared to others (Figure 3.9). Distribution of biochemical parameters among the study participants revealed that the levels of fasting plasma glucose (Figure 3.10), triglycerides (Figure 3.11) are higher in diabetic patients with nephropathy compared to other genotypes of this group of patients as well as even when compared with the same genotype of other group of participants i.e., healthy individuals and type 2 diabetic patients without nephropathy.

Eto et al (1998) reported that the E2 allele frequency was significantly higher in type 2 diabetic patients with nephropathy (7.2%) and with renal failure (9.7%) than in diabetic patients without nephropathy (2.6%). A polymorphism in the ApoE has been linked to the development of diabetic nephropathy (Eto et al., 1995; Shcherbak, 2001). In the present study (Table 3.17) we found that heterozygous E3/E4 genotype and E4 allele played a statistically significant role in developing resistance against type 2 diabetes with and without nephropathy in Bangladeshi population (Odds ratio: 0.38, chi square: 10.96, $p < 0.00$ and odds ratio: 4.6, chi square: 7.44, $p = 0.006$). Lin et al (2014) demonstrated that ApoE2 allele is more likely to increase the risk of diabetic nephropathy, while ApoE4 allele is not associated with the diabetic nephropathy development and susceptibility in an East Asian population. Our findings matched with the report of Kimura et al (1998) who demonstrated that the presence of the apolipoprotein E4 allele is a protective factor, and other alleles entail risk factors (Kimura et al, 1998). Conversely, there are conflicting results regarding the impact of allele E2 and E4 on the development of diabetic nephropathy. According to Ma et al (2008) the ApoE2 allele did not appear to be associated with increased risk of renal impairment in Chinese type 2 diabetic patients while another study indicated that the E4 allele of the ApoE polymorphism is one of the prognostic risk factors involved in the development of diabetic nephropathy with type 2 diabetes mellitus (Ilhan et al, 2007). From genotypic analyses and distribution pattern of lipid profiles with respect to ApoE gene polymorphism it was found that though E4 allele and E3/E4 genotype played a protective role against developing both groups of diabetic patients without and with nephropathy. On the other hand, E4 allele containing E4/E4 genotype had higher levels of glucose and triglycerides in diabetic patients with nephropathy (Figures 3.10, 3.11). In Thai (Chaudhary et al, 2012),

Kashmiri (Afroze et al, 2015), Tunisian (Chaaba et al, 2008) and Spanish (Gomez-Coronado et al, 1999) populations higher levels of triglycerides, LDL cholesterol and lower levels of HDL cholesterol were found to be associated with E4 allele of ApoE gene. In Indians, E3/E4 genotype was found to be associated with lower HDL-C and higher LDL-C concentrations in coronary artery disease patients (Singh et al, 2008).

Structural mutants of apolipoproteins can be used to study the role of the apolipoproteins in lipoprotein metabolism and thus, probable risk of developing macrovascular complications. Zhou et al (2008) demonstrated that HDL isolated from diabetic subjects with microalbuminuria or proteinuria has reduced ability to prevent the oxidation of LDL *ex vivo*. They further reported association of reduced plasma ApoA1 with hypertriglyceridaemia and low level of HDL in diabetic subjects (Zhou et al, 2008). Evidences from clinical trials suggested that the levels of apolipoprotein B (ApoB) and a constituent of atherogenic lipoproteins: ApoA1, a component of anti-atherogenic HDL cholesterol provided better prediction of future macrovascular events. On the other hand, Uniyal et al (2012) demonstrated that microvascular complication diabetic nephropathy as a positive correlation was established between decline of renal function with the ratio of ApoB and ApoA1 (ApoB/ApoA1). The ApoA1 and ApoC3 genes are oriented 'foot-to-foot,' i.e., the 3-prime end of ApoA1 is followed after an interval of about 2.5 kb by the 3-prime end of ApoC3 (Karathanasis et al., 1983). Karathanasis et al. (1983) found that a group of severely hypertriglyceridemic patients with types IV and V hyperlipoproteinemia had an increased frequency of a RFLP associated with the apoA-I gene. Rees et al. (1985) found a strong correlation between hypertriglyceridemia and a DNA sequence polymorphism located in or near the 3-prime noncoding region of ApoC3. Ferns et al. (1985) found an uncommon allelic variant (called S2) of the ApoA-I/C-III gene cluster in 10 of 48 postmyocardial infarction patients (21%). In 47 control subjects it was present in only 2 and in none of those who were normotriglyceridemic. Also, Buraczynska et al. (1985) found association between an *EcoR*I polymorphism of the ApoA1 gene and type 2 diabetes mellitus. On the other hand, Kessling et al. (1985) failed to find an association between any allele of several RFLPs studied and hypertriglyceridemia. However, involvement of ApoA1 gene polymorphism with diabetic

nephropathy with regard to rs121912717 is lacking in Bangladeshi population with type 2 diabetes and diabetes associated nephropathy.

It is well established that disorders of lipoprotein metabolism play a central role among the risk factors for macrovascular diseases caused by diabetes. The apolipoproteins, the protein moieties of the lipoprotein particles, may be even better predictors of atherosclerosis than HDL-cholesterol or LDL-cholesterol (Avogaro et al, 1979). ApoA1 gene provides instruction for making a protein called apolipoprotein A1 which is the major protein component of HDL particle in plasma (Martinez et al, 2003). ApoA1 attaches to cell membrane and promote the movement of cholesterol and phospholipids from inside the cell to the outer surface. Once outside the cell these substances combine with ApoA1 to form HDL. Mutations in the ApoA1 gene cause familial HDL deficiency an inheritant condition characterized by low levels of HDL in the blood and an elevated risk of cardiovascular disease (Storbl et al, 1988). With respect to ApoA1 gene polymorphisms (Figure 3.13), GG genotypes of both healthy individuals and diabetic nephropathy patients (Figure 3.19) had higher ACR than diabetic patients without nephropathy (Figures 3.16). Fasting plasma glucose and HbA1c levels were found evenly distributed in all groups of participants with both homozygous (GG) and heterozygous (GT) genotypes (Figures 3.14, 3.17, 3.20). Furthermore, though HDL levels were found to be significantly lower in diabetic patients with and without nephropathy (Table 3.3), however it's levels were almost evenly distributed in healthy individuals (Figure 3.15), diabetic patients without (Figure 3.18) and with (Figure 3.21) nephropathy. In case of levels of total cholesterol and triglycerides, similar findings were observed.

According to Ensembl database, rs121912717 is present on chromosome 11 at position 116835948 that leads to missense mutation. This SNP has been identified with pathogenic clinical significance as it causes gain of stop codon. Refseq Gene Mapping database demonstrated that change in "G" nucleotide in the allele by an "A" nucleotide causes replacement of glutamic acid residue by a translation termination signal codon (Amber). Lliterature and curated database revealed that the ancestral "G" allele frequency is the highest in the world population (0.99997953838) while presence of "A" mutant allele was reported in 1 out of 244355 individual of the world population. Further analysis showed that the frequency of "G"

allele in South-Asian population was 0.9999675029 and only 1 individual out of 30771 studied populations had T allele but A allele was not recognized in this population. According to SNPdb (https://www.ncbi.nlm.nih.gov/snp), out of total 1242 single nucleotide polymorphism present in human ApoA1 gene, 203 variants are pathogenic. Among these pathogenic SNPs, there are 13 variants that cause gain in stop codon at various positions and rs121912717 is one of the variant that causes gain of stop codon. Analysis of rs121912717 with ApoA1 gene revealed that our population does not carry double mutant TT genotype while more than 95% of the total participants were wild type homozygous (Table 3.20). Further observation unveiled that the GG genotype is almost evenly distributed (>95%, Table-3.20) among all the three group of study participants. While <5% of the population had minor T allele as they were grouped into heterozygous type (GT genotype). Logistic analysis revealed that neither allelic nor genotypic variation with regard to rs121912717 is associated with the risk of diabetes without or with nephropathy (Tables 3.21 and 3.22).

Conclusion

In conclusion, varying distribution of genotypic and allelic frequencies were observed in Bangladeshi unrelated healthy individuals, type 2 diabetic patients with and without nephropathy with regard to ApoE gene polymorphism in Exon 4 and rs121912717 within ApoA1 gene . Only E2/E4 genotype was missing in all the participants while E2/E2 genotype was observed only in individuals with type 2 diabetes. E4 allele and E3/E4 genotype found to be protective in both the patient groups and E3 allele was the most prevalent in all the study participants. We did not find double mutant homozygous TT genotype in the study participants when ApoA1 gene was considered. No association of GG and GT genotypes was established with the risk of nephropathy when different genetic models of ApoA1 gene were considered. Varying distribution of lipid profile along with other biochemical parameters were observed in different ApoE and ApoA1 genotypes. However, a wide scale study with large number of sample is warranted to establish current findings of this study for the association of genetic and allelic variations with diabetic nephropathy with respect to ApoE and ApoA1 gene in Bangladeshi population.

5. References

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