

**Genotypic and functional association of apolipoprotein E and lipoprotein (a) gene polymorphisms with diabetic nephropathy in Bangladeshi population**

**M. Phil. Thesis**

**A thesis is submitted to the University of Dhaka in partial fulfillment of the requirements for the degree of Masters of Philosophy in Biochemistry and Molecular Biology**

**Department of Biochemistry  
and Molecular Biology  
University of Dhaka  
Dhaka-1000, Bangladesh**

**Submitted by:  
Bartholomia Keya Byapari  
Registration no: 206  
Session: 2013-2014**

**October, 2018**

**Genotypic and functional association of apolipoprotein E and lipoprotein (a) gene polymorphisms with diabetic nephropathy in Bangladeshi population**



**A thesis is submitted to the University of Dhaka in partial fulfillment of the requirements for the degree of Masters of Philosophy in Biochemistry and Molecular Biology**

**Department of Biochemistry  
and Molecular Biology  
University of Dhaka  
Dhaka-1000, Bangladesh**

**Submitted by:  
Bartholomia Keya Byapari  
Registration no: 206  
Session: 2013-2014**

**October, 2018**

## **Declaration**

This dissertation has been submitted to the University of Dhaka in partial fulfillment of the requirements for the degree of Master of Philosophy (M. Phil.) in Biochemistry and Molecular Biology. This study has been carried out in the Laboratory of the Department of Biochemistry and Molecular Biology, University of Dhaka. No part of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other learning institutes.

Bartholomia Keya Byapari  
Department of Biochemistry and  
Molecular Biology  
Dhaka University  
Dhaka-1000, Bangladesh.

M. Phil Student

## **Certificate**

This is to certify that Bartholomia Keya Byapari, is a student of Masters of Philosophy (M.Phil.) of the session 2013-2014 at the Department of Biochemistry and Molecular Biology, University of Dhaka. Her registration No. is 206. She has submitted her thesis work entitled “Genotypic and functional association of apolipoprotein E and lipoprotein (a) gene polymorphisms with diabetic nephropathy in Bangladeshi population” for the partial fulfillment for her M.Phil. degree. This original research work was entirely performed by her using the departmental research facilities.

I wish her all the success in life.

A.H. M. Nurun Nabi, *PhD*

## Abstract

**Background:** Type 2 diabetes mellitus (T2DM) is one of the most common diseases with high incidence and prevalence throughout the world. Lipoprotein related metabolism associated with the damage of micro-and macro- vascular disease in T2DM. Apolipoprotein E (ApoE) and lipoprotein (a) (apolipoprotein A1, ApoA1) genes that affect the clearance of lipoproteins and consequently lipid profile in our body are the most important candidate genes, which have been reported to be associated with the diabetes related complications like nephropathy. Thus, the aim of the present study was 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.

**Methods:** A total of 349 unrelated Bangladeshi individuals were enrolled in this. Individuals having HbA1c level  $\geq 6.5\%$  were considered as type 2 diabetic (T2D-DN) patients while individuals having HbA1c level  $\geq 6.5\%$  and microalbumin level  $> 30$  mg/L were considered as type 2 diabetic patients with nephropathy (T2D +DN). Different anthropometric, demographic and biochemical parameters were recorded and measured from the study participants. Genomic DNA was extracted from the white blood cells of the collected blood samples. The amplification-refractory mutation system (ARMS) polymerase chain reaction was used to identify apolipoprotein E gene polymorphism and TaqMan SNP genotyping assay was used to analyze lipoprotein(a) (apolipoprotein A1) gene polymorphism through Real-time polymerase chain reaction. Anthropometric and biochemical parameters were evaluated according to the genotypic frequencies of respective gene of interests studied. Lipid profile measured in the participants was considered as the functional outcome according to their respective genotypes.

**Result:** Out of the total study participants, 123 and 122 individuals were diagnosed as type 2 diabetic patients without and with nephropathy, respectively. Rest of 104 participants was healthy individuals. In this study, healthy individuals were not age and BMI matched with that of patients without or with nephropathy. Systolic and diastolic blood pressure also varied significantly between the study groups ( $p < 0.05$ ). The levels of HbA1c and microalbumin varied significantly between healthy individuals and patient groups confirming their status of T2D without and with nephropathy. Also, levels of microalbumin and albumin creatine ratio (ACR)

differentiate the two patient groups: T2D-DN and T2D + DN ( $7.79 \pm 4.68$  vs  $238.58 \pm 316.07$  and  $10.22 \pm 6.07$  vs  $313.66 \pm 519.87$ , respectively). Levels of lipid profiles varied significantly between the healthy individuals and two groups of patients ( $p < 0.05$ ). Out of three isoforms of ApoE, frequency of isoform E3 allele was higher in all the participants (77.4% in healthy individuals, 85.4% in T2D-DN and 87.3% in T2D +DN patients) followed by E4 and E2 allele (22.1%, 12.2%, 11.9% and 0.5%, 2.4%, 0.8%, respectively). Out of six genotypes with respect to ApoE gene, E2/E4 was not identified in any of the study participants. We did not find any association of neither allele nor genotypes with respect to ApoE gene with the risk of T2D with and without nephropathy. Rather E4 allele and E3/E4 genotype were found to be associated in developing resistance against type 2 diabetes without ( $OR=0.5$ ,  $X^2 = 7.44$ ,  $p < 0.00$  and  $OR=0.38$ ,  $X^2 = 10.96$   $p < 0.00$ , respectively) and with nephropathy ( $OR=0.31$ ,  $X^2 = 17.08$ ,  $p < 0.00$  and  $OR=0.29$ ,  $X^2 = 16.25$ ,  $p < 0.00$ , respectively). However, with respect to rs121912717 within apolipoproteinA1 gene, no association of genotypic and allelic frequencies was found without and with nephropathy. Different distribution pattern of biochemical parameters of glucose, HbA1c, lipid profiles and ACR were observed in different genotypes of ApoE and ApoA1 genes.

**Conclusion:** This study conclude that ApoE gene polymorphism does not determine genetic susceptibility for the development of nephropathy and T2D rather E4 allele and E3/E4 genotype have protective role against T2D with or without nephropathy, while no such association was found in case of ApoA1 gene. Thus E3 allele and E3/E4 genotype can be an important marker to enumerate whether an individual does have any possibility of developing nephropathy with type 2 diabetes. However, a wide scale study with large number of sample is warranted to establish the association of genetic and allelic variations with diabetic nephropathy with respect to ApoE and ApoA1 gene in Bangladeshi population.

## *List of Contents*

<b>Content</b>	<b>Page number</b>
Abstract	i -ii
List of Content	iii -vii
List of table	viii -ix
List of figure	x-xi
List of abbreviation	xii - xv

### **Chapter 1: Introduction**

<b>Sl. Numbers</b>	<b>Contents</b>	<b>Page number</b>
1	Introduction	1
1.1	Overview	1
1.2	Diabetes mellitus	1
1.3	Types of diabetes mellitus	1
1.3.1	Type 1 diabetes	1
1.3.2	Type 2 diabetes	2
1.3.3	Gestational diabetes	3
1.3.4	Latent autoimmune diabetes in adults	4
1.3.5	Monogenic diabetes	4
1.3.6	Brittle diabetes	5
1.3.7	Cystic Fibrosis-related Diabetes (CFRD)	5
1.4	Prevalence in world population	5
1.5	Prevalence in Bangladesh	6
1.6	Diagnosis of diabetes	7
1.7	Pathophysiology of diabetes	8
1.8	Diabetes related complexities	9
1.8.1	Acute phase complications	10
1.8.2	Chronic Phase complications	10
1.9	Prevalence of diabetic nephropathy in world population and in Bangladesh.	12
1.10	Pathogenesis of diabetic nephropathy	13
1.11	Causes of diabetic nephropathy	15
1.12	Genetic association of diabetic nephropathy	15

1.13	Apolipoprotein E and its isoforms	16
1.13.1	Association of ApoE with diabetes	20
1.13.2	Genetic association of ApoE with diabetes and diabetes caused nephropathy	20
1.14	Lipoproteins and its isoforms	21
1.14.1	Genetic association of ApoA1 with diabetes and chronic kidney disease	22
1.15	Rationale of the study	24
1.16	Objectives of the study	25

## Chapter 2: Materials and Methods

Sl. Numbers	Contents	Page number
2	Materials and Methods	26
2.1	Study design	26
2.2	Study subject selection and diagnosis of the patients	26
2.2.1	Questionnaire analysis	28
2.2.2	Sample collection	28
2.2.3	Separation of plasma from blood sample	28
2.3	Genetic analysis	28
2.3.1	Extraction of Genomic DNA from human blood sample	28
2.3.1.1	Reagents required for DNA extraction	29
2.3.1.1.1	EDTA (0.5 M), pH 8.0	29
2.3.1.1.2	Tris HCl (1M), pH 7.6	29
2.3.1.1.3	TE buffer	29
2.3.1.1.4	Red blood cell (RBC) lysis buffer (pH 8.0)	29
2.3.1.1.5	Nucleic acid lysis buffer (pH 8.0)	30
2.3.1.1.6	Working solution of ethidium bromide	30
2.3.2	Procedure for DNA extraction	30
2.3.3	Purity check and quantification of extracted DNA	32
2.3.4	Evaluating the quality of extracted genomic DNA	32
2.3.5	Polymerase chain reaction for ApoE genotyping	33
2.3.6	Agarose gel electrophoresis	34
2.3.7	Genotyping using Real Time Polymerase Chain Reaction	35
2.3.7.1	TaqMan® SNP Genotyping Assay and TaqMan® Genotyping MasterMix	35
2.3.7.2	Protocol for performing genotyping study using TaqMan probe	36
2.3.7.3	Analysis of genotyping	37
2.4	Assay of biochemical parameters	37
2.4.1	Determination of the levels of glucose in plasma	38



2.4.1.1	Required reagents and materials	38
2.4.2	Determination of the level of Urea in the plasma of study participants	38
2.4.3	Determination of the activity alanine aminotransferase (ALT) in the plasma of study participants	39
2.4.4	Determination of the levels of creatinine in plasma	40
2.4.5	Determination of the levels of cholesterol in plasma	41
2.4.6	Determination of the levels of triglyceride in plasma	42
2.4.7	Determination of the levels of HDL in plasma	43
2.4.8	Determination of the levels of creatinine in urine	44
2.4.9	Determination of the levels of microalbumin in urine	44
2.4.10	Determination of urine albumin creatinine ratio	45
2.4.11	Determination of the levels of hemoglobin	45
2.4.12	Determination of the levels of glycated hemoglobin	46
2.4.13	Determination of Plasma Insulin	47
2.4.14	Statistical analyses	48

### Chapter 3: Results

<b>Sl. Numbers</b>	<b>Contents</b>	<b>Page Number</b>
3	Results	49
3.1	Demographic and anthropometric characteristics of the study participants	49
3.2	Biochemical characteristics of the study participants	50
3.3	Determination of lipid profiles in the study participants	51
3.4	Correlation of demographic and biochemical parameters of healthy individuals, type 2 diabetic patients with and without nephropathy	53
3.5	Correlation analyses of the levels of HbA1c and insulin with lipid profiles among the study participants	56
3.6	Demographic and anthropometric characteristics of the male study participants	61
3.7	Biochemical characteristics of the male study participants	62
3.8	Levels of plasma cholesterol, triglycerides, HDL cholesterol and LDL cholesterol in the female study participants	64
3.9	Demographic and anthropometric characteristics of the female study participants	65
3.10	Biochemical characteristics of the female study participants.	66
3.11	Determination of lipid profiles in the female study participants	68

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	70
3.13	Association of genotypic and allelic frequencies in regard to ApoE gene polymorphism with type 2 diabetes without nephropathy among Bangladeshi population	73
3.14	Association of genotypic and allelic frequencies in regard to ApoE gene polymorphism with type 2 diabetes with nephropathy among Bangladeshi population	74
3.15	Genotype distribution of ApoE according to clinical parameters among healthy participants	76
3.15.1	Albumin creatinine ratio	76
3.15.2	Fasting plasma glucose and HbA1c	77
3.15.3	Lipid profiles	77
3.16	Genotype distribution of ApoE according to clinical parameters among diabetic patients without nephropathy	78
3.16.1	Albumin creatinine ratio	79
3.16.2	Fasting plasma glucose and HbA1c	79
3.16.3	Lipid profile	80
3.17	Genotype distribution with clinical data among diabetic patients with nephropathy	81
3.17.1	Albumin creatinine ratio	81
3.17.2	Fasting plasma glucose and HbA1c	82
3.17.3	Lipid profile	83
3.18	Evaluation of allele discrimination plot for genotyping with respect to rs121912717 in ApoA1 gene	84
3.19	Determination of genotype frequency distribution of ApoA1 with respect to rs121912717 in healthy participants, diabetic patients and diabetic patients with nephropathy	85
3.20	Determination of association of genotype frequencies with respect to rs121912717 of ApoA1 gene	86
3.20.1	Association with T2D	86
3.20.2	Association of diabetic nephropathy	88
3.21	Genotype distribution of ApoA1 according to clinical parameters among healthy participants	89
3.21.1	Albumin creatinine ratio	89
3.21.2	Fasting plasma glucose and HbA1c	90
3.21.3	Lipid profile	90
3.22	Genotype distribution of ApoA1 according to clinical parameters among diabetic non-nephropathy patients	91
3.22.1	Albumin creatinine ratio	91
3.22.2	Fasting plasma glucose and HbA1c	92

3.22.3	Lipid profile	93
3.23	Genotype distribution of ApoA1 with clinical data among diabetic nephropathy patients	94
3.23.1	Albumin creatinine ratio	94
3.23.2	Fasting plasma glucose and HbA1c	95
3.23.3	Lipid profile	96

#### **Chapter 4: Discussion:**

<b>Sl. Numbers</b>	<b>Content</b>	<b>Page Number</b>
4.	Discussion	98
	Conclusion	103

#### **Chapter 5: References**

<b>Number</b>	<b>Content</b>	<b>Page Number</b>
	References	104

Appendix

113

Patient record form

## List of Table

Table number	Title of table	Page number
1.1	Tests to diagnose Diabetes	7
2.1	Primers for amplification refractory mutation system polymerase chain reaction.	33
2.2	Reagents required for genotyping of ApoE.	34
2.3	Correlation between fluorescence signal and sequences in the sample	36
2.4	Composition of reaction mixture for real-time PCR.	36
3.1	Demographic and anthropometric characteristics of the study participants.	49
3.2	Biochemical characteristics of the study participants.	51
3.3	Levels of lipid profile in the study participants.	52
3.4	Correlation between demographic and biochemical parameters of healthy control individuals.	54
3.5	Correlation between demographic and biochemical parameters of type 2 diabetic patients without nephropathy.	54
3.6	Correlation between demographic and biochemical parameters of type 2 diabetic patients with nephropathy.	55
3.7	Correlation analyses of the levels of HbA1c and insulin with lipid profiles of healthy control individuals.	56
3.8	Correlation analyses of the levels of HbA1c and insulin with lipid profiles of type 2 diabetic patients without nephropathy.	58
3.9	Correlation analyses of the levels of HbA1c and insulin with lipid profiles of type 2 diabetic patients with nephropathy.	59
3.10	Demographic and anthropometric characteristics of the male study participants	61
3.11	Biochemical characteristics of the male study participants.	63
3.12	Levels of plasma cholesterol, triglycerides, HDL cholesterol and LDL cholesterol in the female study participants	64
3.13	Demographic and anthropometric characteristics of the female study participants.	66
3.14	Biochemical characteristics of the female study participants.	68
3.15	Determination of lipid profiles in the female study participants	69
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.	72
3.17	Association analyses of genotypic and allelic frequencies with type 2 diabetes without nephropathy in regard to ApoE gene polymorphism among Bangladeshi population.	73
3.18	Association analyses of genotypic and allelic frequencies with type 2	74

	diabetic nephropathy in regard to ApoE gene polymorphism among Bangladeshi population.	
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.	75
3.20	Genotype distribution with respect to rs121912717 of ApoA1 gene.	86
3.21	Association of genotype frequencies with regard to rs121912717 of ApoA1 gene with the probable risk of diabetes in Bangladeshi population.	87
3.22	Association of genotype frequencies with regard to rs121912717 of ApoA1 gene with the probable risk of diabetic nephropathy in Bangladeshi population	88

## List of figures

Figure number	Title of figure	Page number
1.1	Schematic mechanisms involved in pathogenesis of two main types of diabetes mellitus	9
1.2	Pathogenesis of diabetic nephropathy	14
1.3	Structure and functional domains of ApoE	14
1.4	Schematic representation of structure and function of ApoE	19
1.5	(a) Structure ApoA1 protein. (b) Structure of HDL	22
1.6	Chromosomal location of ApoA1	23
2.1	Schematic presentation of the overall activities to fulfill the research work	27
2.2	White string of DNA appeared after adding cold ethanol	31
2.3	Image of agarose gel under uv light after electrophoresis of DNA samples	35
3.1	Correlation analyses between levels of HbA1c and plasma HDL cholesterol in healthy individuals	57
3.2	Correlation analyses between HbA1c, levels of plasma cholesterol (A) and LDL cholesterol (B) in type 2 diabetic patients with nephropathy	60
3.3	Graphical representation of distribution of ACR with genotypes regarding ApoE among healthy study participants	76
3.4	Graphical representation of distribution of Fasting plasma glucose and HbA1c with genotypes regarding ApoE among healthy study participants	77
3.5	Graphical representation of distribution of various genotypes of Apo E with cholesterol, triglycerides, dHDL and LDL among healthy participants	78
3.6	Graphical representation of distribution of genotypes regarding ApoE with ACR among diabetic non- nephropathy patients	79
3.7	Graphical representation of distribution of genotypes regarding ApoE with fasting plasma glucose and HbA1c among diabetic non-nephropathy patients	80
3.8	Graphical representation of various Apo E genotypes with cholesterol, triglycerides, HDL and LDL among diabetic non nephropathy participants	81
3.9	Distribution of albumin creatinine ratio among different genotypes in diabetic nephropathy patients	82
3.10	Representation of Association of genotype with fasting plasma glucose and HbA1c among diabetic nephropathy patients	83
3.11	Graphical representation of distribution of the levels of cholesterol, triglycerides, dHDL and LDL among diabetic nephropathy participants according to different genotypes of ApoE gene	84
3.12	Representation of the allelic discrimination plot	85

3.13	Distribution of various genotypes of ApoA1 gene with ACR among healthy study participants	89
3.14	Distribution of various genotypes of ApoA1 gene with Fasting Glucose and HbA1c among healthy study populations	90
3.15	Association of various genotypes of ApoA1 gene with Cholesterol, Triglycerides, HDL and LDL among healthy participants	91
3.16	Distribution of various genotypes of ApoA1 gene with ACR among diabetic non nephropathy participants	92
3.17	Distribution of various genotypes of Apo A1gene with fasting Glucose and HbA1c among diabetic non nephropathy study populations	93
3.18	Graphical representation of distribution of various genotypes of Apo A1gene with Cholesterol, Triglycerides, HDL and LDL among diabetic non nephropathy participants	94
3.19	Graphical representation of distribution of ApoA1 genotype and ACR among diabetic nephropathy patients	95
3.20	Graphical representation of distribution of ApoA1 genotypes with fasting plasma glucose and HbA1c diabetic nephropathy study population	96
3.21	Graphical representation of distribution of ApoA1 genotypes with cholesterol, Triglycerides, HDL, and LDL with diabetic nephropathy study population	97

**List of abbreviation**

ALT	: Alanin transaminase
Arg	: Arginine
ADP	: Adenosine di aminase
ATP	: Adenosine tri aminase
BMI	: Basal Metabolic Index
bp	: Base pair
BP	: Blood pressure
cm	: Centimete
Cys	: Cysteine
CO <sub>2</sub>	: Carbon di oxide
Chol	: Cholesterol
CMIA	: Chemilunescent microparticle immunoassay
DBP	: Diastolic blood pressure
dL	: Deciliter
DMSO	: Dimethyl sulfoxide
dH <sub>2</sub> O	: Distilled water
dHDL	: Direct High density Lipoprotein
DNA	: Deoxyribo nucleic acid
EDTA	: Ethylenediaminetetraacetic acid
Et. Br	: Ethidium bromide
F	: Female
FPG	: Fasting plasma glucose
Gln	: Glutamine
GDM	: Gastetional diabetes mellitus



Glu	: Glucose
g	: Gram
HDL	: High density lipoprotein
Hb	: Hemoglobin
Kg	: kilogram
IGF	: impaired Fasting glucose
IDF	: International diabetes federation
IGT	: Impaired glucose tolerance
IU/ml	: International unit/milliliter
L	: Liter
LDL	: Low density lipoprotein
LDH	: Lactate dehydrogenase
M	: Male
mL	: Mililiter
mg/dL	: Miligram/deciliter
mg	: Miligram
Mg	: Magnesium
MgCl	: Magnesium chloride
mALB	: Microalbumin
mg	: milligram
mmol/L	: Milimole/Liter
mmHg	: Milimeter mercury
N	: Normality
nm	: Nanometer
NDM	: Neonatal diabetes mellitus

ng	: Nano gram
NaCl	: Sodium Chloride
NH <sub>3</sub>	: Amonia
NADH	: Nicotinamide adenine dinucleotide
O.D	: Optical density
PBS	: Phosphate buffered saline
PDH	: Pyruvate dehydrogenase
p.s.i	: Pounds per square inch
PCR	: Polymerase chain reaction
r.p.m	: Rotation per minute
RT	: Real time
RBC	: Red blood cell
RNA	: Ribonucleic acid
SBP	: Systolic blood pressure
SD	: Standard Deviation
SNP	: Single nucleotide polymorphism
SER	: Serine
TG	: Triglycerides
T2D	: Type 2 diabetes
TAE	: Tris- acetate-EDTA
TE	: Tris-EDTA
UV	: Ultra violet
U/L	: Unit/ liter
VLDL	: Very low density lipoprotein
WHO	: World health organization

WBC	: White blood cell
Yr	: Year
$\mu\text{g}$	: microgram
$\mu\text{g/L}$	: Microgram/liter
$\mu\text{L}$	: Microliter
$\mu\text{m/L}$	: Micromole/liter
%	: Percent
$^{\circ}\text{C}$	: Degree Centigrade
>	: greater than
<	: Less than
$\leq$	: Less than or equal to
$\geq$	: Greater than or equal to