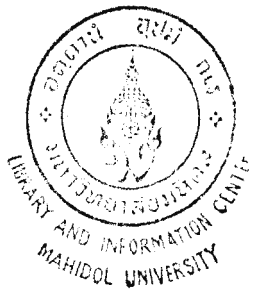
15 JUL 2000



POLYMORPHISMS AND MUTATIONS OF APOLIPOPROTEIN B AND APOLIPOPROTEIN E GENES IN THAI SUBJECTS

SAIPHON POLDEE

ดีเด่นวิทยานิพนธ์
จาก
บัณฑิตวิทยาลัย มหิดล

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE (BIOCHEMISTRY)

FACULTY OF GRADUATE STUDIES

MAHIDOL UNIVERSITY

2000

ISBN 974-663-909-9

COPYRIGHT OF MAHIDOL UNIVERSITY

Copyright by Mahidol University

TH
S157p
2000
C.2

44852 c.2

Thesis
Entitled

POLYMORPHISMS AND MUTATIONS OF APOLIPOPROTEIN B AND APOLIPOPROTEIN E GENES IN THAI SUBJECTS

Saiphon Poldee
Mr.Saiphon Poldee
Candidate

Atip Likidlilid
Assoc. Prof. Atip Likidlilid M.Sc.
Major-advisor

Klai-upsorn Pongrapeeporn
Asst. Prof. Klai-upsorn Pongrapeeporn, Ph.D.
Co-advisor

N. Tirawanchai
Asst. Prof. Nednapis Tirawanchai, Ph.D.
Co-advisor

Liangchai Limlomwongse
Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate Studies

R.W. Kalpravidh
Assoc. Prof. Ruchaneekorn Kalpravidh, Ph.D.
Chairman
Master of Science Program in Biochemistry
Faculty of Medicine, Siriraj Hospital

Copyright by Mahidol University

Thesis
Entitled

POLYMORPHISMS AND MUTATIONS OF APOLIPOPROTEIN B AND APOLIPOPROTEIN E GENES IN THAI SUBJECTS

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Biochemistry)
on
May 3, 2000

Saiphon Poldee
Mr.Saiphon Poldee
Candidate

Atip Likidlilid
Assoc. Prof. Atip Likidlilid M.Sc.
Chairman

N. Tirawanchai
Asst. Prof. Nednapis Tirawanchai, Ph.D.
Member

Klai-upsorn Pongrapeeporn
Asst. Prof. Klai-upsorn Pongrapeeporn, Ph.D.
Member

P. Patmasiriwat
Assoc. Prof. Pimpicha Patmasiriwat, Ph.D.
Member

Chamras Promptmas
Asst. Prof. Chamras Promptmas, Ph.D.
Member

Liangchai Limlomwongse
Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

C. Tuchinda
Prof. Chanika Tuchinda M.D., M.S., FAAP.
Dean
Faculty of Medicine, Siriraj Hospital
Mahidol University

Copyright by Mahidol University

ACKNOWLEDGEMENT

I would like to express my sincere gratitude and deepest appreciation to Assoc. Prof. Atip Likidlilid my supervisor for his creative guidance, invaluable advice, supervision and encouragement throughout this thesis work, he was never lacking in kindness and support which will never be forgotten.

I would like to thank my co-supervisor, Asst. Prof. Dr. Klai-upsorn Pongrapeeporn, Asst. Prof. Dr.Nednapis Tirawanchai, for their kindness and valuable suggestions and for their guidance, supervision and continuous discussions throughout this work. They all were nice and friendly.

Sincere appreciation is expressed to Associate Professor Boonchuay Dulyasukdi, Mrs. Anchalee Amornratta (Department of Preventive and Social Medicine) for coordinating the collection of blood samples and Mr.Somchat Maneenoi (Siriraj computer center) for performing statistical analyses. Miss Aree Futakul for her kindly guidance, suggestions, and help; especially teaching several molecular techniques for this thesis.

I am particularly indebted to the Faculty of Medicine Siriraj Hospital Mahidol University for the scholarship which enabled me to undertake this study.

Finally, I would like to express my gratitude to my parents, and my wife for their infinite love, attention, understand and encouragement which will never be forgotten.

Saiphon Poldee

Copyright by Mahidol University

4037755 SIBC/M : MAJOR : BIOCHEMISTRY ; M.Sc. (BIOCHEMISTRY)
KEY WORDS : APOLIPOPROTEIN B/ APOLIPOPROTEIN E/ POLYMORPHISMS

SAIPHON POLDEE: POLYMORPHISMS AND MUTATIONS OF APOLIPOPROTEIN B AND APOLIPOPROTEIN E GENE IN THAI SUBJECTS. THESIS ADVISORS: ATIP LIKIDLILID M.Sc., KLAI-UPSORN PONGRAPEEPORN Ph.D., NEDNAPIS TIRAWANCHAI Ph.D. 183 p. ISBN 974-663-909-9

To study the impact of apolipoprotein B and E polymorphisms and mutations on plasma lipid levels, the 171 subjects were obtained from Dyslipoproteinemia Clinic at Siriraj Hospital. The study of exon 1 in apolipoprotein (apo) B signal peptide insertion (I)/ deletion (D) polymorphism by PCR in 171 Thai population has shown that the allele frequencies of I and D were 0.78 and 0.22, respectively. The Thai population was subdivided into 103 hyperlipidemia, and 68 normolipidemia groups. The D allele frequencies in hyperlipidemia (I=0.74, D=0.26) was significantly higher than that in normalipidemia (I=0.83, D =0.17) ($p < 0.05$). Comparison of these allele frequencies with those reported for several other population samples showed that the Caucasian populations were significantly higher in D allele frequencies but similar in Asian populations. To detect point mutation in exon 26 of apo B-100 by PCR-RFLP in Thai population, the Arg3500Gln and Arg3531Cys were not identified in 171 Thai samples. However, the heterozygotes of Arg3611Gln were identified in 2 hyperlipidemic subjects which showed no significant reduction on lipid profiles after treatment with lipid-lowering drugs. The apo E polymorphisms were also studied by PCR-RFLP and found that the apo E2, E3, and E4 allele frequencies were 0.08, 0.80, and 0.12, respectively. Comparison of these allele frequencies between the hyperlipidemia (E2=0.08, E3=0.76, E4=0.16) and normolipidemia (E2=0.08, E3=0.85, E4= 0.07) showed that the E4 allele frequencies were significantly higher by an increase of 86% than that of normolipidemia ($p<0.05$). We also compared these allele frequencies with those reported for several other population samples. The results showed that there were marked significant differences in Thai and other racial populations, e.g. Finnish, Sudanese, Swedish, Trinidadian and Greenlander ($p<0.05$). These differences in apo E allele frequencies appeared to be mainly due to differences of an increased E4 allele frequencies in Finnish, Sudanese, and Swedish. The difference of Trinidadian from Thai populations was due to an increase in apo E2 allele frequency (60%) whereas there was a decrease (70%) in the Greenlander population. From the study of the relationship between apo B Ins/Del polymorphism and lipid profiles in plasma, the results showed that significant differences of these polymorphisms and lipid profiles were observed in only ID genotypes of hyperlipidaemia. These significant differences may be due to the influence of exacerbating factors which is particularly strong in old age. However, comparison between before and after treatment with lipid-lowering drugs for 3-5 years showed that II and ID genotypes significantly decreased TC and LDL-C after treatment. ($p<0.001$). DD genotypes showed no decreased in plasma lipids. The association of apo E polymorphism and plasma lipid profiles showed that the E4 allele was associated with the higher levels of TC and LDL-C ($p<0.05$) whereas E2 allele was associated with a higher level of TG ($p<0.001$) as compared with the normal E3. We also compared the effects of lipid-lowering drugs before and after treatment on plasma lipid levels in individuals with various apo E polymorphisms. The results showed that only homozygous E3/E3 significantly reduced TC, LDL-C, and TG after treatment for 3-5 years, (TC, LDL-C; $p<0.001$; TG; $p<0.01$). The genotypes containing E4 allele showed significantly decreased TC and LDL-C levels ($p<0.05$). The genotype containing E2 allele showed no significant differences of lipid levels before and after treatment.

Copyright by Mahidol University

4037755 SIBC/M : สาขาวิชา: ชีวเคมี; วท.ม. (ชีวเคมี)

สายฝน ผลดี : ความหลากหลายและการกลายพันธุ์ของยีน apolipoprotein B และ apolipoprotein E ในคนไทย (POLYMORPHISMS AND MUTATIONS OF APOLIPOPROTEIN B AND APOLIPOPROTEIN E GENE IN THAI SUBJECTS). คณะกรรมการควบคุมวิทยานิพนธ์ : อธิป ลิขิตลิลิต, M.sc., คล้ายอัปสร พงศ์รพีพร, Ph.D., เนตรนภิส ธีระวัลย์ชัย, Ph.D. 183 หน้า ISBN 974-663-909-9

จากการศึกษาความหลากหลายของยีน apolipoprotein (apo) B ด้วยวิธี PCR ในคนไทย 171 คน พบว่าความถี่ของยีนใน exon I ชนิด insertion (I) และ deletion (D) เป็น 0.78 และ 0.22 ตามลำดับ และเมื่อแบ่งกลุ่มของคนไทยนี้ตามระดับไขมันพบว่ากลุ่มที่มีไขมันในเลือดปกติจะมี 68 คนและกลุ่มที่มีไขมันในเลือดสูงจะมี 103 คน เมื่อเปรียบเทียบความถี่ของยีนนี้ระหว่างกลุ่มไขมันปกติ (I=0.83, D=0.17) และกลุ่มไขมันในเลือดสูง (I = 0.74 , D=0.26) พบว่ากลุ่มที่มีไขมันสูงจะมี D สูงกว่ากลุ่มไขมันปกติอย่างมีนัยสำคัญ ($p< 0.05$) เมื่อเปรียบเทียบความถี่ยีนนี้กับชาติอื่นพบว่า กลุ่มที่เป็น Caucasian เช่น ฟินแลนด์, ฝรั่งเศส, นอรเวย์ และสหรัฐอเมริกาจะมีความถี่ของยีน D สูงกว่าในประชากรจากประเทศในแถบเอเชียเช่น ไทย, จีน, และอินเดียอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) สำหรับการศึกษาการกลายพันธุ์ของ ยีน apoB-100 ใน exon 26 ด้วยวิธี PCR-RFLP จะไม่พบการกลายพันธุ์ที่ตำแหน่งกรดอะมิโน Arg3,500Gln และ Arg3,531Cys ในประชากรไทยที่ศึกษา ขณะที่การกลายพันธุ์ที่ตำแหน่งกรดอะมิโน Arg3,611Gln จะพบ 2 ราย ซึ่งเป็นชนิด heterozygotes และทั้งสองรายพบในกลุ่มไขมันในเลือดสูง การศึกษาความหลากหลายของยีน apo E ด้วยวิธี PCR-RFLP ในประชากรไทย 171 คนพบว่ามี ความถี่ของยีน E2 , E3 และ E4 เป็น 0.08, 0.80 และ 0.12 ตามลำดับ เมื่อศึกษาความถี่ของยีนนี้ในกลุ่มที่มีไขมันในเลือดสูงพบว่าความถี่ของยีน E2=0.08, E3=0.76 และ E4=0.16 ส่วนกลุ่มไขมันในเลือดปกติจะมี E2=0.08, E3=0.85 และ E4=0.07 เมื่อเปรียบเทียบความแตกต่างของทั้งสองกลุ่มโดยใช้ค่า χ^2 ของความถี่แต่ละยีน เทียบกับค่า χ^2 รวม พบว่าความถี่ของยีนชนิด E4 ในกลุ่มที่มีไขมันในเลือดสูงจะสูงกว่า (86%) กลุ่มคนปกติอย่างมีนัยสำคัญ ($p<0.05$) จากการเปรียบเทียบความถี่ของยีน apo Eในประชากรไทยกับกลุ่มประชากรของต่างประเทศพบว่า ความถี่ของยีน apo E ในคนไทย แตกต่างจากกลุ่มประชากรในประเทศฟินแลนด์, ซูดาน, สวีเดน, ทรินิแดด และ กรีนแลนด์ อย่างมีนัยสำคัญ ($p<0.05$) และยังพบว่าในกลุ่มประชากรจากประเทศฟินแลนด์, ซูดาน, และสวีเดนนั้นมี ความถี่ของยีน E4 สูงกว่าในคนไทย ในขณะที่ในกลุ่มประชากรจากประเทศทรินิแดด มี ความถี่ของยีนของยีน E2 สูงกว่าในประชากรไทย แต่ในชาวกรีนแลนด์กลับพบว่ามี apo E2 ต่ำกว่าในประชากรไทย จากการศึกษาถึงความสำพันธ์ระหว่าง ยีโนไทป์ และระดับไขมันในเลือดในคนไทย 171 คน จะพบความสัมพันธ์เฉพาะยีน ID ในกลุ่มที่มีระดับไขมันสูง แต่เมื่อเปรียบเทียบระดับไขมันในเลือดระหว่างก่อนการรักษาด้วยยาลดไขมันพร้อมด้วยการควบคุมอาหารกับหลังการรักษา เป็นเวลา 3-5 ปี ในกลุ่มที่มีระดับไขมันในเลือดสูงพบว่ากลุ่มที่มีที่มี ยีโนไทป์ เป็น II และ ID จะมีระดับ TC และ LDL-C ลดลงอย่างมีนัยสำคัญ ($p<0.001$) ในขณะที่กลุ่มที่มียีโนไทป์ เป็น DD ระดับไขมันจะไม่ลดลงแต่ระดับของ HDL-C ลดลงอย่างมีนัยสำคัญ ($p<0.05$) สำหรับความสัมพันธ์ของความถี่ยีน apo E กับระดับไขมัน พบว่าชนิด E4 มีระดับ TC และ LDL-C สูงกว่าชนิด E2 และ E3 อย่างมีนัยสำคัญ ($p<0.05$) และเมื่อเปรียบเทียบระดับไขมันก่อนและหลังรักษาพบว่า ยีโนไทป์ ชนิด E3/E3 เท่านั้นที่พบว่ามีระดับ TC , LDL-C, และ TG ลดลงอย่างมีนัยสำคัญ (TC, LDL-C; $p<0.001$, TG; $p<0.01$) และยีโนไทป์ผสมที่มี E4 พบว่ามีระดับ TC และ LDL-C ลดลงอย่างมีนัยสำคัญ($p<0.05$) ขณะที่ยีโนไทป์ ผสมที่มี E2 ไม่พบความแตกต่างของระดับไขมันก่อนและหลังการรักษา

Copyright by Mahidol University

CONTENTS

Copyright by Mahidol University

Page

Copyright by Mahidol University

Page

Copyright by Mahidol University

Page

Copyright by Mahidol University

LIST OF TABLES

Copyright by Mahidol University

Page

Copyright by Mahidol University

Page

Copyright by Mahidol University

LIST OF FIGURES

Copyright by Mahidol University

Page

Copyright by Mahidol University

LIST OF ABBREVIATIONS

A	=	adenine
ACAT	=	acyl-CoA : cholesterol O-acyltransferase
ANOVA	=	analyses of variance
Apo B-48	=	apolipoprotein B-48
Apo B-100	=	apolipoprotein B-100
Apo E	=	apolipoprotein E
bp	=	base pairs
C	=	cytosine
CHD	=	coronary heart disease
°C	=	degree celcius
cDNA	=	complementary deoxyribonucleic acid
CETP	=	cholesteryl ester transfer protein
DNA	=	deoxyribonucleic acid
dATP	=	deoxyadenosine 5'-triphosphate
dCTP	=	deoxycytidine 5'-triphosphate
dGTP	=	deoxyguanosine 5'-triphosphate
dTTP	=	deoxythymidine 5'-triphosphate
dNTP	=	deoxynucleotide triphosphate
EM	=	electron microscopy
ER	=	endoplasmic reticulum
EDTA	=	ethylenediamine tetraacetic acid
FDB	=	familial defective apolipoprotein B-100

Copyright by Mahidol University

FH	=	familial hypercholesterolemia
FFA	=	free fatty acids
Fig.	=	figure
G	=	guanine
g	=	gramme
HDL	=	high density lipoprotien
HMG-CoA	=	3-hydroxy-3-methyl-glutaryl-CoA reductase
h	=	hour
IDL	=	intermediate density lipoprotein
kb	=	kilobase
LCAT	=	licithin:cholesterol acyltransferase
LDL	=	low density lipoprotein
LDL-C	=	low density lipoprotein cholesterol
LDLR	=	low density lipoprotein receptor
L	=	litre
M	=	molar
Mr	=	relative molecular weight or relative molecular mass
?l	=	microlilitre
mg/dl	=	milligramme/decilitre
min	=	minute
ml	=	millilitre
mM	=	millimolar
mRNA	=	messenger ribonucleic acid

Copyright by Mahidol University

ng	=	nanogramme
nt	=	nucleotide
PCR	=	polymerase chain reaction
pmole	=	picomole
RFLP	=	restriction fragment length polymorphisms
SE	=	standard error
sec	=	second
SDS	=	sodium dodecyl sulfate
T	=	thymine
TBE	=	tris-EDTA-borate buffer
TC	=	total cholesterol
TG	=	Triglyceride
Tris	=	Tris (hydroxymethyl)-aminomethane
U	=	uracil
UV	=	ultraviolet
VLDL-C	=	very low density lipoprotein cholesterol
w/v	=	weight/volume

Copyright by Mahidol University

CHAPTER I

INTRODUCTION

Introduction

Coronary heart disease is the result of progressive thickening of the arterial wall, due in part to lipid infiltration and smooth muscle cell proliferation, presumably in response to injury to the endothelium[1]. Atherosclerosis and coronary heart disease make major increasing contributions to the morbidity and mortality of developing populations. The higher rate of coronary heart disease has also currently been reported in Thai population. Epidemiological studies and clinical trials have determined that plasma lipid levels are significant risk factors for the development of disease. Information about the plasma apolipoprotien levels may be the better predictors of this disease risk. The score for severity of atherosclerosis is strongly associated to the low-density lipoprotein (LDL) cholesterol and apolipoprotein (apo) B concentrations. Increasing evidence suggests that triglyceride rich lipoprotein such as very-low density lipoprotein (VLDL) cholesterol and intermediate density lipoprotein (IDL) cholesterol or VLDL remnant play a significant role in the pathogenesis of atherosclerosis[2]. The level of high-density lipoprotein (HDL) cholesterol was found to inversely correlated with the severity of disease. LDL, IDL and VLDL concentrations are elevated in a number of diseases, e.g. type III hyperlipoproteinaemia, renal disease, hyperthyroidism, and diabetes that the developing stages were associated with the

Copyright by Mahidol University

rapid and premature development of atherosclerosis. These lipoproteins have high capacity for interacting with cells like skin fibroblasts and arterial smooth muscle cells leading to the accumulation of cholesterol esters and triglyceride in the wall of arteries and forms bulky plaques called atherosclerotic plaques. This plaques obstruct the flow of blood and might lead to a heart attack (coronary heart disease or CHD) or a stroke (cerebrovascular accident or CVA). In general, the lipoproteins that subdivided into VLDL and IDL was primarily derived through triglyceride metabolism whereas LDL was primarily derived through cholesterol metabolism. The hepatic recognition and subsequent metabolism of VLDL, IDL, and LDL are largely dependent on the presence of apo E and apo B, respectively. The quantitative importance of a partial deficiency of apo E and apo B may reflect to the catabolism of VLDL, IDL, and LDL leading to the development of coronary artery disease. These defects may come from the fact that these two lipoproteins can not mediate the clearance of VLDL, IDL, and LDL from plasma by binding to its B/E (LDL) receptors at the liver and extrahepatic tissues. Of all the established risk factors that mentioned above for coronary artery disease are the strongest predictor of atherosclerotic plaque initiation and progression. However, aging as an index of changes in lipid metabolism may influence the onset of coronary artery disease. At present, traditional epidemiological studies of hyperlipidaemia, the risk factors of coronary artery disease, have focused on identifying intermediate biochemical factors that may contribute to the prediction of presence of disease[3]. Thus, a central question of genetic studies of coronary artery disease is whether knowledge about variation in the genome (polymorphism) or gene mutation will be useful for the prediction of disease onset, rate of progression and severity[4]. Measured genotype

Copyright by Mahidol University

information has the potential to contribute the ability to predict hyperlipidaemia onset, rate of progression and severity because genotypes are typically not altered by the disease process or time, and may represent information about a biochemical trial that can not be measured. In addition, measured genotype information may be easier and less expensive to obtain than biochemical and physiological information[5]. The genetic variations in apolipoproteins are therefore associated with the abnormal lipid metabolism causing in the susceptibility to coronary artery disease.

Objectives of this study

1. To investigate the genotypes and allele frequencies of polymorphisms and mutations of apo B and apo E genes in Thai general population.
2. To investigate the genotypes and allele frequencies of apo B and apo E polymorphisms between genders in this general populations.
3. To study the influence of genetic variations in apo B and apo E genes on quantitative variation of plasma lipid levels in Thai general population.
4. To compare apo B, apo E genotypes and allele frequencies between Thai and other populations.
5. To study the effect of lipid-lowering drugs in hyperlipidaemic subjects with genetic variation of apo B and apo E genes on plasma lipid levels between before and after treatment.

Copyright by Mahidol University

CHAPTER II

LITERATURE REVIEW

Lipoproteins

Lipoproteins are the macromolecular complexes of lipid and protein, an major function of which is to transport lipids through the vascular and extravascular body fluids[6]. Classical studies by using the analytical ultracentrifuge in the 1950s and 1960s revealed for the first time of the heterogeneous nature of the complexes in plasma that were responsible for lipid transport[7].

Lipoprotein classes[1, 8-10].

The five major classes of lipoproteins are named either by their density (table 2.1) or by electrophoretic mobility (Figure 2.1).

(1) Chylomicrons which are in origin on electrophoresis are triglyceride-rich lipoproteins synthesized from intestinal absorption of triacylglycerol; they have a density (d) of approximately 0.98 g / ml.

(2) Very low-density lipoproteins (VLDL) (d < 1.006 g/ml) are triglyceride-rich lipoproteins derived by the liver for the export of triacylglycerol; on electrophoresis they show prebeta mobility.

(3) Intermediate-density lipoproteins (IDL) (d = 1.006-1.019 g/ml) are produced by the catabolism of VLDL.

Copyright by Mahidol University

(4) Low-density lipoproteins (LDL) (d = 1.019-1.063 g/ml), which are derived by catabolism of IDL, are major cholesterol-carrying lipoproteins of serum and representing a final stage in the catabolism of VLDL; they have beta mobility on electrophoresis.

(5) High-density lipoproteins (HDL) (d =1.063-1.21 g/ml) which have alpha mobility, comprise several components derived from various sources such as liver, intestine, other lipoproteins, and other tissues.

Table 2.1 Classification of lipoproteins[6]

CLASSIFICATION OF LIPOPROTEINS		
LIPOPROTEIN	DENSITY(g/ml)	SOURCES
Chylomicrons	~0.98	intestine
Very low-density lipoproteins (VLDL) (prebetalipoprotiens)	~1.006	liver
Intermediate-density lipoproteins (IDL)	1.006-1.019	catabolism of VLDL
Low-density lipoproteins (LDL) (betalipoproteins)	1.019-1.063	catabolism of IDL
High-density lipoproteins (HDL) (alphalipoproteins)	1.063-1.21	liver, intestine, other tissues

Copyright by Mahidol University

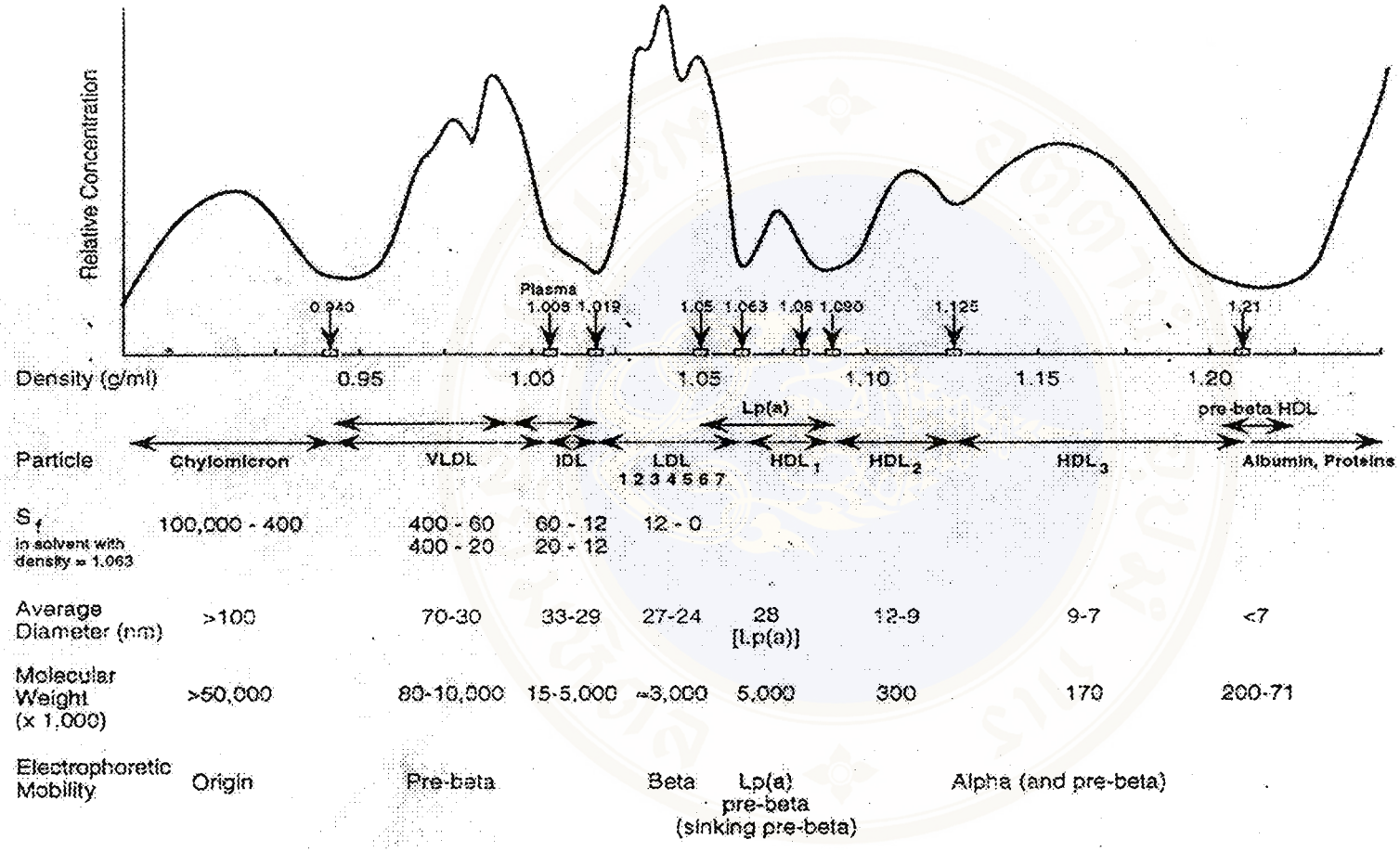


Figure 2.1 Physical properties of the major lipoprotein[10].

Copyright by Mahidol University

Lipoprotein structure. [1, 8-10]

The basic structures of all lipoproteins are similar; they all contain a core of neutral lipids consisting of cholesterol esters and triglycerides, a surface coat of more polar lipids such as unesterified cholesterol, phospholipids and apoproteins (Figure 2.2)

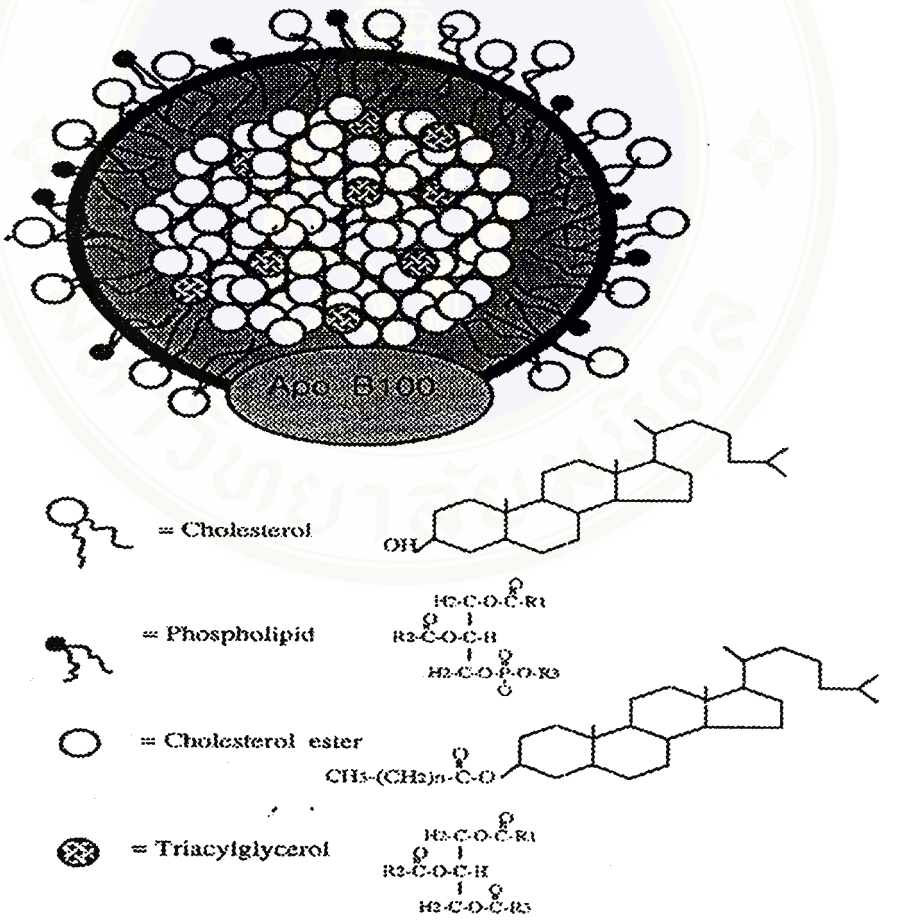


Figure 2.2 Lipoprotein structure[1]

Copyright by Mahidol University

Lipoprotein function [1, 8-10]

Lipoproteins carry out three main functions. One is transport the dietary fat from the intestinal mucosa, where it is absorbed, to the other tissues. Chylomicrons and chylomicron remnants perform this function. The second is to transfer triglyceride from the liver to other tissues, where the lipid can be either stored or oxidized for energy. VLDLs carry out this role. After the VLDLs deliver their triglyceride to the tissues, their remaining constituents are returned to the liver in the form of IDL and LDL. The third system mediates reverse cholesterol transport. This system, which involves HDL and LDL, returns excess cholesterol from extrahepatic tissues to the liver. However, the lipoprotein composition is considerably greater complexity. The major lipoprotein classes are heterogeneous as shown in Table 2.2

Apolipoprotein classes [7-10, 12, 13]

The surface coat of lipoprotein particle, whose lipids provide a covering structure that resembles the typical plasma membrane of cell, serves as an interface between the aqueous plasma and the inner nonpolar lipid core. This polar surface thus make possible the transport of the highly insoluble cholesterol esters and triglycerides in plasma. One or more apolipoproteins are present in each lipoprotein. According to the ABC nomenclature, the major apolipoprotein of HDL (alpha lipoprotein) is designated A. The major apolipoprotein of LDL (beta lipoprotein) is apolipoprotein B and is also found in VLDL and chylomicrons. Apolipoprotein C-I, C-II and C-III, are smaller polypeptides and are freely transferable between several different lipoproteins. The properties and characteristics of each lipoprotein were shown in table 2.3.

Copyright by Mahidol University

Table 2.2 Composition of Human Lipoproteins[1, 9]

		Percentage by Mass											
			Cholesterol			Apoproteins (% of Protein mass)							
Paticle	Mass(mDa)	TG	Total[a]	Free	CE	PL	Prot	AI	AII	B	C	D	E
Chylomicron	150-1000+	88	3	1	3	5	1-2	5-20	3-5	2-30	50-60	0	6-9
VLDL	5-130	59	12	5	12	16	7-10	1-2	<1	50-60	30-40	<0.1	5-8
IDL	3.5-4.0	20	29	9	3	20	11-17	<1	<1	80-90	10-15	<0.1	1-3
LDL	2.5-3.0	4	35	11	41	21	23	0.5	0.25	90-95	3-5	<0.1	1-4
HDL_2	0.36-4.0	5	13	5	13	35	33-42	85	8	0	7	1-2.9	0
HDL_3	0.20	3	12	3	15	23	56	72	22	0	5	1.5-2	0.4
Lp(a)	5.5	3	30	9	36	18	34	0	0	80-90[b]	0	0	0
β-VLDL(hep)	38	39	22	7	26	19	9	1	<1	55	27	-	17
β-VLDL(int)	82	43	26	7	32	14	4	0	0	34[c]	31	-	34
Lpx	5-10	3	28	25	5	61	6	1-2	<1	0	50	2-4	0

[a] Calculated from free and esterified cholesterol fractions. Cholesterol contribution from cholesterol ester =CE x387/650 where 387 is the molecular weight of cholesterol and 650 is the average molecular weight of cholesterol esters. [b]Also contains apoprotein (a) at 10-20%. [c] Present as B48. Other lipoproteins contain B100. TG. triglyceride; CE, cholesterol ester; PL, phospholipid; Prot, protein; VLDL,very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL , high density lipoprotein; Lp(a), lipoprotein (a); LpX, lipoprotein X ; hep, hepatic origin; int, intestinal origin.

Copyright by Mahidol University

Table 2.3 Apolipoproteins of human plasma lipoproteins.[9-11]

Apolipoprotein	Molecular Mass (Da)	Comments
Apo A-I	28,000	Main structural protein of HDL and ligand for HDL receptor. Required activator of lecithin cholesterol acyltransferase (LCAT). Identical to prostacyclin stability factor. Inhibits lysosomal cholesterol ester hydrolase
Apo A-II	17,000	Structural protein in HDL. May inhibit cholesterol mobilization from cells. Enhances hepatic triglyceride lipase activity.
Apo A-IV	46,000	Associated with the formation of triacylglycerol-rich lipoprotein. Found in HDL. May mediate binding of HDL to hepatocytes and peripheral cells. Can enhance lecithin cholesterol acyltransferase activity.
Apo B-100	550,000	Major structural protein for VLDL and LDL. Ligand for LDL receptor. Synthesized in liver.
Apo B-48	260,000	Major structural protein for chylomicron. Made only in intestine in man. Lacks binding site for LDL receptor.
Apo C-I	7,600	Minor activator of lecithin cholesterol acyltransferase. Inhibits clearance of β-VLDL by LDL receptor-related protein (a remnant receptor). Blocks phospholipase A-II.
Apo C-II	8,916	Required activator of lipoprotein lipase. Inhibits removal of triglyceride-rich lipoproteins from plasma.
Apo C-III	8,750	Stabilizes surface. Provide negative charge. Inhibits lipoprotein lipase. Inhibits removal of triglyceride-rich lipoproteins from plasma.
Apo-D	19,300	Appears to be involved in cholesterol ester exchange with pre-β HDL.
Apo-E	34,000	Binds to remnant and apo B-E (LDL) receptors. Normal function required for conversion of VLDL to LDL. Three major isoforms are seen (E2, E3 and E4). Defect or deficiency results in accumulation of β-VLDL in type III hyperlipidaemia.

Copyright by Mahidol University

The apolipoproteins of the surface coat serve several important functions [1]

1. They are required for the synthesis and secretion of specific lipoproteins.
2. They act to stabilize the surface coat and hence the whole lipoprotein particle.
3. They are cofactors in the activation of enzymes that modify the lipoproteins.
4. They can interact with specific cell surface receptors that remove lipoproteins from the circulation

Lipoprotein metabolism[1,10,13]

1. Chylomicron catabolism

Over 98% of dietary fat is typically absorbed. This occurs in the duodenum and jejunum. After a fatty meal, pancreatic lipase hydrolyzes ingested triglycerides to free fatty acids, mono-and diglycerides, and glycerol. Together with bile acids, these hydrolysis products form micelles in the lumen of the gut. The micellar contents are then absorbed into mucosal epithelial cells. Triglycerides are then resynthesized in the mucosal cells while most absorbed cholesterol is esterified. The cholesterol esters and triglycerides in the ER are then packaged into the core of chylomicra. After completion of assembly with proteins in the Golgi apparatus, nascent chylomicrons are secreted into the interstitium of intestinal villi and enter lacteals and then entry into the blood. There, on the surface of the chylomicron are found phospholipids (primarily lecithin), free cholesterol and apolipoproteins B- 48, A-I, A-II and possibly, small amounts of A-IV all acting as detergents to solubilize the hydrophobic core molecules for transport in the aqueous blood stream. Apo B-48 is a structural protein that remains with the

Copyright by Mahidol University

particle during its sojourn through the plasma. Its unique structure allows portions to dip down into the hydrophobic core while hydrophilic parts of the protein interact with the aqueous environment. Apo A-I and A-II can be removed, and other apoproteins such as apo C and apo E are acquired or exchanged during subsequent steps.

Newly synthesized chylomicra collect in intestinal lymphatics and are transported into the blood stream via the thoracic duct. Once in the blood stream, chylomicra travel to capillaries of tissue that either burn or store fat mainly, skeletal muscle, heart, and adipose. Within the lumen of such capillary beds, chylomicra encounter lipoprotein lipase, a hydrolytic enzyme bound to the endothelial wall by a heparin-like molecule. Lipoprotein lipase cleaves free fatty acids from the core triglycerides. Most of the free fatty acids are taken up into the tissue and reesterified into triglyceride while some free fatty acids are swept away into the blood stream after binding to albumin.

During lipolysis of chylomicra, components making up the surface shell of the chylomicron particle become redundant and pinch off to become new particles in the plasma or adsorb onto other lipoproteins. HDL are formed in this way utilizing the redundant phospholipids, free cholesterol, and apo A-I and A-II from the surface of chylomicra. A good deal of cholesterol is also stored in adipose tissue. Apo C-II is required for activation of lipoprotein lipase and is supplied by HDL prior to the chylomicron's encounter with lipoprotein lipase. After removal of most of the core lipids, apo C-II and C-III are recycled back to HDL. During their trip through the blood stream, chylomicra also acquire apo E which has transfered from HDL. After repeated encounters with lipoprotein lipase, a remnant particle is formed. Once

Copyright by Mahidol University

sufficiently reduced in size, chylomicron remnants can penetrate to the sinusoids of the liver and interact with receptors on liver parenchymal cells. Both the apo B-E receptor (also called the LDL receptor) and another remnant receptor (possibly LDL receptor-related protein) appear to remove large amounts of chylomicron remnants rapidly from the circulation. In addition to its role in hydrolysis of core triglycerides in triglyceride-rich lipoproteins, lipoprotein lipase may adsorb onto the lipoprotein and become an important cofactor for binding to the LDL receptor-related protein, thus promoting rapid removal of remnants. Apo C-III inhibit removal of remnants, thereby possibly protecting nonhydrolyzed particles from premature removal into the liver. Apo C-I was shown to specifically inhibit binding of chylomicron and VLDL remnants to the LDL receptor-related protein. Once in the liver, the chylomicron remnant is degraded to its constituent parts in lysosomes, releasing free fatty acids, free cholesterol, and amino acids after degradation of the lipoproteins.

2. VLDL catabolism

Between meals, another means must be available to provide fat fuel. This involves, essentially, transport of fat from adipose storage to active tissues such as muscle, liver, and heart. Rather than mobilize free fatty acids directly from adipose to the general circulation for utilization by heart and muscle, most of the fatty acids released from adipose between meals travel to the liver for packaging into very low density lipoproteins (VLDL). Virtually all of the necessary free fatty acids for VLDL triglyceride synthesis are provided by free fatty acid flux to the liver, mainly from adipose tissue. In fact, only 25 to 30 % of the free fatty acids taken up by the liver daily need to be esterified into VLDL triglyceride to supply the 30 to 100 g of VLDL

Copyright by Mahidol University

triglyceride flux observed daily in normal man. Most free fatty acids in the liver are burned as fuel or are partially burned, forming ketone bodies. Together with cholesterol ester, triglycerides make up the core of the VLDL, while free cholesterol, phospholipids, and apoproteins form the solubilizing surface constituents. For newly secreted VLDL, the structural apolipoprotein is B-100, while other apoproteins secreted with the nascent VLDL include the apo C and apo E. The size of secreted VLDL may be influenced by the availability of triglyceride, with larger VLDL being secreted when relatively more triglyceride is available. Increased cholesterol in the hepatocyte may also stimulate VLDL secretion. However, cholesterol may stimulate net secretion of VLDL because of reduced early removal of nascent VLDL by the LDL receptor, rather than supply a limiting substrate for secretion.

VLDL undergoes a virtually identical degradation process as the chylomicron, whereby core triglycerides are stripped out of the particle as free fatty acids and glycerol. Interactions of VLDL with HDL particles are also shown in Figure 2.3. Once most of the triglyceride has been removed from VLDL they become intermediate density lipoproteins (IDL). Further conversion to LDL can occur when VLDL are incubated in vitro with lipoprotein lipase. However, in the intact animal or in humans, conversion of VLDL to LDL appears to require the liver and normal apo E. Much, of the cholesterol ester found in VLDL and LDL is acquired from HDL via the action of cholesterol ester transfer protein. Cholesterol ester transfer protein mediates the transfer of cholesterol esters from HDL in exchange for triglycerides and phospholipids. A portion of VLDL or IDL cholesterol may be removed by the liver possibly mediated by hepatic lipase and / or apo E at the step of conversion to LDL.

Copyright by Mahidol University

Some VLDL or IDL is removed and degraded directly in the liver by way of the LDL pathway after binding to the LDL receptor. Larger VLDL appear to be preferentially removed by the liver prior to conversion to LDL.

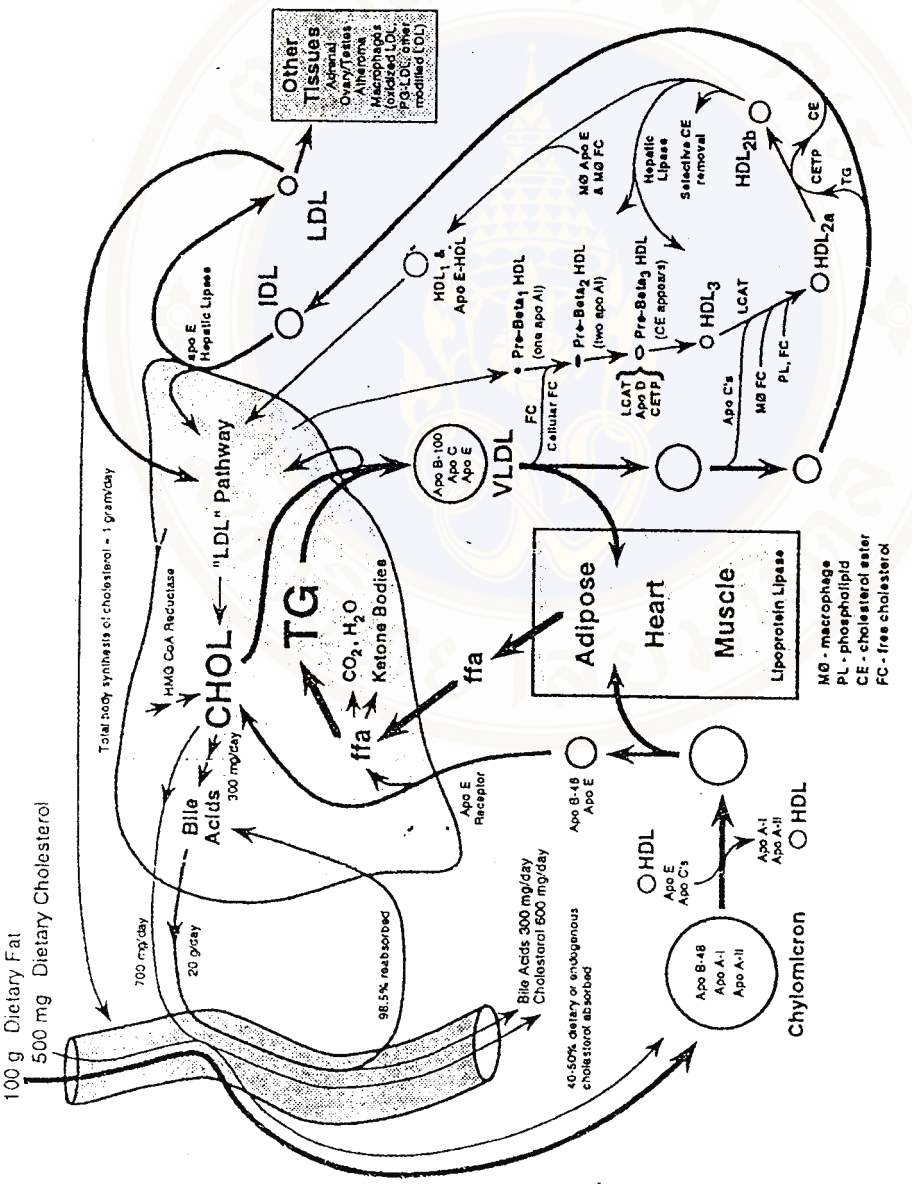


Figure 2. 3 An overview of lipoprotien metabolism[10].

Copyright by Mahidol University

3. LDL catabolism

Once LDL has been formed from VLDL, very little triglyceride remains in the particle, and the only apoprotein is B-100. Thus, VLDL gives up all its C apolipoproteins and apo E either to HDL or other lipoproteins before conversion to LDL. LDL carries about two-thirds of the total cholesterol in normal plasma. LDL may be used by any tissue requiring cholesterol such as for cell membranes during proliferation, for synthesis of steroid hormones, or for production of cholesterol-containing lipoproteins by the liver or intestine. A portion may also be removed by macrophages after modifications, such as oxidation or aggregation with proteoglycans or antibodies. In this fashion, LDL is thought to contribute significantly to the accumulation of foam cells in atherogenesis.

The overall removal rate of LDL is directly proportional to the LDL concentration with no obvious saturation in human studies. Nevertheless, a large fraction, about 50 to 70% of LDL, has been shown to be removed in man by way of a saturable mechanism called the LDL receptor pathway. The most important tissue for bulk removal of LDL is liver, possibly followed by intestine. Both these organs use cholesterol in production of lipoproteins. Most of the rest of LDL is removed by way of so-called scavenger pathways, which do not exhibit feedback control like the LDL pathway.

The LDL pathway for LDL uptake and degradation has been elegantly described by Michael Brown and Joseph Goldstein [13]. LDL uptake and degradation by way of the LDL receptor is closely coupled to cholesterol needs within each cell. When cholesterol is plentiful in a cell, an oxidized cholesterol derivative migrates to the

Copyright by Mahidol University

nucleus and interacts with a specific DNA-binding protein acting at sites called sterol regulatory element, thereby suppressing the synthesis of new LDL receptors. When the cell is depleted of cholesterol, new LDL receptors are synthesized and migrate to the cell membrane. After LDL receptors are transported to the surface of cells, and from there they migrate to special regions on the cell surface called coated pits, where they aggregate and wait for LDL particles to arrive. When the LDL receptors bind to a specific amino acid sequence on apo B-100 in circulating LDL, these calthrin-rich areas of cell membrane invaginate and pinch off to form phagocytic vacuoles within the cytoplasm. The vacuoles then fuse with lysosomes and the receptor-ligand complexes are internalized into lysosomes. A rapid drop in pH within the vacuole releases the LDL from its receptor. The receptors are recycled back to the cell surface to be used again. The cholesterol esters of LDL are hydrolyzed to unesterified cholesterol by cholesterol ester hydrolase, and apo B-100 is degraded to amino acids. If excess free cholesterol accumulates, it is esterified by the enzyme acyl-CoA: cholesterol acyl-transferase (ACAT). ACAT is also regulated by sterol regulatory elements, as is HMG-CoA reductase, the rate-limiting step in cholesterol synthesis. Linoleic acid is most commonly found esterified to cholesterol in LDL, whereas intracellular ACAT esterifies oleic acid to cholesterol. When cholesterol is needed again, the cholesterol ester is hydrolysed by cholesterol ester hydrolase.

4. HDL metabolism and reverse cholesterol transport.

HDL are found in various sizes with two or three principal density subtypes. Both the liver and small intestine secrete small or nascent HDL particles. These include

Copyright by Mahidol University

both a disc-shaped form of HDL and a round form sometimes called small HDL_3. The liver secretes particles containing apolipoprotiens AI, AII, and apo E. The intestinal product contains only apo AI and some apo AIV. Nascent HDL (disc and spheres) are rapidly converted in plasma to HDL_3. This occurs by adsorption of free cholesterol and phospholipids onto the surface of the HDL with subsequent synthesis of cholesterol ester by the enzyme lecithin-cholesterol acyltransferase, which requires apo AI as an activator. Lecithin-cholesterol acyltransferase is complex with HDL and transfers a fatty acid from lecithin to free cholesterol to form cholesterol ester, which is then sequestered within the core of the growing HDL particle. HDL_3 is more active than HDL_2 in promoting cholesterol removal from cells. Free cholesterol appears to be the main form of cholesterol removed by HDL_3. This occurs by passive diffusion of free cholesterol from the cell membrane to the HDL particle. A key determinant of this diffusion process is the ratio of free cholesterol to phospholipid in both the donor cell and the acceptor particle with free cholesterol diffusing down the gradient of this ratio. Lecithin-cholesterol acyltransferase plays a key role in maintaining a low free cholesterol concentration in HDL, favoring free cholesterol efflux from cells to HDL. After repeated interactions with cells and lecithin-cholesterol acyltransferase, HDL_3 expands to become HDL_2. Some investigators divide the HDL_2 subfraction into still smaller divisions of HDL_{2a} and HDL_{2b}. The HDL_{2a} appears to arise from HDL_3 after accumulation of additional cholesterol. When HDL_{2a} interacts with plasma cholesterol ester transfer protein, cholesterol ester is transferred from HDL to triglyceride-rich particles such as chylomicra, VLDL, or remnants. Triglyceride is transferred to HDL in exchange for the cholesterol ester. The enrichment of HDL_{2a} with triglyceride and

Copyright by Mahidol University

depletion of cholesterol ester may produce HDL_{2b}. These triglyceride-rich particles are then thought to interact with hepatic lipase, resulting in removal of excess triglyceride and regeneration of HDL_3. Furthermore, degradation of HDL phospholipids by hepatic lipase may drive free cholesterol into hepatic cells. Another possible fate for HDL_2 is the acquisition of apo E, phospholipid, and cholesterol from cholesterol-laden macrophages, thus yielding apo E-HDL or HDL_c (HDL_1). HDL_c is found in the blood of diet-induced hypercholesterolaemic animals. It is rich in cholesterol and its sole apolipoprotein is apo E. This HDL_c has an even greater affinity for the LDL receptor than LDL. Therefore, these particles can be directly remove and degraded by the liver of other cells with an active LDL receptor pathway without regeneration of HDL_c. In rabbits, 90% of plasma cholesterol ester was found to originate in the HDL fraction (from esterification of free cholesterol by lecithin-cholesterol acyltransferase). About 70% of HDL cholesterol ester was then cleared from the plasma compartment after transfer to LDL and VLDL (via cholesterol ester transfer protein or CETP). The another 20% of HDL cholesterol ester was cleared by "selective uptake". This process involves transient uptake of HDL particles in cholesterol needy cells, removal of some cholesterol ester, and subsequent release of the particle without degradation of the apoproteins. The remaining 10% of cholesterol ester in HDL was cleared in HDL particle containing apo E by direct degradation. In man , with about 25% of the cholesterol ester transfer activity of rabbits, aproportionately smaller portion of HDL cholesterol transport would be expected to occur by way of transfer to other particles, and a larger portion may occur by way of selective uptake.

Copyright by Mahidol University

Hyperlipidaemias[12]

Hyperlipidaemia is a conditions reflected an increase in blood lipoprotein levels which is either derived from increased synthesis due to a diet high in saturated fat and /or a genetically determined reduction in removal from blood (Table 2.4) as followings:

1. An increase in chylomicron or VLDL leads to increase in plasma triglyceride.
2. An increase in IDL or LDL leads to increase in plasma cholesterol.

Diseases of lipoprotein metabolism [2, 12]

The most common lipoprotein abnormalities due to the gene polymorphism or defect in lipoprotein metabolism will lead to the accumulation of excessive lipids in the blood plasma. These diseases collectively are known as the hyperlipidaemias, or more properly, the hyperlipoproteinaemias which cause the development of coronary artery disease.

Table 2.5 lists the phenotypic abnormalities of some apoprotein gene polymorphisms occur in patients with hyperlipoproteinaemias. Several of these diseases are risk factors for atherosclerosis and coronary heart disease. Table 2.6 shows the diseases of lipoprotein metabolism caused by single gene defects. However, these single gene defects will cause specific lipoprotein abnormalities and also are the risk factors of atheroscerlosis and coronary heart disease.

Copyright by Mahidol University

Table 2. 4 Hyperlipidaemia.[2,12]

	Typical lipid levels (mmol/L)					
	WHO pheno-type	Inherit-ance	Choles-terol	Triglyce-ride	Lipopoteins	Clinical signs
Lipoprotein lipase deficiency	I	AR	<6.5	10-30	Chylmicra↑	Eruptive xanthoma, xanthelasma, lipaemia retinalis, Hepatosplenomegaly
Polygenic hyper - cholesterolaemia	IIa	Polygenic	6.5-9	<2.3	LDL↑	Xanthelasma, corneal arcus
Familial hyper cholesterolaemia	IIa	AD	7.5-16	<2.3	LDL↑	Tendon xanthomas, corneal arcus, xanthelasma
Familial defective apoprotein B-100	IIa	AD	7.5-16	<2.3	LDL↑	Tendon xanthomas, corneal arcus, xanthelasma
Familial combined hyperlipidaemia	IIa,IIb, IV or V	AD	6.5-10	2.3-12	LDL↑, VLDL↑ HDL↓	Arcus, xanthelasma
Remnant particle disease	III	Polygenic	9-14	9-14	IDL↑	Palmar striae, tuberoeruptive xanthomas
Familial hypertrigly-ceridaemia	IV,V	AD	6.5-12	10-30	VLDL↑ chylomicra↑	Eruptive xanthelasma, lipaemia retinalis, Hepatosplenomegaly

WHO, World Health Organization; AR, autosomal recessive; AD, autosomal dominant; LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins; HDL, high-density lipoproteins.

Copyright by Mahidol University

Table 2.5 Apoprotien gene polymorphism[2,12]

Gene lucus	Associated abnormalities
Apo-A-I, C-III, A-IV	Hypertriglyceridaemia, low HDL
	Coronary heart disease
	Familial combined hyperlipidaemia
Apo-A-II	Hypertriglyceridaemia
Apo-B	Hypercholesterolaemia
	Coronary heart disease
	Hypertriglyceridaemia
Apo-E, C-I,C-II	Dysbetalipoproteinaemia
	Coronary heart disease

Table 2.6 Disease of lipoprotein metabolism caused by single gene defects[2]

Disease	Lipoprotein abnormality	Lipid abnormality	Metabolic basis
Familial hyper-cholesterolemia	LDL elevated	Cholesterol elevated	Decrease clearance of LDL from plasma, deficiency or abnormality in LDL receptor
Familial defective apolipoprotein B-100	LDL elevated	Cholesterol elevated	Decrease clearance of LDL from plasma. Familial result from genetic defect in apoB
Famial hypertri-glyceridemia	LDL elevated	Triglyceride elevated	Uncertain; VLDL overproduction or decreased catabolism
Familial dysbetali-poproteinemia	β-VLDL and IDL elevated	Cholesterol and triglyceride elevated	Decreased clearance of remnants; defective binding of apo E to LDL receptor
Familial lipoprotein lipase deficiency	Chylomicrons and VLDL elevated	Triglyceride elevated	Deficiency of lipoprotein lipase or apo C-II

Copyright by Mahidol University

Apolipoprotein B (apo B) and apolipoprotein E (apo E) as previously described are two of most a dozen protein constitutions of plasma lipoproteins that serve various function. They include the maintenance of the structure of lipoprotein particles and thus play a crucial role in lipid metabolism of several different lipoproteins. Not only their important roles in lipid metabolism, but apo B and apo E particularly in their polymorphisms also show the association with cardiovascular conditions[14, 15]. The epidemiological studies in world-wide populations have been shown that the rates of cardiovascular morbidity and mortality are increasing in each year[16]. Since the increase of blood lipid levels leading to hyperlipidaemia is multifactorial, it results from the interaction between a lifestyle and the genetic constitution. From the reason that, the genetic information is not altered by the disease process, time, or lifestyle, so the distribution of apoB and apo E allele frequencies and genotypes should be examined as genetic markers in Thai population. This may be the advantage to prevent the onset of hyperlipidaemia.

The genetic disorder of apo B and apo E gene are two of many the genetic disorder of hyperlipidaemia. Therefore, diagnosis based on genetic information of apo B and apo E genes have been a prime target for this study.

Copyright by Mahidol University

Apolipoprotein B

Apolipoprotein B is the main apolipoprotein of chylomicrons, very low density lipoprotein (VLDL) and low density lipoproteins (LDL). In 1980 it was found in the plasma in 2 main forms, apoB-48 and apoB-100. The two isoforms are important proteins in human lipoprotein metabolism. Apo B-48, so named because it appears to be about 48 % the size of apo B-100 on sodium dodecyl sulfate (SDS)-polyacrylamide gels, is synthesized by the intestine in humans[17]. Knott et al.[18] has reported the primary structure of apo B. The precursor contains 4,563 amino acids; the mature apo B-100 has 4,536 amino acid residues. This represents a very large mRNA of more than 16 kb. Apo B-48 is necessary for the assembly of chylomicrons and therefore has an obligatory role in the intestinal absorption of dietary fats and fat soluble vitamins. Apo B-100, which is produced in the liver in humans, is required for the synthesis and secretion of VLDL, LDL which contain about two thirds of the cholesterol in human plasma. The most of LDL apoprotein particles are metabolic products of VLDL. Apo B-100 is virtually the only protein component of LDL. Elevated concentrations of cholesterol and LDL cholesterol in plasma are recognized as risk factors for developing atherosclerotic coronary artery disease[19, 20].

Since, the two isoforms of apo B in lipoprotein are the central roles in metabolism and in the atherogenic potential of apo B-containing lipoproteins, apo B has been a prime target for study in the lipoprotein and arteriosclerosis field.

Copyright by Mahidol University

Over the past 10 years, significant progress has been made in understanding apo B, including the determination of the primary structure of the two isoforms of apo B, the organization of the apo B gene, and the delineation of the important functional domains of apo B[21].

A substantial amount of heterogeneity in the apo B gene has been documented, and multiple apo B gene mutations that affect the plasma cholesterol level have been reported. In the past few years, progress has been made in understanding the kinetics of apo B synthesis and secretion by cells and also the cellular pathways for the catabolism of apo B-containing lipoproteins. Additionally, in the past 10 years it has become apparently that biological modifications of apo B-containing lipoproteins may be important in understanding their atherogenicity.

Structure of apo B

In 1980 it was found that there are two primary forms of apo B in human plasma and thoracic duct lymph. One, found in LDL and VLDL, had an apparent molecular mass of 549 kDa, wherease the other, found in chymicrons of thoracic duct lymph and plasma had an apparent molecular mass of 264 kDa. The amino acid compositions were distinct, but immunochemical cross-reactivity was prominent. In order to facilitate comparison of apparent molecular weights of apo B species, a centile system of nomenclature was proposed in which the predominant form of apo B in human LDL is termed "apo B-100" and each other species or fragment of apo B is assigned a centile designation reflecting its apparent Mr relative to apo B-100. In this system the smaller species of human apo B was designated "apo B-48"[17].

Copyright by Mahidol University

In 1986 Cladaras et al [22] has reported the complete nucleotide sequence for the apo- B complementary DNA (cDNA) , along with the deduced amino acid sequence. The cDNA is 14,121 nucleotides in length, with 5' and 3' untranslated regions of 128 and 304 base pairs (bp) , respectively. The cDNA codes for a 27 -amino-acid hydrophobic signal peptide, which is cotranslationally cleaved, followed by a mature protein of 4,536 amino acids. Visvikis and Chan[23] have confirmed the existence of a 3-amino-acid insertion/deletion polymorphism in the apo B signal peptide, which is of no human functional significance. The sequence of the apo B-100 polypeptide chain is unique, although computer-assisted searches have identified remote but statistically significant internal amino acid repeats within apo B-100 . Computer sequence alignment programmes have also identified remote but statistically significant amino acid homologies with other lipproteins. These sequence homologies were predicted to form amphipathic helices. In addition, Weisgraber and Rall[24] have shown that apo B-100 binds strongly to heparin. Heparin binding sites on apo B may serve to promote the binding of triglyceride-rich lipoproteins to the capillary endothelium, where there lipid cores are digested by lipoprotein lipase.

Apo B-100 contains numerous hydrophobic domains throughout its length that are believed to be important in lipid binding[25]. In addition, Knott et al.[18] and others [26] have identified nine amphipathic helices, each one contains 22 amino acids in length, that are similar to amphipathic α-helices found in the putative lipid-binding domains of other apolipoproteins. Computer analysis of the predicted secondary structure of the protein showed that some of the potential α helical and β sheet structures are amphipathic. Cladaras et al[22] concluded that, these regions may

Copyright by Mahidol University

contribute to the formation of lipid binding domains of apo B-100. However, all of the apo B-100 sequences having the capacity to form amphipathic α-helices are located in the carboxyterminal containing half of the apo B-100 molecule. In addition, apo B contains multiple proline-rich sequences predicted to form amphipathic β-sheets and β-turns; these are located throughout the apo B sequence except for the amino terminal 1,000 amino acids. Although amphipathic β-sheets are not found in other apolipoproteins, these structures are thought to have high lipid-binding potential. Thus, apo B has many potential lipid-binding regions throughout its length, a result thought to be adequate for explanation of the fact that apo B never exchanges between lipoprotein particles, in contrast to the other apolipoproteins, which have one or two putative lipid-binding domains and readily exchange between lipoproteins[26]. Chen and coworkers[27] have reported experimental evidence that many different apo B peptides are capable of binding to lipoprotein particles.

In 1989 Phillips et al.[28] and others[29] examined the apo B-100 of LDL particles by electron microscopy (EM) after the particles had been treated with glutaraldehyde and their lipid extracted; the images thus produced strongly suggestion that apoB-100 may indeed encircle LDL particles, as shown in Figure 2.4.

New EM techniques have recently been developed the visualize apo B on the surface of LDL particles. The binding of apo B-specific monoclonal antibodies whose epitopes have been precisely localized can also be directly visualized. These studies should improve our understanding of apo B on LDL particles.

Copyright by Mahidol University

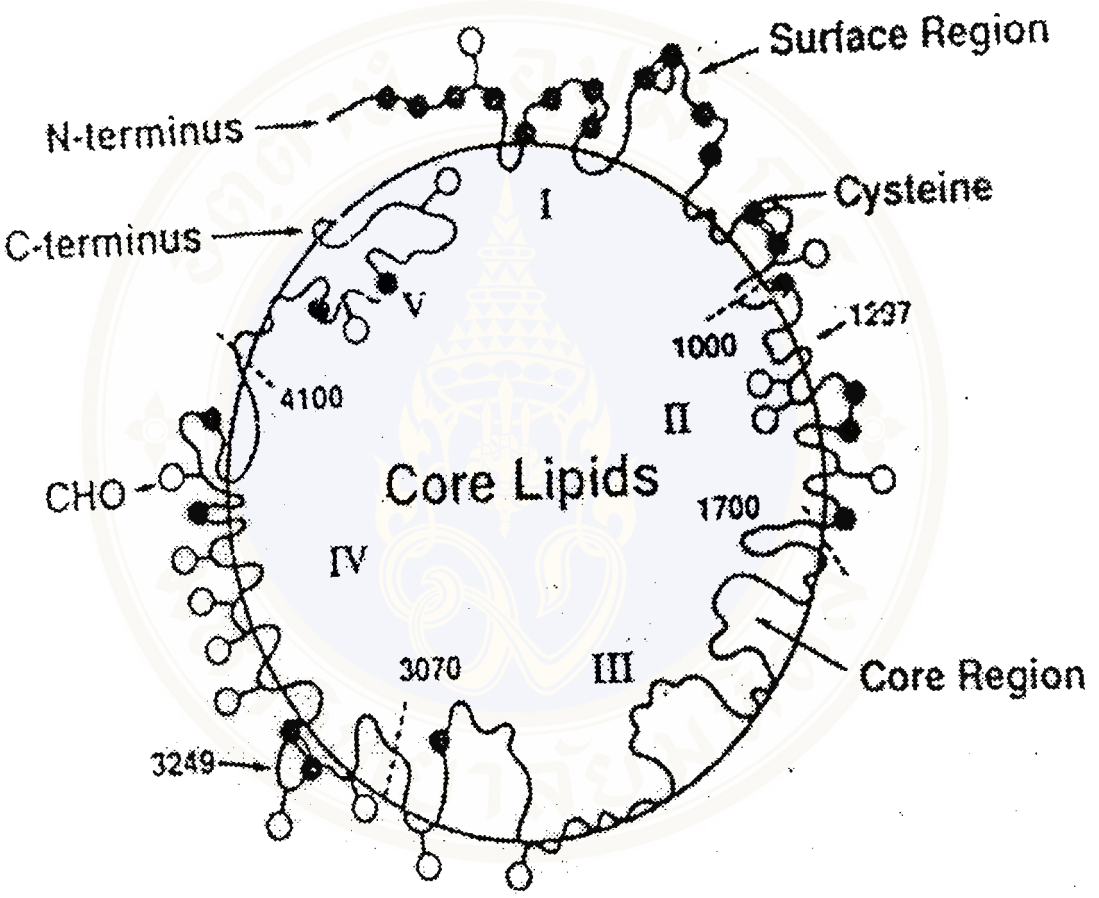


Figure 2.4 Schematic of apolipoprotein (apo) B-100 structure on low-density lipoprotein particle[28].

Copyright by Mahidol University

For the apo B-100 binding domain of LDL receptor, it has been observed in the carboxyterminal portion of the molecule. The receptor-binding region of apo B-100 has been recently studied in detail by Milne and coworkers[30]. They determined the location of the epitopes of more than 30 apo B-specific monoclonal antibodies and which of the antibodies were capable of inhibiting the binding of LDL particles to the LDL receptor. The antibodies with epitopes located between amino acids 2,980 and 3,780 completely block the specific binding of LDL to the LDL receptor when bound to LDL. Antibodies that bind to epitopes immediately flanking this region partially block binding of LDL to its receptor, whereas monoclonal antibodies binding elsewhere in the molecule had for the most part little or no receptor-blocking activity. A summary of the efforts of Milne and coworkers is shown in Figure 2.5. The regions of apo B containing the clusters of positively charged amino acids are known to be evolutionarily well conserved. Positively charged regions of apo B are thought to bind to the many negatively charged amino acid residues within the cysteine-rich repeats of the ligand-binding domain of the LDL receptor[12].

Finally, as discussed above, the missense Arg→Gln, Arg→Cys, and Arg→Gln mutations in the codon for apo B-100 at amino acid 3,500, 3,531 and 3,611, respectively are associated with defective binding of LDL to the LDL receptor.

Copyright by Mahidol University

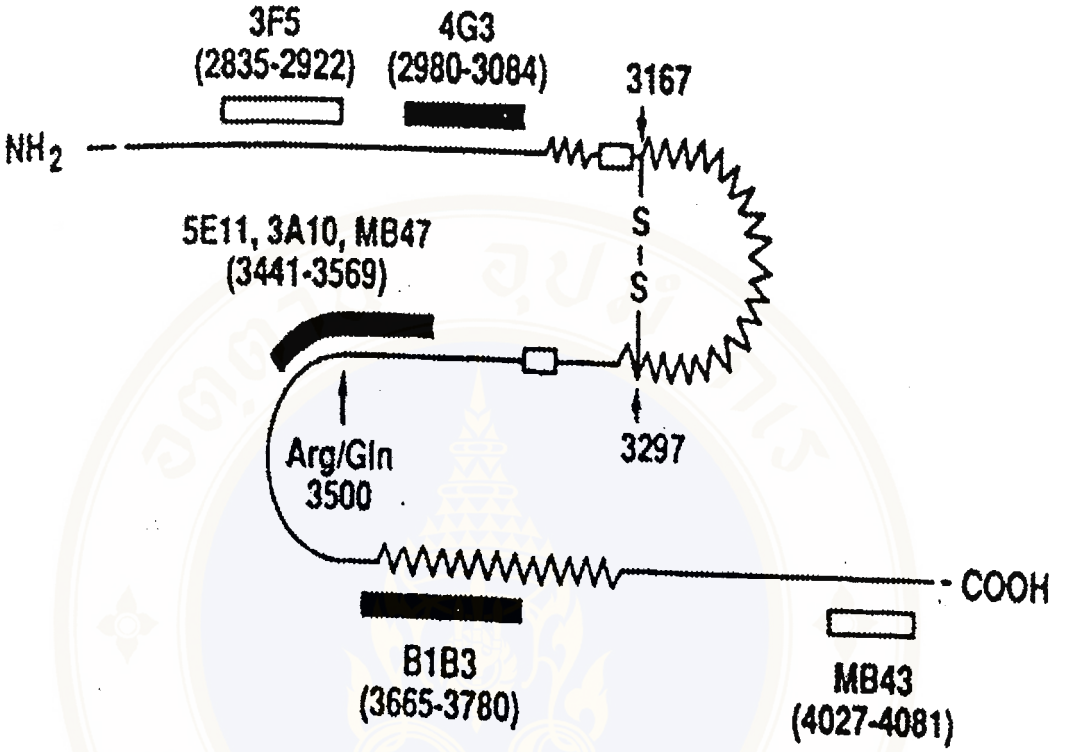


Figure 2.5 Schematic of proposed structure of the putative binding domain of apolipoprotein (apo) B[30]

Specificity of Apo B synthesis in liver and intestine.

The nature of the B apoproteins synthesized by human liver and intestine has been the subject of several studies. Deeb et al[31] used a hybridization probe to detect homologous sequences in both flow-sorted and in situ metaphase chromosomes. They found that RNA isolated from monkey small intestine contained sequences homologous to the cDNA of apolipoprotein B-100. These results were interpreted as indicating that intestinal (apo B-48) and hepatic (apo B-100) forms of apo B are coded by a single gene.

Copyright by Mahidol University

Apo B-100 (the full-length form of the protein) is by far the predominant form of apo B in plasma LDL and VLDL. Only apo B-100 has been found to accumulate in the culture medium of a human hepatoma cell line (Hep G2)[32]. Furthermore, newly synthesized apo B in samples of human liver consists exclusively of apo B-100, in fetuses as well as adults [33]. Apo B-48 is the predominant form of apo B in human thoracic duct lymph chylomicrons[17]. However, from several data we conclude that the specific of apo B-100 and apo B-48 are synthesize in liver and intestine, respectively.

Apo B Gene

In 1985 Lusis et al[34] identified cDNA clones for human apoB; examination of a somatic cell panel indicated that the apo B gene resides on chromosome 2. Mehrabian et al[35] localized APOB to 2p24-p23 by somatic cell hybridization and in situ hybridization. Filter hybridization studies with genomic DNA and with hepatic and intestinal mRNA suggested that hepatic and intestinal apoB are derived from the same gene. The apo B gene, which is located on chromosome 2, spans approximately 43 kb and contains 29 exons and 28 introns; two of the exons, exons 26 and 29, are extremely long (7,572 and 1,906 bp, respectively) (Figure 2.6). Exon 26, which codes for amino acids 1,379-3,903, is threefold longer than any previously reported exon of an mammalian gene. Whether the two large exons arose from fusion of smaller exons is unknown. Six " Alu-type" repetitive sequences are present in all the introns of the gene. The first there are in the 5'→ 3' orientation, and the other there in the opposite orintationThere is an AT-rich hypervariable region within 200 bp of the polyadenylation site of the last amino acid codon that consists of a variable number of

Copyright by Mahidol University

15-bp hypervariable elements. There are at least 14 different 3' hypervariable minisatellite region alleles containings 25-52 repeats, and about 75% of the population is heterozygous for different alleles at this site. The number of repetitive elements at the hypervariable regions can be rapidly and accurately assessed with polymerase chain reaction-based techniques; Therefore, the utility of these regions for epidemiological and family studies is large.

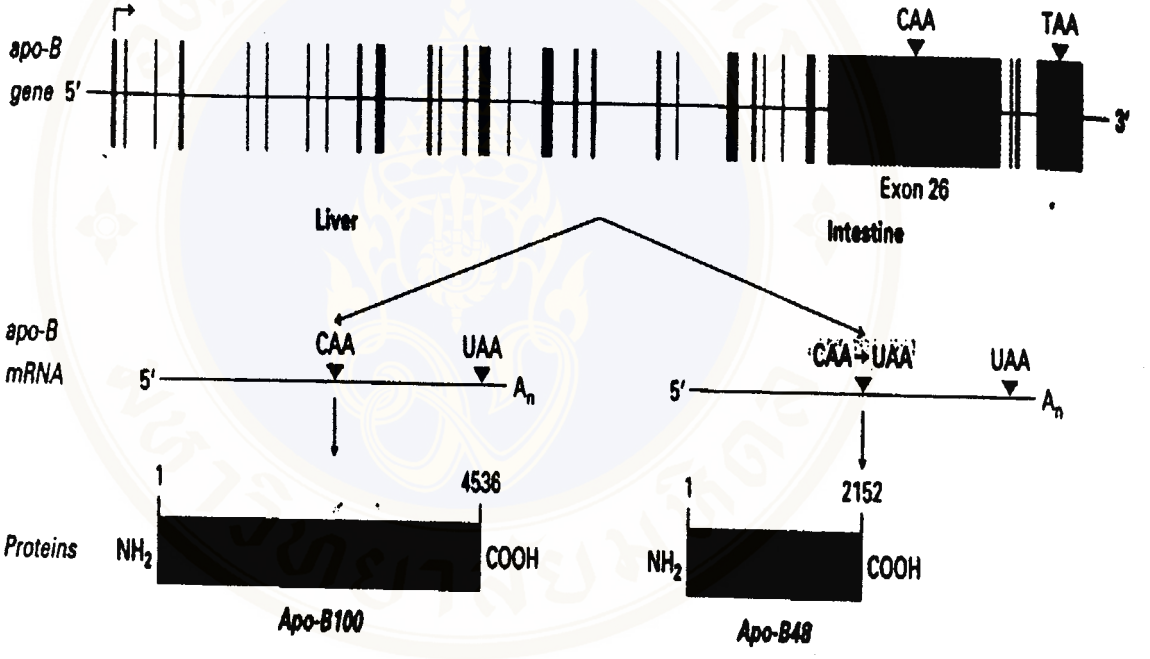


Figure2.6 RNA editing of apo-B mRNA. The apo-B mRNA produced in the liver has the same sequence as the exons in the primary transcript. This mRNA is translated into Apo B-100, which has two functional domains: a N-terminal domain (black) that associates with lipids and a C-terminal domain that binds to LDL receptors in cell membranes. In the apo B mRNA produced in the intestine, the CAA codon in exon 26 is edited to a UAA stop codon. As a result, intestinal cells produce apo B-48, which corresponds to the N-terminal domain of apo B-100[36].

Copyright by Mahidol University

LIBRARY AND INFORMATION CENTER MAHIDOL UNIVERSITY

Mechanism of apo B-48 formation: Two proteins from a single gene

In addition to the structural relation of apo B-48 to apo B-100, the mechanism for apo B-48 formation was almost simultaneously determined in 1987 by Chen et al[37] and Powell et al[38] and later by Hsopattankar et al[39]. These investigators demonstrated that apo B-48 is produced from the apo B-100 gene by a novel mechanism involving mRNA editing. In sequencing the apo B cDNA isolated from human intestinal cDNA libraries, they found that the intestinal apo B cDNA contained a T at nucleotide 6,666, in contrast to the C found at the position in the liver apo B cDNA clones (Figure 2.6). The substitution of the T for a C at nucleotide 6,666 yields an in-frame stop codon (TAA) that replaces the CCA codon specifying apo B-100 amino acid Gln at position 2,153. The location of the stop codon predicts that apo B-48 found in plasma would contain the amino terminal 2,152 amino acids of apo B-100; this prediction was strongly supported by the protein-sequencing efforts of Chen et al[37] as well as by the studies of Hardman et al[40]. This substitution at nucleotide 6,666 was present in the intestinal mRNA but not in intestinal genomic DNA, so it was clearly the result of a specific form of posttranscriptional editing of the intestinal apo B mRNA. Apo B mRNA editing to create an in-frame stop codon has been shown to occur in rat and rabbit intestinal apo B mRNA and rat liver, which is known to produce both apo B-48 and apo B-100. A fraction of the human intestinal apo B mRNA is 7-8 kb in length because of the use of alternate polyadenylation signals after the edited nucleotide (Figure 2.6). The process in humans appears to be developmentally regulated; intestinal organ cultures drawn from fetuses in early gestation primarily make apo B-100, whereas the adult intestine makes apo B-48 and very little, if any, apo B-100. The

Copyright by Mahidol University

44852 ; เวชศาสตร์

efficiency of apo B mRNA editing in rat liver has recently demonstrated modulation by fasting / refeeding and thyroid hormone.

The exact mechanism for apo B mRNA editing remains unclarified, but the most plausible hypothesis is that a tissue-specific enzyme recognizes a specific sequence within the apo B mRNA and deaminates position 4 of cytosine-6,666, creating a uracil residue. A reasonable hypothesis suggests that a specific RNA editing process would require conservation of the nucleotide sequence surrounding nucleotide 6,666 through evolution. Indeed, sequencing studies of the apo B gene from several species have shown that the sequence of the region flanking nucleotide 6,666 is highly homologous (90%) among the mouse, rat , rabbit, and human genes. However, Chen et al[37] have recently used site- directed mutagenesis techniques to introduce 22 different mutations into a nine-base region flanking nucleotide 6,666. Unexpectedly, they discovered that most of the constructs were edited, implying that the editing mechanism may be at least partially lax. Two groups have reported data on the length of the apo B sequence required for apo B editing. Bostrom and coworker[41] inserted two lengths of the apo B cDNA flanking nucleotide 6,666 into the protein - coding sequence of an apo E expression vector. When a 354-bp segment of apo B cDNA flanking nucleotide 6,666 was inserted into the expression vector and the expression vector transfected into an intestinal carcinoma cell line, editing of the mRNA at nucleotide 6,666 occurred. However, no editing was observed when a 63-bp portion of apo B cDNA flanking nucleotide 6,666 was inserted into the expression vector. Davies and coworkers[42] have also examined the question of sequences required for the apo B editing process. They transfected constructs containing eight different

Copyright by Mahidol University

lengths of apo B cDNA flanking nucleotide 6,666 (ranging in size from 26 to 2,385 bases) into McArdle 7777 cells, a rat hepatoma cell line that synthesizes and secretes apo B-48. They found that mRNA editing occurred with each of the constructs, revealing that as few as 26 nucleotides surrounding base 6,666 were sufficient for mRNA editing to occur. Davies et al[42] reported that an RNA-folding computer programme predicted that the edited nucleotide exists in a conserved eight-nucleotide loop in an mRNA stem loop structure.

Driscoll and coworkers[43] have demonstrated that the RNA editing mechanism can be observed in vitro with cytoplasmic extracts from McArdle 7777 cells. They inserted various lengths of the apo B cDNA surrounding base 6,666 into appropriate plasmid vectors, and various lengths of the apo B RNA were synthesized in vitro. Editing of the synthetic RNAs containing as few as 55 bases of the apo B sequence was demonstrated with cytoplasmic extracts, Editing was specific for RNA but not DNA and was destroyed by proteinase K, suggesting that the editing activity involves a protein. Results of gel retardation assays using extracts from an apo B-48-producing cell line have suggested the presence of a factor that binds specifically to the appropriate region of the apo B mRNA . Efforts to purify this activity are under way in several laboratories. Scott and coworkers[44] have recently developed transgenic mice containing a 2-3- kb construct spanning the apo B-48 editing site. Apo B mRNA was identified in multiple tissues, and substantial amounts of editing activity were documented in the intestine, spleen, and lung. Lower levels of editing activity were documented in the liver, brain, and heart. The physiological rationale for editing in this

Copyright by Mahidol University

broad spectrum of tissues is not known, but these data certainly present the possibility that editing might occur for gene products other than apo B.

Editing of the intestinal apo B mRNA to create an in-frame stop codon was unprecedented in the molecular biology of mammals, and its existence raises many questions. Is the C at base 6,666 simply deaminated to produce a U, or is it possible that the base is modified in some other way so that it is read by the ribosome and reverse transcriptase as a U? Outside the nine-base region examined by Chen et al, [45] what are the nucleotide specifications of the editing process? What process signals the use of alternate polyadenylation sites after the edited nucleotide in the human intestine? Is RNA editing the result of a single protein or a complex comprising several proteins? Does the RNA editing process exist only for the apo B mRNA , or is it a mechanism that is used for other transcripts in other tissues? These questions are important , and at least some of the answers will probably forthcoming in the next 2-3 year. However, a more fundamental question for the lipoprotein metabolism field should also be considered. Why is there a need to make two forms of apo B, and why has the process been so conserved through evolution? Currently, there is no clear answer to third question. Apo B-100 seems perfectly capable of participating in the assembly of large, triglyceride-rich particles. What physiological role does apo B-48 have in the intestine that would not be equally well served by apo B-100? Perhaps apo B-48 can accommodate more apo E molecules than can apo B-100 particles, resulting in more efficient and rapid delivery of dietary fats to the liver. Scott et al [44] recently postulated that apo B-48 may have evolved for the purpose of efficient delivery of antioxidants to the liver, where they may protect nascent hepatic lipoproteins from free radical attack.

Copyright by Mahidol University

Apo B gene polymorphism

The gene coding for apoB is highly polymorphic, including the insertion/ deletion polymorphisms and some other mutations [46]. An insertion or deletion of three codons involving three amino acids, leucine-alanine-leucine[47], within exon 1 of the apoB gene cause variation in the length of the signal peptide of the apoB protein (Figure 2.7). This amino terminal cleavable signal sequence directs the emerging protein to translocate through the endoplasmic reticulum membrane. Thus this signal peptide polymorphism within the apoB gene may play a role in affecting the translocation of the apoB polypeptide into the endoplasmic reticulum. Formally, the only way to prove that a detected DNA sequence change is of functional importance is by in-vitro expression studies, and to date, these have only been carried out on apo B signal peptide variants. Using a yeast expression system, constructs were made in which signal peptide variants (SP27 and SP24) were fused to the yeast secretory protein, invertase, and the secreted invertase activity and overall secretion efficiency were measured. Relative to SP27, the SP24 variants mediated inefficient translocation into the endoplasmic reticulum. One prediction from these data would be that plasma lipid levels in individuals with the SP24 allele are less responsive to increase in dietary fat[48-52]. The SP24 allele showed a much smaller change in plasma lipids in response to dietary fat manipulation compared with individuals with the SP27 allele. The signal peptide length polymorphism have shown an increased frequency of the SP24 allele in patients compared with control individuals. The interpretation of these inconsistencies is that the apoB signal peptide variation is associated with and may be of direct

Copyright by Mahidol University

functional importance in determining differences in plasma lipids [52-55]. Several previous studies have investigated the impact of this polymorphism on plasma lipid levels. Some studies have found the correlations between the ins/del polymorphism and lipid levels[56-59].

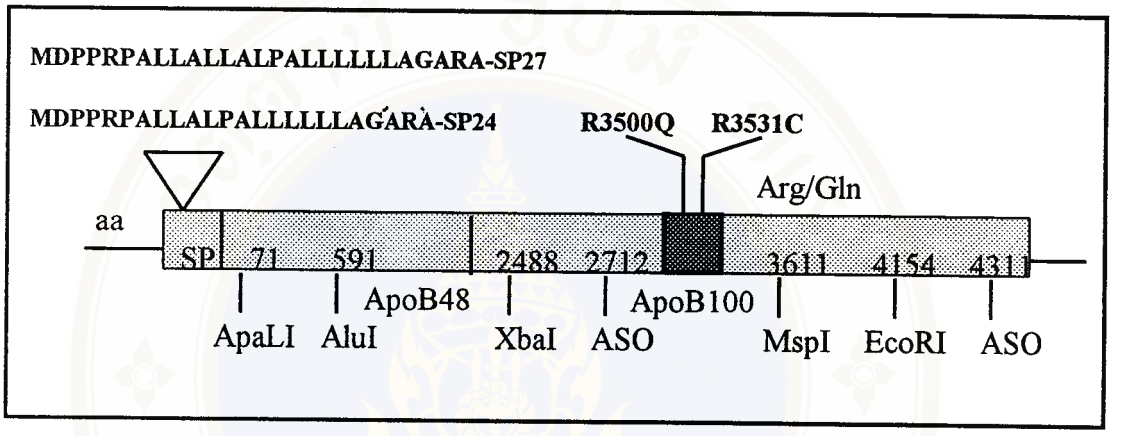


Figure 2.7 Cartoon of the apolipoprotein B (apoB). The filled box depicts the LDL receptor binding domain. SP represents the signal peptide of the 27 and 24 amino acid (aa) variants of the peptide. Restriction fragment length polymorphism detected by restriction enzymes (RE) are ApaLI, AluI, XbaI, MspI and EcoRI. R3500Q, the substitution of Arg by Gln at aa residue 3500; R3531C, the substitution of Arg by Cys at aa residue 3531. The method of detecting the base change at the DNA level is by RE, which is specified[53].

Mutation in apo B gene associated with high blood cholesterol levels

The majority of LDL particles are removed from plasma by the interaction of apo B with the cellular LDL receptor. Goldstein, Brown, and coworkers[12] demonstrated that the gene for mutation in the LDL receptor protein can retard the

Copyright© by Mahidol University

clearance of LDL particles and lead to an accumulation of LDL particles in the plasma. Vega and Grundy[60] showed that some patients have reduced clearance of LDL not because of decreased activity of LDL receptors but because of a defect in the structure (or composition) of LDL that reduces its affinity for receptors. In 5 of 15 patients, turnover rates indicated that clearance of autologous LDL was significantly lower than that for homologous normal LDL. In these 5 patients, autologous LDL appeared to be a poor ligand for LDL receptors.

A defect in the LDL metabolism not only is there delayed receptor-mediated clearance of LDL particles from plasma, but the rate of LDL particle production is increased. LDL production rate is increased because the apo E-mediated hepatic uptake of LDL precursors is delayed as a result of decreased LDL receptor activity; consequently, an increased fraction of these precursor particles is metabolize to a LDL. Most patients with familial hypercholesterolaemia have total cholesterol levels well above 300 mg/dl. For years lipoprotein investigators speculated that a specific inherited defect in apo B could abolish its ability to bind to the LDL receptor and therefore cause delayed LDL clearance and an accumulation of LDL particles in the plasma. Such a defect would not be expected to produce the same degree of increase in the plasma LDL concentration as that observed in familial hypercholesterolaemia. Although the removal of LDL from the plasma would be retarded because of the defect in the apo B protein on LDL particles. The predicted production rate of LDL would be essentially normal because the apo E-mediated removal of LDL precursor particles would be predicted to proceed at a normal rate.

Copyright by Mahidol University

The genetic defect in apo B that interferes with its capacity to bind to the LDL receptor has been characterized. The several observations were made by Vega and Grundy[60] during lipoprotein turnover studies that were designed to understand the metabolic basis for moderate hypercholesterolaemia. For their study, they selected subjects with total cholesterol levels of 250-300 mg/dl, deliberately excluding subjects with overt clinical signs of familial hypercholeaterolaemia. In metabolic ward studies, they compared the fractional catabolic rate of each patient's autologous LDL with that of homologous LDL obtained from normolipidaemic control subjects. In five subjects, the clearance of the patient's own LDL was significantly slower than that of the control LDL, resulting in the speculation that these patients had abnormal LDL that was not removed at a normal rate by the LDL receptor. Later, in collaboration with Vega and Grundy[60], Innerarity and coworkers[61] examined the ability of these five subjects showed defective binding to the LDL receptor, this defect having only about 32% of the normal receptor-binding activity. Studies with partially delipidated LDL showed an identical binding defect, strongly suggesting that the defect resided in the protein moiety of apo B on LDL particles. An identical binding defect was identified in the LDL of several hypercholesterolaemic family members. In 1987 FDB (Familial defective apolipoprotein B-100) was first described as a genetic disorder by Innerarity and coworkers[61]. They proposed that all of the affected family members were heterozygotes for a defect in apo B that prevented its interaction with the LDL receptor, and they designated the disorder familial defective apo B-100 (FDB), characterized by delayed clearance of LDL from plasma. Weisgraber et al[62] found an antibody, whose epitope is between residues 3350 and 3506 in exon 26 of apoB, that

Copyright by Mahidol University

distinguishes abnormal LDL from normal LDL in this disorder; the antibody MB47 bound with a higher affinity to abnormal LDL. Thus, an assay was provided for screening large populations for this disorder. Illingworth et al[63] found that LDL cholesterol was reduced after administration of lovastatin in 12 hypercholesterolemic patients from 10 unrelated families with familial defective apo B-100. By extensive sequence analysis of the 2 alleles of the apo B gene of a subject heterozygous for familial defective apolipoprotein, Soria et al[64] demonstrated a mutation in the codon for amino acid 3500 that results in the substitution of glutamine for arginine. This same mutant allele was found in 6 others, unrelated subjects and in 8 affected relatives in 2 of these families. A partial haplotype of this mutant apo B-100 allele was constructed by sequence analysis and restriction enzyme digestion at positions where variations in the apo B-100 are known to occur. This haplotype was found to be the same in 3 probands and 4 affected members of 1 family and lacks a polymorphic XbaI site whose presence has been correlated with high cholesterol levels. Thus, it appears that the mutation in the codon for amino acid 3500 (CGG-to-CAG), a CG mutational 'hotspot' defines a minor apo B-100 allele associated with defective low density lipoproteins and hypercholesterolaemia. Ludwig[65] and Motti et al[66] used 10 markers for haplotyping at the apo B locus in cases of familial defective apolipoprotein B-100: 8 diallelic markers within the structural gene and 2 hypervariable markers flanking the gene. In 14 unrelated subjects heterozygous for the mutation, 7 of 8 unequivocally deduced haplotypes were identical, and 1 revealed only a minor difference at one of the hypervariable loci. The genotypes of the other 6 affected subjects was consistent with the same haplotype. Familial defective apolipoprotein B-100 (FDB) results from a G-

Copyright by Mahidol University

to-A transition at nucleotide 10708 in exon 26 of the APOB gene. These data are consistent with the existence of a common ancestral chromosome.

In a screening for the Apo B-3500 mutation by PCR amplification and hybridization with an allele-specific oligonucleotide. Loux et al[67] have found only 1 case among 101 French subjects with familial hypercholesterolaemia. The son of this individual, a 45-year-old man, was also found to have the mutation. Bryong et al[68] was found 9 heterozygous in 432 hyperlipidaemic subjects, which attended in lipid clinics in Scotland and Wales. Haplotype analysis revealed strict identity to that previously reported by Ludwig and McCarthy[65], thus supporting a unique European ancestry. The family lived in the southwest of France and had no knowledge of Germanic origin. Rauh et al[69] stated that the frequency of the arg3500-to-gln mutation has been found to be approximately 1/500 to 1/700 in several Caucasian populations in North America and Europe. On the other hand, Friedlander et al[70] found no instance of this mutation in a large screening programme in Israel. They pointed out that the mutation has also not been found in Finland and is said to be absent in Japan[71]. Tybjaerg-Hansen and Humphries[72] gave a review suggesting that the risk of premature coronary artery disease in the carriers of the mutation is increased to levels as high as those seen in patients with familial hypercholesterolaemia; at age 50, about 40% of males and 20% of females heterozygous for the mutation have developed coronary artery disease. Data from ^{13}C MRI studies suggest that the mutation has effects on the disposition of charged residues in apo B of much greater potential consequence to ligand function than would be expected with the loss of a single unit of cationic charge at a receptor-interactive site[73] Definitive proof of this will ultimately require the characterization of

Copyright by Mahidol University

the apo B-containing lipoproteins produced by cells transfected with a human apo B-100 expression vector and the lipoproteins produced by cells transfected with an apo B expression vector differing only in the codon for amino acid residue 3,500. In dual-label fibroblast binding assay, LDL from the eight subjects with the mutation had an affinity for the LDL receptor that was 63% that of control LDL. LDL from eight unaffected family members had an affinity of 91%. By way of comparison, LDL from six patients heterozygous for the Arg3500Gln mutation had an affinity of 36%[74].

To date, all subjects with familial defective apo B-100 have been heterozygous for the mutation; typical total cholesterol levels for these subjects are 250-300 mg/dl elevated, the average of levels reported for 61 individuals in four surveys was 260 mg/dl but lower than that of familial hypercholesterolaemia heterozygotes. However, a recent study from Europe has demonstrated that some affected subjects may have the extremely elevated cholesterol level characteristic of patients with familial hypercholesterolaemia. As a result of the high cholesterol levels, it is most probable that these individuals will have an increased incidence of atherosclerotic disease. However, the exact frequency of the amino acid substitution at the residue 3,500 mutation has been reported between 1 in 500 and 1 in 700 individuals of European and North American descent [69]. A high prevalence of FDB in Switzerland [75] (1 in 210 individuals), suggests the origins of this mutation to be in central Europe. From the screening of miscellaneous populations, many investigators have reported that one in every 500 people of this mutation has been found[76-77]. This frequency might be in the same range as the LDL receptor gene mutations. The mutation in amino acid residue 3,500 as in several of the mutations caused by a mutation in a CG dinucleotides. CG

Copyright by Mahidol University

dinucleotides are hot spots mutation in higher animals, and base substitution in CG dinucleotides are important in the pathogenesis of many inherited diseases in humans. An important question about the amino acid 3,500 mutation is whether this mutation occurred independently in different human populations, as has been documented with specific thalassemia mutations, or only once in a single "founder" and then subsequently propagated among subgroups in the general population. In a patient homozygous for the Arg3500Gln mutation, Schaefer et al[78] found LDL cholesterol and apo B concentration approximately twice normal, whereas apo E plasma level was low. Using a stable-isotope labeling technique, they obtained data showing that the in vivo metabolism of apo B-100-containing lipoproteins in FDB is different from that in familial hypercholesterolaemia, in which LDL receptors are defective. Although the residence times of LDl apo B-100 appeared to be increased to approximately the same degree, LDL apo B-100 synthetic rate was increased in FH and decreased in FDB. The decreased production of LDL apo B-100 in FDB may originate from enhanced removal of apo E-containing LDL precursors by LDL receptors, which may be upregulated in response to the decreased flux of LDL-derived cholesterol into hepatocytes. Almost all individuals with familial defective apo B-100 are of European descent, and in almost all cases the mutation is on a chromosome with a rare haplotye at the apo B locus, suggesting that all probands are descended from a common ancestor in whom the original mutation occurred. Distribution of the mutation is consistent, with an origin in Europe 6,000 to 7,000 years ago. Ludwig and McCarthy [65] have performed apo B haplotyping studies with the amino acid 3,500 mutation on nine unrelated subjects and their families have determined that this mutation is invariably found on the same apo B

Copyright by Mahidol University

haplotype. These data suggest that the mutation has probably occurred only once. If there was only one founder for the amino acid 3,500 mutation, then it is probable that future studies will reveal that this mutation will occur with greater regularity in selected countries or ethnic populations.

The region of the apo B molecule interacting with the LDL receptor may be quite large (Figure 2.5); thus, it seems probable that other apo B amino acid substitutions could interfere with its ability to bind to the LDL receptor, thereby producing the familial defective apo B-100 phenotype[79]. The other mutations in exon 26 of apo B -100 gene have been identified such as Arg3531Cys and Arg3611Gln, which are associated with a minor decrease in LDL receptor binding [74, 80]

Hypercholesterolemia due to ligand-defective apolipoprotein B (apo B) Arg3531Cys.

Suspecting that mutations in the APOB gene other than the arg3500-to-gln mutation may cause familial hypercholesterolaemia, Pullinger et al.[74] used single-strand conformation polymorphism analysis to screen genomic DNA from patients attending a lipid clinic and looked for mutations in the putative LDL receptor-binding domain of apoB-100. They found a novel arg3531-to-cys mutation, caused by a C-to-T transition at nucleotide 10800, in a 46-year-old woman of Celtic and Native American ancestry with primary hypercholesterolaemia and pronounced peripheral vascular disease. After screening 1,560 individuals, one unrelated 59-year-old man of Italian ancestry was found to have the same mutation. He had coronary heart disease, a total cholesterol of 310 mg/dl, and an LDL cholesterol of 212 mg/dl. A total of 8 individuals

Copyright by Mahidol University

were found with the same defect in the families of these 2 patients. The age- and sex-adjusted TC and LDL-C were 240 and 169, respectively, for the 8 affected individuals, as compared with 185 and 124, respectively, for 8 unaffected family members. In a dual-labeled fibroblast binding assay, LDL from the 8 subjects with the mutation had an affinity for the LDL receptor that was 63% that of control LDL. LDL from 8 unaffected family members had an affinity of 91%. By way of comparison, LDL from 6 patients of heterozygotes for the arg3500-to-gln mutation had an affinity of 36%. Deduced haplotypes using 10 APOB gene markers showed the arg3531-to-cys alleles to be different in the 2 kindreds and indicated that the mutations arose independently. This was the second reported cause of familial ligand-defective apoB.

Arg3611Gln of apo B-100 gene

The study of an MspI polymorphic site in exon 26 of the apo B gene was reported by Huang et al[81]. The DNA change from CGG to CAG causes an amino acid substitution of glutamine for arginine at residue 3611 of the mature apo B and causes loss of an MspI restriction site. Demant et al[82] found a significant association between a particular RFLP of the apo B gene and the total fractional clearance rate of LDL. Presumably, this effect acts through variable binding of this region to the LDL receptor (LDLR) and is a significant factor in the rate of catabolism of LDL. Williams et al[83] demonstrated association of specific alleles for the apo B gene with obesity, high blood cholesterol levels, and increased risk of coronary artery disease. This variations in serum cholesterol level were associated with functional allele corresponding to amino acid variants at positions 3611, which lie near the LDLR binding region of apo B. Products of the apo B gene with high or low affinity for the MB-19 monoclonal

Copyright by Mahidol University

antibody can be distinguished. Gavish et al[84] used this antibody to identify heterorzygotes and detect allele specific differences in the amount of apo B in the plasma. A family study confirmed that the unequal expression phenotype was inherited in an autosomal dominant manner. However, in the repot of Xu et al[80] no significant association was found between this RFLP and serum cholesterol and apo B level. From these studies, a simple relationship between apo B mutation at codon 3611 and atherosclerotic risk has not yet been clearly established.

Apolipoprotein E (apo E)

Human apolipoprotein E (apo E) is a constituent of plasma very low density and high density lipoproteins and is important in modulating the catabolism of remnants of triglyceride-rich lipoproteins. Apo E is recognized by hepatic B/E or LDL receptors which mediates the clearance of apo E containing lipoprotein particles[85].

Apolipoprotein E (apo E) polymorphism is a genetic determinant of plasma lipid levels and of coronary heart disease risk. Three common alleles E2, E3, and E4 control the polymorphism of apolipoprotein E. These code for proteins which differ in functional properties, e.g. receptor binding activity and in vivo catabolism. This explains the significant effect of the apoE gene locus on the variability of plasma lipoprotein concentrations and moreover the implication of apoE alleles in the aetiology of multifactorial forms of hyperlipidaemia, e.g. familial type III hyperlipidaemia (apo E 2; arg158--cys) and polygenic hypercholesterolaemia (apo E 4; cys112--arg)[86-89]

Copyright by Mahidol University

Apolipoprotein E gene

The apo E gene occurs on chromosome 19 and is closely linked to apo C-I, and apo C-II. In addition, the LDL receptor has also been mapped to this chromosome, but apparently they are not closely linked to apo E. The apo E gene is 3.7 kilobase in length and contains four exons. The apo E messenger RNA (mRNA) is 1163 bp in length. The primary translation product comprises 317 amino acids, with the 18 N-terminal amino acids serving as a signal peptide. Thus, the mature apo E is secreted as 299 amino acid protein with a *Mr* of 34,200[90].

Structure and function of apolipoprotein E

Apolipoprotein E, a protein with a relative molecular mass (*Mr*) of 34,200, is a constituent of several plasma lipoproteins, including chylomicrons, chylomicrons remnants, VLDL, IDL, and a subclass of HDL, which participates in the transport of lipids among various tissues and cells. A major physiological role for apo E in lipoprotein metabolism is to mediate the high affinity interaction of apo E containing lipoprotein with lipoprotein receptors, including the LDL receptor (apo B, E receptor) and a postulated chylomicron remnant or apo E receptor[91,92].

Lipoprotein binding to the receptors initiates the cellular uptake and degradation of lipoproteins, which leads to the use of the lipoprotein cholesterol in the regulation of intracellular cholesterol metabolism. Apolipoprotein E shares this function with apo B, the protein constituent of LDL particle[93].

The predicted secondary structure of apo E, based on the Chou-Fasman algorithm, is shown in Fig 2.8. The α-helix, β-sheet, β-turn, and random structure are

Copyright by Mahidol University

predicted to make up 62%, 9%, 11%, and 18% of the protein, respectively. The α-helical content has been determined experimentally for human and rabbit apoE to be ~65% and ~70%, respectively[93].

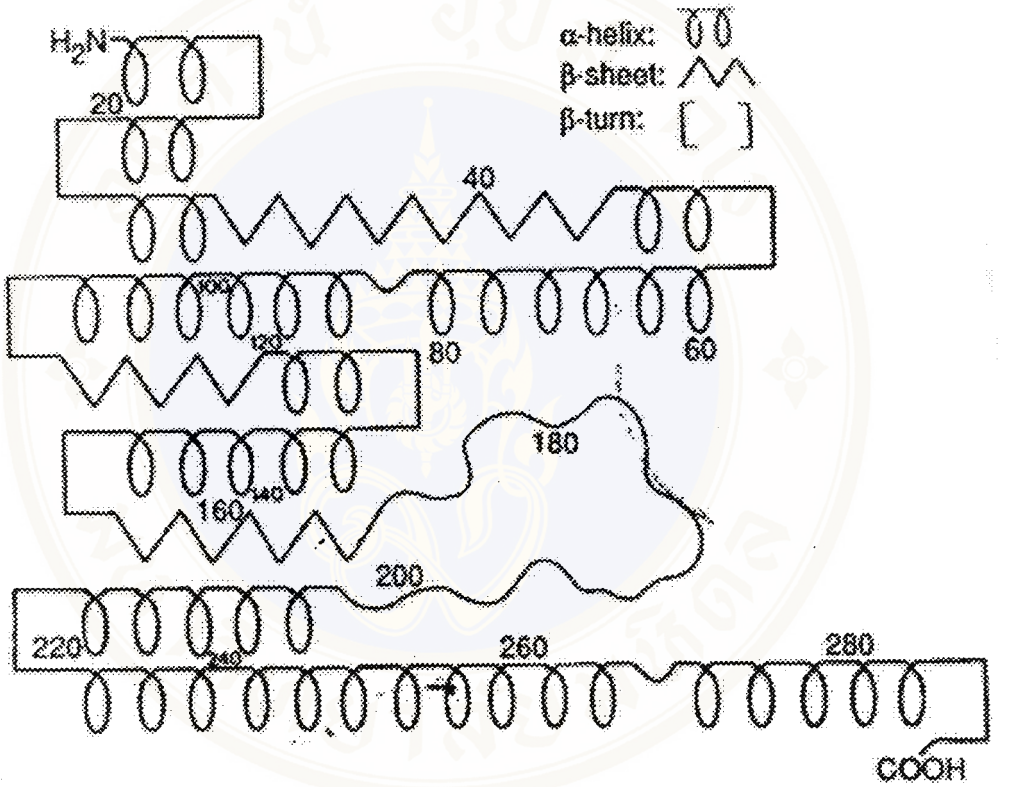


Figure 2.8 Predicted secondary structure of apo E, showing α -helixes, β -sheet structure, and β -turns. The remainder of the molecule is predicted to have a random structure[93].

The three dimensional structure of apo E3 and variants apo E2 and apo E4 is providing unique insights into the structure of the receptor binding domain and other functional domains of apo E. The N-terminal two-thirds (residues 1 to 191) of the apo E-3, containing the receptor binding domain, occurs as a four-helix bundle[94].

Copyright by Mahidol University

An important observation is that residue158 of apo E (the site for the common variant that is defective in receptor binding) lies outside the patch of positive potential. Previously, Innerarity et al[95] had speculated that the substitution of cysteine for arginine at residue 158 disrupts receptor binding secondarily by altering the interaction of this part of the molecule with the critical basic residues in the 136 to 150 region. The crystal structure of apo E3 versus apo E2 (the variant) confirms this postulate[96]. In apo E3 the arginine at residue 158 is involved in two salt bridges, one with aspartic acid 154 in helix 4 and the other with glutamic acid 96 in helix 3. However, in apo E2, where cysteine is substituted for the normally occurring arginine at residue 158, the aspartic acid 154 now forms a salt bridge with arginine 150 and swings the side chain of argingine 150 into the new plane. The reorganization of the salt bridges undoubtedly exerts an impact on the structure within the 136 to 150 region. Thus, it is envisioned that basic residues within the 136 to 150 region interact directly with the LDL receptor.

The structural properties of apo E also affect the distribution of its different isoforms among the different plasma lipoproteins. Apo E3 and apo E2 are preferentially associated with HDL, whereas apo E4 is preferentially associated with VLDL. At residue 112, apo E4 differs from apo E3 and apo E2. Apolipoprotein E4 has arginine at this position; apo E3 and apo E2 have cysteine. Thus, it appears that residue 112 modifies lipoprotein distribution[97]. However, it is known that the major lipid binding domain of apo E occurs in the C-terminals of the molecule (residues ~200 to 280)[98]. These observations have led to the postulate that the N-terminal and C-terminal domains of apo E interact in some way to determine lipid binding specificity

Copyright by Mahidol University

and that residue 112 or residues in the vicinity of 112 are involved in this interaction. The crystal structure of apo E4 reveals that the arginine-cysteine interchange at residue 112 causes a profound local change in the structure of the molecule. The occurrence of arginine at residue 112 in apo E4 results in the formation of a salt bridge between arginine 112 and glutamic acid 109. This ionic interaction causes the side chain of arginine 61 in the adjacent helix to swing into a new plane. By comparison, the cysteine at residue 112 of apo E3 results in a different orientation for glutamic acid 109 and arginine 61. These structural changes could influence the interaction of the N- and C-terminal domains of apo E and thus alter lipoprotein specificity. The association of the specific isoforms with different lipoproteins would be expected to alter lipoprotein metabolism. In association with the occurrence of specific apo E mutations that affect receptor binding, the distribution of apo E (VLDL vs. HDL preference) has an impact on the expression of type III hyperlipoproteinaemia.

Polymorphism of apolipoprotein E

Studies of apo E phenotype and allelic frequency among various populations around the world have revealed interesting ethnic differences and demonstrated that the apo E genotype has major effect on plasma lipid levels and possibly on cardiovascular risk[15, 99-100]. The polymorphic nature of apo E was establised by Utermann and coworkers[101] using isoelectric focusing, as further clarified by Zannis and Breslow[102-103] using two-dimension electrophoresis. The three major isoforms of apo E, refered to apo E2, E3, and E4, which are products of three alleles E2, E3 and E4 at a single gene locus.

Copyright by Mahidol University

The molecular basis for apo E polymorphism has been established by Mahley and coworker[104]. They determined the primary structure of apo E and found that the isoforms E4, E3 and E2 differed from one another by single amino acid substitutions at two sites in the protein (Figure 2.9). The existence of the single amino acid substitutions confirmed that E2, E3 and E4 arose from separate alleles at a single gene locus. These substitutions also explain the single unit change differences among the three isoforms since they involve the substitution of the neutral amino acid cysteine for the basic amino acid arginine.

Amino acid substitutions accounted for the differences among apo E4, E3, and E2. Apolipoprotein E4 differs from apo E3 in that in apo E4 arginine is substituted for the normally occurring cysteine at amino acid residue 112. The most common form of apo E2 differs form apo E3 at residue 158, where cysteine is substituted for the normally arginine. The charge differences among the three isoform detected by isoelectric focusing is explained by the single amino substitutions. A secondary form of apo E polymorphism is explained by posttranslational glycosylation. The glycosylated (sialylated) isoforms of apo E apparent in plasma resulted from the attachment of a carbohydrate chain to a single site at theronine residue 194 in apo E. The asialo isoform, which accounts for about 80 percent of the total apo E in plasma, was found to have no carbohydrate moiety at all. It is unclear whether the major apo E isoform without carbohydrate represents product of deglycosylation. It is possible that the minor glycosylated isoforms, including those sialylated, are products of a subset of cells synthesizing apo E.

Copyright by Mahidol University

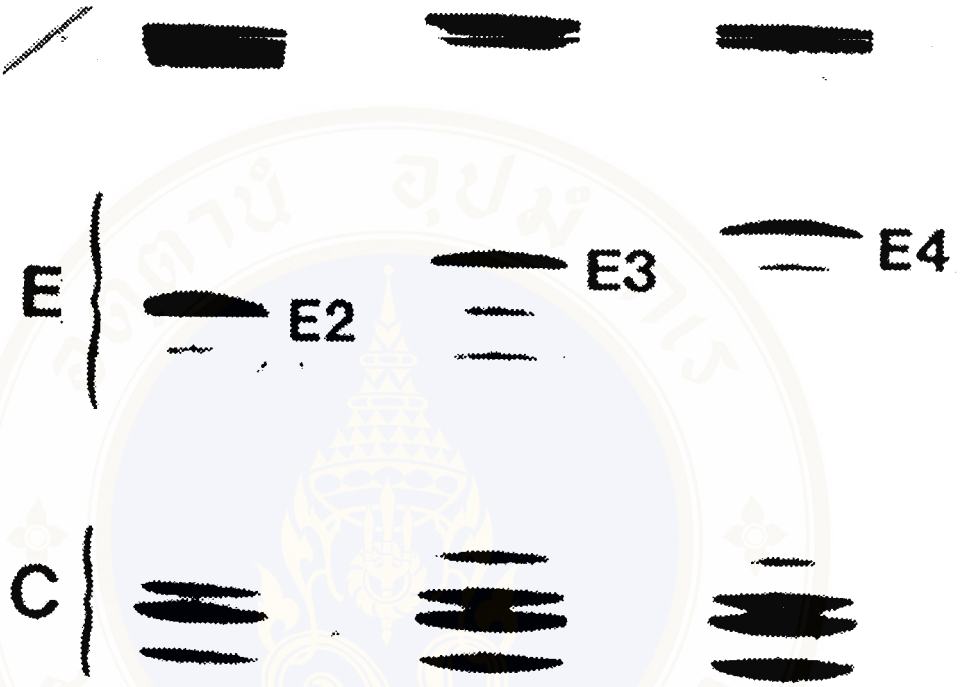


Figure 2.9. Isoelectric focusing of apo-VLDL demonstrating the three homozygous apo E phenotypes. The amino acid substitutions that account for the charge differences among the isoforms are shown. The minor, more acidic apo E isoforms in each case represent sialylated isoforms. The E2/E2 phenotype is from a type III hyperlipidaemic subject[103].

Three isoforms of apo E are commonly produced by genetic polymorphism, which are referred to as apo E2, apo E3 and apo E4. The corresponding gene polymorphisms are termed ε2, ε3, and ε4. There is one allele on each chromosome containing the apo E gene, so that an individual may possess three similar (homozygotes) or three different (heterozygotes) apo E gene polymorphisms. The

Copyright by Mahidol University

following genotypes are thus possible: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4. The corresponding lipoprotein phenotypes are E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, E4/E4. The distribution of the phenotypes in general populations of other countries are shown in table 2.7.

Table 2 .7 Apolipoprotein E phenotype and frequencies in various vopulations

	Phenotype						Allele		
	E4/E4	E4/E3	E3/E3	E3/E2	E2/E2	E4/E2	ε4	ε3	ε2
USA (n=152)	3.0	14.0	58.0	22.0	1.3	2.0	11.0	76.0	13.0
Canada (n=102)	3.9	20.6	61.8	9.8	2.0	2.0	15.2	77.0	7.8
Germany (n=1000)	2.3	20.2	62.7	11.0	0.8	3.0	13.9	78.3	7.8
Germany (n=1031)	2.8	22.9	59.8	12.0	1.0	1.5	15.0	77.3	7.7
Austria (n=469)	2.1	17.3	64.0	13.2	1.3	2.1	11.7	78.9	9.0
Scotland (n=400)	1.0	24.8	58.3	12.8	0.5	2.8	15.0	77.0	8.0
Hungary (n=202)	1.0	22.8	65.3	7.9	2.0	1.0	12.9	80.7	6.4
Iceland (n=185)	3.2	23.2	60.0	10.3	0	3.2	16.5	76.8	6.8
Finland (n=615)	6.3	31.9	54.0	6.7	0.3	0.8	22.7	73.3	4.1
Newzealand (n=426)	0.9	25.1	51.4	20.0	1.4	1.2	14.1	73.9	12.0
Sigapore (Malay) (n=118)	3.4	16.1	60.2	16.9	1.5	0.8	11.9	76.7	11.4
Sigapore (Indian) (n=142)	1.4	21.8	68.3	7.0	0.7	0.7	12.7	82.7	4.6
Sigapore (Chinese) (n=190)	1.1	11.6	69.5	15.3	1.6	1.1	7.4	82.8	9.7
Japan (n=319)	1.3	11.3	72.1	13.8	0.6	0.9	7.4	84.6	8.0
Sudan (n=103)	8.7	35.9	39.8	9.7	1.0	4.9	29.1	61.9	8.1

The most common phenotype is apo E3/E3 (typically ~50 to 70 percent of the population) and the most common allele is ε3 (typicaly ~70to 80 percent); therefore apo E3 is considered to be the parent form of the protein. The less frequently occurring ε4 and ε2 alleles nonetheless contribute significantly to the gene pool (typically with 10 to 15 percent for ε4 and ~5 to 10 percent for ε2). As a result of the lower frequencies

Copyright© by Mahidol University

of the ε4 and ε2 alleles, the phenotypes E4/E4, E4/E2, and E2/E2 are relatively rare (Table 2.7).

Initially, the prominence of apo E3 suggested that it was the wild-type of the protein and that apo E4 and apo E2 were variants. However, it now appears that E4 is most likely the ancestral allele. Almost all animals including the higher primates such as the baboon, possess the equivalent of apo E4 homozygosity (arginine at the residue corresponding to amino acid 112 in the sequence). In addition, aboriginal human groups, such as the Huli of the Papua New Guinea highlands, have ε4 as the most common allelic form. The evolution of the allelic forms of apo E is of considerable interest to population geneticists and suggests that there is a selective advantage to the occurrence of apo E3 and possibly apo E2 in humans. Alternatively, genetic drift could be responsible for the disparities in allelic frequency between the lower species and humans.

Boerwinkle et al[105] and others[106-107] also observed statistically significant differences in apo E allele frequencies among different ethnically and/ or geographically distinct population. As illustrated in Table 2.7, allelic frequency has interesting variations in different racial groups. The apo E gene frequency in most European and North American populations is rather homogeneous (E3, 76 to 79 percent; E4, 11 to 15 percent; and E2, 6 to 9 percent). An obvious exception is the Finnish population[108-109], in which E4 is much more prevalent (23 to 24 percent). On the other hand, Asians [106, 110] (specifically Chinese and Japanese) have a relatively higher frequency of E3 (~82 to 85 percent). By contrast, African nationals from the Sudan[110] and Nigeria[111] have lower E3 (~61 percent) and higher E4 (~30 percent). A study comparing the apo

Copyright by Mahidol University

E genotype in black and white men in the United states[112] revealed that blacks resembled the Sudanese and Nigerians (E3, 80.3 percent in white vs. 65.3 percent in blacks; E4, 11.9 percent in whites vs. 23.2 percent in blacks; E2, 7.7 percent in whites vs. 11.5 percent in blacks)[113,114]. One of the interesting population studies compares the apo E phenotypes of two isolated cultural groups in Papua New Guinea, the Huli and the Pawaia[115]. The Huli are characterized by an extremely high frequency of the E4 allele and low frequency of the E3 allele (E4, 49.0 percent; E3, 35.6 percent; E2, 15.4 percent). The Pawaia have a very different apo E phenotype pattern, more similar to that of blacks (E3, 60.3 percent; E4, 25.9 percent; E2, 13.8 percent).

Apolipoprotein E polymorphism is one of the common genetic factors responsible for inter-individual differences in lipid and lipoprotein levels. While the E3 allele showed no deviations from the populations mean, Sing and Davignon[116] found that the ε4 and ε2 alleles had significant effects on various lipid and lipoprotein parameters.

The ε2 allele has been shown to be associated with lower levels of plasma cholesterol, LDL cholesterol as compared with levels in individuals with ε3. Conversely, the ε4 allele is associated with higher levels of total cholesterol and LDL cholesterol. Utermann et al[101] have reported that apo E2 is found significantly more frequently in hypertriglyceridaemic subjects, while apo E4 is observed more frequently in hypercholesterolaemic subjects. The original observations made by Utermann et al [101] and Davignon et al.[117] have evaluated the data in seven different studies that included Caucasian and Asian populations and have demonstrated a remarkably consistent pattern: individuals with the E2/E2 phenotype have the lowest plasma

Copyright© by Mahidol University

cholesterol levels, while those with the E4/E4 phenotype have the highest (the difference between cholesterol levels for E2/E2 vs. E4/E4 individuals ranges from 25 to 66 mg/dl).

For these same populations, Davignon et al[117] calculated the average cholesterol effect of each of the common alleles (ε2, ε3, and ε4). The difference in the average effects for ε2 versus ε4 ranged from 13 to 30 mg/dl. Compared to the cholesterol-elevating effect of the ε4 allele, the ε2 allele was shown to have a greater impact on lowering plasma cholesterol. This is considered to be an unusually large effect from a single gene on an individual trait. Because of the two different high affinity to bind to receptor of lipoproteins that contain the difference apo E genotype. The situation in apo E2 homozygotes seems to be paradoxical. Because of the failure of apo E2 (Arg158 to Cys) to bind to LDL (B/E) and apo E receptors, an accumulation of remnant lipoproteins and hence hyperlipidaemia as expected to result. However, most of E2 homozygotes have subnormal rather than elevated cholesterol and low LDL. This may be explained by the fact that because of the delayed catabolism of lipoprotein that contain apo E, less cholesterol of exogenous origin (remnants) and from the periphery (apo E-HDL) enters the liver through apo E mediated uptake. For compensation LDL (B/E) receptors may be up regulated, resulting in an enhanced uptake LDL and hence a lowering of LDL in plasma. Furthermore, a delay in the interconversion of IDL to LDL may also contribute to the low LDL in the plasma of E2 homozygotes. Because of this, it has been proposed that apo E2 may even be protective against development of cardiovascular disease in the absence of exacerbating factors. Some of these exacerbating factors include high fat and calorie diet, age,

Copyright by Mahidol University

obesity, hyperthyroidism, diabestes, and (in woman) oestrogen status. A similar thought of opposite mechanism was account for the association of the E4 allele with hypercholesterolemia. The in vivo turnover studies by Gregg et al.[118] have demonstrated that apo E4 has a greater preference for association with triglyceride-rich lipoproteins compared with apo E2 and E3, which both prefer HDL. As this result, apo E4 is catabolized more rapidly than apo E3. Because of the enhanced catabolism of lipoproteins that contain apo E4, more cholesterol is delivered to liver cells by apo E4 mediated uptake in subjects with an E4 allele. This theoretically should result in a down regulation of LDL (B/E) receptors in apo E4/E4 and possibly also E4/E3 subjects and in turn in an elevation of LDL in their plasma. Thus the gene E4 may be one factor involved in the pathogenesis of polygenic or multifactorial forms of hypercholesterolaemia. In every study, the ranking of the apo E phenotypes from the lowest to the highest cholesterol levels was E2/E2, E3/E2, E3/E3, E4/E2, E4/E3, and E4/E4. Dallongeville and coworkers [119] have confirmed and extended these observations using meta-analysis to compare data in 45 pupulations in 17 countries. Consistently, regardless of differences in ethnicity and in metabolic conditions, they confirmed that the E2 allele was associated with lower plasma cholesterol levels, and that compared to the E3 allele, E4 was associated with higher cholesterol levels.

In a number of studies, E2 has been shown to be significantly associated with hypertriglyceridaemia, and Sing and Davignon[116] have found that the E4 allele was associated with lower plasma triglyceride levels. Gregg and coworkers[1125] have shown that apo E2 is catabolized slower in vivo than apo E3. An intriguing observation is that individuals with the E4/E3 phenotype also have elevated triglyceride levels.

Copyright by Mahidol University

Apolipoprotein E4, which is preferentially associated with triglyceride-rich lipoproteins may interfere with lipolysis of lipoprotein lipase or with higher uptake of these lipoproteins.

Copyright by Mahidol University

CHAPTER III

MATERIALS AND METHODS

1. Subjects

All of the subjects used for this study were selected mainly based on the plasma total cholesterol or triglyceride levels as followings:

1.1 Control subjects

Sixty-eight healthy normal control subjects obtained from the Department of Preventive and Social Medicine, Faculty of Medicine Siriraj Hospital , Mahidol University, Bangkok, Thailand, were recruited with: Total cholesterol ≤ 200 mg/dl and Triglyceride ≤ 200 mg/dl, subdivided into 44 women and 25 men aged from 21 to 76 years (mean age 38.3±12.4 years)

1.2 Hyperlipidaemic subjects

One hundred and three primary hyperlipidaemic subject obtained from Dyslipoproteinaemia and Obesity clinics, Department of Preventive and Social Medicine, Faculty of Medicine Siriraj Hospital , Mahidol University, Bangkok, Thailand were selected with : either Total cholesterol > 200 mg/dl or Triglyceride > 200 mg/dl , subdivided into 68 women and 35 men aged from 33 to 76 years (mean age 52.26±9.07 years). Subjects with hypertension, diabetes mellitus, coronary heart disease, renal failure, and endocrine or other metabolic disorders were excluded.

Copyright by Mahidol University

2. Chemicals

Chemicals used in this study were analytical and molecular biological grades

Chemical name	Chemical structure	Molecular weight	Company	Country
1. Absolute ethanol	C_2H_5OH	46.07	E. Merck	Germany
2. Acrylamide	-	-	Biorad	USA
3. Agarose	-	-	Sigma	USA
4. Ammonium chloride	NH_4Cl	53.49	Sigma	USA
5. Ammonium persulfate	$(NH_4)_2S_2O_8$	228.19	BDH	England
6. Boric acid	H_3BO_3	61.85	E.merck	Germany
7. Bromophenol blue	-	-	BDH	England
8. Deoxyribonucleotide triphosphates (dNTP)	-	-	Qiagen	Germany
9. Ethidium bromide	$C_{12}H_{20}N_3Br$	394.31	Sigma	USA
10. Ethylene diamine tetraacetic acid(EDTA)	$C_{10}H_{14}N_2Na_2O_8$	32.24	E.merck	Germany
11. Guanidine Hydrochloride	CH_5N_3HCl	95.53	Sigma	USA
12. Isopropanol	C_3H_8OH	60.10	Sigma	USA
13. Magnesium chloride	$MgCl_22H_2O$	303.30	BDH	England
14. N,N'-methylenebis acrylamide	-	-	Biorad	USA
15. Mineral oil	-	-	Sigma	USA
16. Sodium acetate	CH_3COONa	82.3	BDH	England
17. Sodium chloride	NaCl	58.4	BDH	England

Copyright by Mahidol University

Chemical name	Chemical structure	Molecular weight	Company	Country
18. Sodium citrate	$C_6H_5Na_3O_7$	294.1	Sigma	USA
19. Sodium dihydrogen phosphate	$NaH_2PO_4.2H_2O$	156.01	BDH	England
20. Sodium dodecyl sulfate(SDS)	$C_{12}H_{25}O_4SNa$	288.38	Sigma	USA
21. Sodium hydroxide	NaOH	40	BDH	England
22. N,N,N',N',-tetramethyl - ethylene diamine (TEMED)		116.21	Fluka	Japan
23. Tris [hydroxymethyl- aminomethane]	$C_4H_{11}NO_3$	121.10	Sigma	USA
24. Tris [hydroxymethyl- aminomethane]hydrochloride	$C_4H_{11}NO_3.HCl$	157.6	Sigma	USA
25. Urea	CH_4N_2O	60.06	Sigma	USA

3. Enzymes

Restriction enzymes used were as followings:

1) HhaI, Pharmacia, USA

2) MspI,Pharmacia, USA

3) Proteinase K, Gibco BRL, USA

4) Taq DNA polymerase, Qiagen, Germany

5) FspI, Pharmacia, USA

6) TaqI, Pharmacia, USA

Copyright by Mahidol University

LIBRARY AND INFORMATION CENTER MAHIDOL UNIVERSITY

4. Oligonucleotides

1) Oligonucleotide primers are listed in Table 3.1

Table 3.1 The oligonucleotide primers of apo B and apo E

Set of primer	Code	Type	Site of annealing	Sequence 5'.....3'
Signal peptide of apo B				
BID	P1	Sense	120-141	-CAGCTGGCGATGGACCCGCCGA-
	P2	Antisense	213-191	-ACCGGCCCTGGCGCCCGCCAGCA-
Arg3531Cys of apo B				
BM31	P5	Sense	10,551-10,569	-GACCACAAGCTTAGCTTGG-
	P6	Antisense	10,884-10,866	-GGGTGGCTTTGCTTGTATG-
	P7	Sense	10,774-10791	-GAGAAGCCACACACTCAAAT-
Arg3500Gln and Arg3611Gln of apo B				
BMP	P3	Sense	10,675-10,698	-CTTACTTGAATTCCAAGAGCACCC-
	P4	Antisense	11,151-11,128	-GTAGGATGATATTTTTGAGGAACC-
Apo E polymorphism				
EMP	E1	Sense	3,650-3,669	-AACAACTGACCCCGGTGGCG-
	E2	Antisense	3,941-3,922	-ATGGCGCTGAGGCCGCGCTC-
	E3	Sense	3827-3846	-CCCACCTGCGCAAGCTGCGC-

Copyright by Mahidol University

44852

5. Equipments

1) DNA thermocycler 480, Perkin Elmer Cetus, USA

2) Hot-plate/stirrer, Branstead/thermolyne,USA

3) Microcentrifuge, Fotodyne, USA

4) Mini-PROTEAN II electrophoresis cell, Biorad,USA

5) pH meter, Beckman, USA

6) Pipetman, Gilson, France

7) Polaroid camera, Fotodyne, USA

8) Sterilizer-autoclave, Tomy Seiko, Japan

9) UV-transilluminator, Fotodyne, USA

10) Vortex mixer, Scientific Industries, USA

11) Water bath, Lab-Line, USA

6. Miscellaneous

1) Microcentifuge tube Treff AG, Switzerland

2) Pipette tips (for Pipetman P20, P100, P200), Treff AG, Switzerland

3) pipette tips (for Pipetman P1000), Elkary products, USA

4) Polaroid films, Berle Jucker ,Thailand

7. Reagents

All of the solutions were prepared in sterile distilled water, unless otherwise indicated. They were sterilized by autoclaving, except organic solvents and unsterilizable solutions.

Copyright by Mahidol University

7.1 Reagents for DNA extraction from human blood sample

1) Absolute ethanol

2) 0.5 M EDTA, pH 8.0: EDTA sodium salt 93 g, distilled water 400 ml. NaOH pellets 10 g, adjust pH to 8.0 with 10 N NaOH. Autoclave.

3) Lysis buffer: pH 7.2 ; Weigh NH_4Cl 6.35 g, EDTA 1.33 g and Trizma base 0.92 g. Add 500 ml of distilled water, adjust the pH to 7.2 and add distilled water up to 1.0 L

4) Proteinase K solution: 10 mg/ml (stored at -20 °C)

5) Protein precipitate: 7.5 M Guanidine HCl, pH 7.6, 1 M Tris HCl, pH 7.6, filter with 0.2 μm Nalgene filter and transfer into labeled glass container.

6) 10% (w/v) SDS solution: 10 g of SDS was dissolved in 100 ml sterile distilled water.

7) 10x TBE buffer, pH 8.0: Trizma base 108 g, Boric acid 55 g, 0.5 M EDTA, pH 8.0; 40 ml, add distilled water up to 1,000 ml, mixed well.

8) 1 M Tris-HCl, pH 7.6: Weigh Tris-HCl 60.6 g, add 400 ml of distilled water, add approximately 13 ml of concentrated HCl, then to adjust the pH to 7.6, add distilled water up to 500 ml. Sterilized by autoclaving.

7.2 Reagents for polymerase chain reaction (PCR)

1) Deoxyribonucleotide triphosphate mixture:100 mM dGTP,100 mM dATP,100 mM dCTP, and 100 mM dTTP

2) 10 x PCR buffer : 100 mM Tris-HCl , 500 mM KCl, 15 mM $MgCl_2$, pH 8.7

3) Q-solution: 5x concentrated (Qiagen, Germany)

4) $MgCl_2$ solution : 25 mM

Copyright by Mahidol University

5) Mineral oil

6) Primer: The oligonucleotide primers were synthesized by Gibco BRL , USA. Each primers was later dissolved in sterile distilled water.

7) Taq DNA polymerase (5 units/μl) : commercially from Qiagen, Germany.

7.3 Reagents for agarose and polyacrylamide gel electrophoresis

7.3.1 Reagents for agarose gel electrophoresis

1) Agarose

2) Electrophoresis running buffer : 1x TBE buffer, diluted 100 ml of 10x TBE with 900 ml of distilled water for use in tanks and gels.

3) Ethidium bromide (1 mg/L in sterile distilled water)

4) Gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in 50 ml of water.

5) 10x TBE buffer, pH 8.0: Trizma base 108 g, Boric acid 55 g, 40 ml 0.5 M EDTA, pH 8.0 adjust to 1000 ml. with distilled water.

7.3.2 Reagents for polyacrylamide gel preparation

1) N,N'- methylenebis-acrylamide

2) 10% (w/v) ammonium persulfate (APS)

3) Electrophoresis running buffer : 1x TBE buffer, dilute 100 ml of 10x TBE in 900 ml of distilled water.

4) Ethidium bromide (1 mg/l in sterile distilled water)

5) Gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in 50 ml of water.

6) N,N,N',N'-tetramethylethylene diamine (TEMED)

Copyright by Mahidol University

7)10x TBE buffer, pH 8.0 : Trizma base 108 g, Boric acid 55 g, 0.5 M EDTA, pH 8.0 40 ml, add distilled water upto 1,000 ml. Mix well.

Methods

1. Determination of lipid profiles

Lipid profiles were analyzed by Clinical Laboratory Service Department, Faculty of Medical Technology, Mahidol University. Venous blood 10 ml was taken after 12-14 hours of fasting. Plasma total cholesterol and triglyceride levels were determined by Hitachi 917 Autoanalyzer. The concentration of plasma HDL was measured after precipitation of LDL and VLDL fractions with dextran sulfate and $MgCl_2$, and plasma LDL level was calculated using the formula described by Friedewald et al [4] and VLDL level was calculated by this formula: VLDL-C = Total-cholesterol – (LDL-C + HDL-C).

2. DNA extraction from whole blood

DNA was extracted from peripheral blood leucocytes using UCLA method. EDTA can be used as anticoagulant. All of the processes must be absolutely aseptic. Five to ten millilitres of anticoagulated blood was centrifuge at 1,800 rpm for 10 minutes, plasma was removed and transfer 250-500 µl of buffy coat of ficoll pellet to a 1.5 ml microcentrifuge tube. Added 1.0 ml of red blood cell lysis reagent, vortexed and let stand for 2 minutes. The nuclei pellet was collected by centrifugation at 5,000 rpm for 10 minutes, discarded the supernatant, repeated this step until all red blood cells were lysed,

Copyright by Mahidol University

however, did not repeat this step more than 3 times. The nuclei were vortexed to prevent clumping. Approximately 40 µl of Proteinase K (10mg/ml) was added, vortexed the sample and added 800 µl of distilled water, vortexed it again. Three hundred µl of 10% sodium dodecyl sulphate (SDS) was added to the solution. This detergent lysed the nuclear membrane and the solution became viscous as the DNA was released. The sample was mixed gently by rocking the tube. The protein was precipitated by adding 7.5 M Guanidine HCL , mixed the sample gently and incubated at 68-70 °C for 10 minutes. After 10 minutes, the sample was mixed vigorously obtaining the homogeneous mixture. Tried to avoid creating bubbles while mixing with pipettes and incubated the sample at 68-70°C for additional 5 minutes. After the 5 minutes, the sample was spinned at 10,000 rpm for 4 minutes (If the pellet is compacted and the supernatant is clear and free of debris, continue to the next step. If the pellet is diffused and the supernatant is cloudy, repeat incubated and spin it again). The supernatant was transferred to a clean 50 ml plastic tube by decanting or pipetting. The DNA was precipitated by slowly adding approximately 4 ml of cold absolute ethanol. The solution of absolute ethanol was kept at -20°C overnight to complete DNA precipitation. The tube was gently rocked the tube back and forth until cotton-like strands of DNA appeared. (If the amount of DNA was enough to be visualized by naked eyes after precipitation). The precipitated DNA was collected by transfer the DNA to another labeled 1.5 ml Eppendorf tube with drawing 800 µl of DNA-ethanol, spinned at 10,000 rpm for 2 minutes and discarded the alcohol supernatant. The ethanol was removed by adding 500 µl of 70% ethanol to the sample, vortexed to loosen the pellet and let the sample stand for 1 minute. The DNA was collected by centrifugation at 10,000 rpm for 2 minutes and discarded as much of the

Copyright by Mahidol University

supernatant as possible. The DNA pellet was dissolved with 200 µl sterile distilled water, vortexed and incubated at 68-70°C for 5 minutes with the cap open to evaporate the ethanol. Finally, the quality of DNA was verified by 0.7% agarose gel electrophoresis.

3. Polymerase Chain Reaction (PCR)

Principle

The polymerase chain reaction (PCR) is method of amplifying a target sequence of DNA. PCR provides a sensitive, selective, and extremely rapid means of amplifying a desired sequence of DNA. Specificity is based on the use of two oligonucleotide primers that hybridize to complementary sequences on opposite strands of DNA and flank the target sequence. The DNA sample is first heated to separate the two strands, the primers are allowed to bind to the DNA, and each strand is copied by a DNA polymerase, starting at the primer site. The two DNA strands each serve as a template for the synthesis of new DNA from the two primers. Repeated cycles of heat denaturation, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase result in the exponential amplification of DNA segments of defined length. This process is repeated for multiple cycles [4]

3.1 Analysis of apo B signal peptide insertion/deletion polymorphism

The rapid and efficient method of typing an insertion/deletion polymorphism in the signal peptide region of the human apo B gene are the polymerase chain reaction and synthetic oligonucleotides closely flanking the region of interest, alleles differing by only nine base pairs were readily distinguishable[23,50]. The primer set BID (P1/P2) was used

Copyright by Mahidol University

to amplify 93 bp (insertion) and 84 bp (deletion) of DNA fragment covering a part of noncoding and encoding 5' region. This segment located within nucleotide 120 to nucleotide 213 in exon 1 of apo B gene.

To generate double-stranded amplified DNA, the polymerase chain reaction(PCR) was modified from the methods of Visvikis et al[23] and Saha et al[50] This procedure was carried out in 100 µl final volume in 0.6 ml microcentrifuge tube. The reaction mixture contained 0.5-1.0 µg of genomic DNA, 1x PCR buffer and 0.65 µg of each oligonucleotide primer (Gibco BRL ,USA). The four dNTPs (dGTP, dATP, dCTP, and dTTP) were present in final concentration of 150 µM , then added 1.5 units of Taq DNA polymerase. The mixture was mixed for 2-3 second by vortexing and overlaid with 60 µl of mineral oil to avoid evaporation during amplification. The optimized procedure for amplification was carried out using a DNA thermal cycler. (Perkin Elmer Cetus Instruments). A total of 50 cycles were run under a following condition: denaturation at 96°C for 6 minutes in the first cycle and 95 °C for 1 minute in each subsequent cycle. The annealing and extension step was carried out simultaneously for 1.5 minutes at 65°C . The annealing temperature at 65°C was used for sets of primers BID with good outcomes, the specific DNA fragments as defined by the primers were amplified and the length of the amplified DNA products would be determined by the extension between the two primers.

3.1.1 Examination of the PCR products of insertion/ deletion in signal peptide of apo B gene by electrophoresis

Electrophoresis in an polyacrylamide gel separates the DNA fragments by size. DNA is negative charge and will migrate towards the anode when an electrical field is

Copyright by Mahidol University

applied. Smaller DNA fragment migrate through the polyacrylamide matrix more quickly than larger fragments. Amplified DNA was subjected to electrophoresis in 8% polyacrylamide gels. Before pouring the gel, the gloves must be worn when preparing and handing the gel solutions since acrylamide was a known neurotoxin. Twenty ml of 8% polyacrylamide solution, containing 4 ml of bis-acrylamide, 1.6 ml of 10x TBE buffer, 14.4 ml of distilled water, 160 µl of 10% ammonium persulfate (To initiated free radicals which an important for chain reaction) and 15 µl of TEMED were mixed gently. TEMED accelerated the polymerization of acrylamide and bis-acrylamide by catalyzing the formation of free radicals from ammonium persulfate. The free radicals would drive the polymerization. The gel was poured as soon as possible after TEMED had been added to the beaker. The polyacrylamide solution was slowly pushed by using syringe into the gel mold. The solution was poured in continuous stream to prevent the formation of air bubbles. The comb was immediately placed to make the sample wells into the top of the gel. The gel was left to polymerize at room-temperature or incubated for 30-60 minutes. After the gel had polymerized, the comb was carefully removed. The gel mold was attached to the electrophoresis apparatus. Then 1,000 ml of 1x TBE buffer was prepared and 500 ml was filled to the lower chamber and approximately 300 ml to the upper chamber. The solution of DNA markers (1µg/µl of 10 bp DNA Ladder, Gibco BRL, USA) was mixed with 2 µl of gel loading buffer and applied into the first well using an automatic pipette. Ten µl of the amplified DNA was similarly mixed with 2 µl of the gel loading buffer and applied into a well in the same manner. When all of the samples had been loaded, the electrode was connected to power supply and electrophoresis was carried on at 90 volts for 90 minutes at room

Copyright by Mahidol University

temperature. After the electrophoresis was ended, the gel was soaked in a solution of ethidium bromide (1mg/L in distilled water) for 1-2 minutes. The stained agarose gel was then placed on a short wavelength UV transilluminator (254 nm). The amplified DNA segment was observed and photograph of the gel might be taken. The size of the amplified DNA was estimated by comparing it with that of the DNA markers.

3.1.3 Statistical analysis for apo B ins/del polymorphisms

Statistical analyses were performed using the software package SPSS and Statview. Allele or gene frequencies was determined by using the gene counting method. Chi-square analysis was carried out to test deviation of genotype frequencies from these predicted by the Hardy-Weinberg equilibrium hypothesis. The data obtained were compared within the varions groups in this study or with the other published frequencies. Chi-square analysis was also tested the significant variation among these frequencies. Differences in mean lipid level, among Ins/Del polymorphism of each subgroup were evaluated by non-parametric test of Kruskal-Wallis. Mean and standard errors of adjusted quantitative parameters of difference of serum lipid levels in before and after treatment with lipid-lowering drug were estimated by the pair Student's t-test. The nominal level of statistical significance for all analyses was $p< 0.05$.

3.2 Determination of Arg3531Cys mutation by polymerase chain reaction.

The Arg3531Cys mutation is caused by the substitution of thymine for cytosine at position10,791, in exon 26 of apo B gene. The presence of this mutation was determined in pooled samples by the polymerase chain reaction (PCR). The primers set BM31 (P5,P6 and P7) as shown in table 3.1 was used to amplify a common band of

Copyright by Mahidol University

334 bp and a mutation-specific band 111 bp in case of Arg3531Cys. The DNA segment located within nucleotide 10,551-10,884 for common band and nucleotide 10,774 - 10,884 for a mutation at Arg3531Cys, in exon 26 of apo B gene.

The polymerase chain reaction (PCR) to amplification of Arg3531Cys was modified from the methods of Anne Tygjaer-Hensen, et al[20]. This procedure was carried out in a final volume of 50 µl containing approximately 0.1-0.2 µg of genomic DNA and 200 µmol of each deoxynucleoside triphosphate per litre, 1.5 mmol of magnesium chloride per litre, 1x PCR buffer (200 mM Tris- hydrochloride, pH 8.4, and 500mM potassium chloride) and three oligonucleotide primers as shown in Table 8 with 0.025 µmol per litre for P5, 1.0 µmol per litre for P6, and 1.0 µmol per litre for P7 and 1 unit of the Taq polymerase. The mixture was mixed by vortexing and overlaid with 30 µl of mineral oil before amplification. The optimized procedure for amplification was carried out using a DNA thermal cycler (Perkin Elmer Cetus Instruments). A total of 35 cycles was run under the condition: denaturation at 96 °C for 5 minutes in the first cycle and 95 °C for 1 minute in each subsequent cycle, annealing at 55°C for 1 minute. The extension of oligonucleotide chains was performed at 72 °C for 1 minute in all cycles and for 7 minutes in the last cycle.

3.2.1 Examination of the PCR products by electrophoresis

The amplified DNA was analyzed by electrophoresis in 4 % agarose gel. Practically, a 4% agarose gel was prepared by boiling 4 g agarose powder in 100 ml of agarose gel buffer (1x TBE buffer). After agarose was melted, let it cool to about 55° C . Then 10 ml of the gel was poured into a plastic mold (7 cm x10 cm) in which a comb with 8 teeth (1 mm thick, and 6 mm wide) was previously placed to make the

Copyright by Mahidol University

sample wells near one edge of the gel. The gel was left to polymerize at room temperature for 30 minutes and the solid gel with 6 mm thick was formed. The comb was then removed and the gel was transferred into a electrophoretic chamber filled with 1x TBE. The gel was submerged under the buffer. The solution of DNA markers, (25 bp DNA Ladder, Gibco BRL,USA) was mixed with 2 µl of the gel loading buffer and applied into the first well using an automatic pipette. 10 µl of the amplified DNA were similarly mixed with 2 µl of the gel loading buffer and applied into a well in the same manner. When all of the samples had been loaded, the electrode was connected to power supply and electrophoresis was carried on at 90 volts for 1 h at room temperature in submarine fashion. After the electrophoresis was end, the gel was soaked in a solution of ethidium bromide (1mg/L in distilled water) for 1-2 minutes. The stained agarose gel was then placed on a short wavelength UV transilluminator (254 nm). The amplified DNA segment was observed and photograph of the gel might be taken. The size of the amplified DNA was estimated by comparing it with that of the DNA markers.

3.3. Detection of mutation Arg3500Gln and Arg3611Gln which cause familial defective apolipoprotein B-100 by Mutagenic Polymerase Chain Reaction Primers and Restriction Fragment length Polymorphism

3.3.1 Restriction endonuclease digestion of PCR products

Digestion of DNA with restriction endonuclease generates different restriction maps or patterns of DNA fragments from homologous genes that contain different base sequence. This phenomenon is termed "Restriction Fragment Length

Copyright by Mahidol University

Polymorphism or RFLP''. The differences in DNA sequence site can result in variation of restriction sites and thus in the length of restriction fragments. An inherited difference in the pattern of restriction is known as a restriction fragment length polymorphism. RFLPs result from single base changes on DNA into a restriction fragment and are providing to be useful for diagnostic tool [4].

A single nucleotide mutation in codon 3500 in exon 26 of the apo B gene (CGG to CGA) leads to substitution of glutamine for arginine in the variant gene product. Individuals carrying the mutant allele in the heterozygous state are affected by a disorder termed "familial defective apo B-100" (FDB). FDB are characterized by delayed clearance of LDL particle from plasma, hypercholesterolemia, and high concentration of apo B in plasma. This mutation supposedly alters the structural conformation of the protein in the vicinity of the receptor-binding domain, thus it may be preventing the binding of LDL particle to the LDL receptor.

The method that further simplifies and rapid detection of the point mutation on codon Arg3500Gln of the apo B gene was used in this study. This method is based on the selective creation of an artificial MspI restriction site in the wide-type allele, but not in the mutation allele, by the use of sequence modifying PCR primers. In addition, because an *MspI* restriction fragment length polymorphism (RFLP) of apo B has been identified in codon 3611.[66] We used a single PCR to combine the detection of both polymorphic sites, allowing rapid and reliable decision on the cis- or trans- localization of the two mutations on chromosome 2 without a pedigree analysis. In the cis-localization both mutant recons are on one homologue and both wild type are on the other (- -/+ +). The phenotype observed is wild type. In the trans- localization each

Copyright© by Mahidol University

homologue has a mutant and a nonmutant recon (- +/- +), and the mutant phenotype is observed.

Two oligonucleotides 24 base long used as primers (BMP of P3 and P4 in table 3.1) for PCR amplification, they were synthesized on a Gibco BRL, USA. The sequence of 5' PCR primer, which correspond to nucleotides 10,675-10,698 of the apo B sequence was modified in the next to last base of its 3' end (nucleotide 10,697), where a **C** was introduced instead of an **A**. The terminal 3' nucleotide corresponds to the first base of codon 3500. The 3' PCR primers includes nucleotides 11,128-11,151. A polymerase chain reaction was carried out in 100 µl reaction volumes containing approximately 0.3-0.5 µg of genomic DNA and 200 µmol per liter of each deoxynucleotide triphosphate and 0. 1 µmol of each of the two primers, 1x PCR buffer. The reaction mixture was denatured at 96°C for 6 minutes before the addition of 2.5 U of Taq polymerase. The mixture in each tube was overlaid with 50 µl of mineral oil to prevent condensation during amplification. PCR amplification was done by 30 repetitions of denaturation at 96°C for 2 minutes, annealing at 60°C for 1 minute, and primer extension at 70°C for 1 minute. A further extension time of 7 minutes was added at the end of the last cycle. PCR was performed in an automated Perkin Elmer Cetus Instruments. The total length of the resulting 477 bp of PCR product was estimated by compairing it with that of the DNA markers on polyacrylamide gel electrophoresis. A 10 µl aliquot of the PCR product was mixed with 1.5 µl of restriction enzyme buffer [100mmol/L Tris-HCl buffer containing 50 mmol of $MgCl_2$, 1 mol of NaCl , 10 mmol of 2-mercaptoethanol per liter, pH 8 at 37 °C (Pharmacia)]

Copyright by Mahidol University

and brought to a final volume 20µl with sterile distilled water and added 0.5 µl (5.5 U) of *MspI* (Pharmacia, USA), and incubated at 37°C for 2 h or overnight.

3.3.2 Examination of MspI restriction fragment length polymorphism reaction product by electrophoresis.

Ten µl of the reaction product was subjected to electrophoresis in 5% polyacrylamide gels: 25 ml of 5% polyacrylamide solution containing 3.125 ml of bis-acrylamide, 2.5 ml of 10x TBE buffer, 19.375 ml distilled water, 160 µl of 10 % ammonium persulfate and 15 µl of TEMED. When all of the samples and 25 bp DNA ladder marker(Gibco BRL), had been loaded , the electrode was connected to power supply and electrophoresis was carried on at 90 volts for 1.5 h. After the electrophoresis was end, the gel was soaked in a solution of ethidium bromide. The stained polyacrylamide gel was then placed on UV transilluminator. The result of an electrophoretic fragment length after cleavage with *MspI* was estimated by comparing it with that of the DNA ladder markers.

3.4 Determination of apo E phenotypes by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The three common alleles ε2,ε3 and ε4 are inherited co-dominantly and code for three apo E proteins (Isoforms): E2,E3 and E4. The isoforms differ at amino residues 112 and 158 . Isoform E2 has Cysteine residues at both sites, E4 has Arginine residues at both sites, and E3 has a Cysteine at position 112 and an Arginine at position 158. The molecular biology techniques which specific and rapid of determination of apo E polymorphism are the Polymerase Chain Reaction followed by HhaI digestion[120,121].

Copyright by Mahidol University

Two specific sequences of exon 4 were designated on the basis of the known nuclotide sequence of the apo E gene. The primer set EMP (E1/E2) as shown in table 13.1 was used to amplify the PCR1, 292 bp DNA segment. This segment located within nucleotide 3,650 to nucleotide 3,941 and PCR 2 was done with primer E3 and E2 to amplify 115 bp DNA segment, located within nucleotide 3,827 to nucleotide 3,941 in exon 4 of apo E gene.

The amplification consisted of PCR buffer (Qiagen, Germany), 0.2-0.3 μg of genomic DNA, 40 pmol of each primer, 8 mmol of each dNTP, 100 ml/L dimethyl sulfoxide, 1x Q solution (Qiagen) and 3.5 U of Taq DNA polymerase (Qiagen) in final volume of 100 μl. The reaction mixture was denatured at 96 °C for 12 minutes before the addition of 3.5 U of Taq DNA polymerase. The mixture in each tube was overlaid with 30 μl of mineral oil to avoid evaporation during amplification. The amplification began by 5 cycle of 1 minute at 95°C (for denaturation), followed by 3 minutes at 72° C (for primer annealing and extension). Then series of 30 cycles (95 °C for 1 minute, 65°C for 1 minute and 72°C for 1 minute) was carried out. The total length of resulting 292 bp (PCR1) and 115 bp (PCR2) of PCR product was estimated by comparing with the 25 bp DNA ladder markers (Gibco BRL) on 10% polyacrylamide gel. It was then placed on ultraviolet light, and photographed on Polaroid film to distinguish the expected amplimers from nonspecific amplifications.

3.4.1 Endonuclease restriction digestion of PCR products.

Each PCR product (90 μl) after amplification was precipitated by 3 mol/L sodium acetate (4.5 μl) and frozen absolute ethanol (180 μl) and left for a night at -20 °C. The precipitate was washed with frozen 700 ml/L ethanol, solubilized in steriled water.

Copyright by Mahidol University

The amplified products were 292 bp and 115 bp for PCR1 and PCR2, respectively. The PCR 1 and PCR 2 were digested with 250 kU/L enzyme *Hha*I at 37°C for 4 h. The digested PCR product was then loaded on a 100 g/L polyacrylamide gel electrophoresis for 1h at 25 W constant . The DNA fragments separated after digestion were revealed by ethidium bromide under ultraviolet illumination. Their sizes were determined by comparison with 25 bp DNA ladder markers.

3.4.2 Statistical analysis of apo E polymorphisms

Statistical analyses were performed using the software package SPSS and Statview. Allele or gene frequencies were estimated using the gene-counting method. The genotype frequencies were analysed for adherence to Hardy-Wienberg equilibrium hypothesis by a chi-square test. Differences in apo E genotype distribution between different population samples were also determined by chi-square analysis. These data obtained were compared with the other published frequencies for the apo E genotypes and tested for significant variation by chi-square analysis. Differences in mean lipid levels between apo E phenotypic groups were evaluated by non-parametric test of Kruskal-Wallis (H-test). Mean and standard errors of adjusted quantitative parameters of difference of serum lipid levels in before and after treatment with lipid-lowering drug were estimated by the pair Student's t-test. The nominal level of statistical significance for all analyses was $p < 0.05$.

Copyright by Mahidol University

CHAPTER IV

RESULTS

Lipid levels in Thai population

Table 4.1 showed the mean lipid levels of Thai population which subdivided into normolipidaemia and hyperlipidaemia. The lipid profile of total cholesterol, LDL-cholesterol, triglycerides, and VLDL-cholesterol were highly significant increased in hyperlipidaemia than that in normolipidaemia ($p<0.001$). HDL-cholesterol showed no significant difference between these two groups. In general, high levels of cholesterol and triglyceride have been considered to play a part in coronary heart disease. Additional risk factors also include high blood pressure, tobacco smoking, age, male gender, obesity (particularly abdominal obesity), lack of exercise, drinking soft as opposed hard water, and genetic defects of apolipoprotein.

Table 4.1. Plasma total cholesterol, LDL-cholesterol, triglyceride, HDL-cholesterol in Thai population (data expressed as mean ± SE, TC = total cholesterol, and TG = triglyceride).

Type of subjects	TC (mg/dl)	LDL-C (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	VLDL-C (mg/dl)	TC/HDL-C
Thai population (n= 171)	237±5	159±4	144±7	50±1	29±1	4.7
Normolipideamia (n=68)	175±3	105±2	99±5	51±2	20±1	3.52
Hyperlipidaemia (n=103)	278±4*	195±5*	173±9*	49±1	35±2*	5.67

* level of significance ($p<0.001$) as compared with normolipidaemia estimated by unpaired Student's t-test.

Copyright by Mahidol University

Elevation of plasma free fatty acids will also lead to increase VLDL secretion in liver, involving extra triglyceride and cholesterol output into the circulation. Factors leading to higher or fluctuating levels of free fatty acids include emotional stress, nicotine from cigarette smoking, caffeine from coffee drinking, and partaking of a few large meals of diet high in saturated fat and calories, high in sugar, low in fruits and vegetables rather than continuous feeding. Premenopausal woman appear to be protected against many of these deleterious factors, possibly because they have higher concentrations of HDL due to the influence of oestrogen than to men and post- menopausal women. From the other study [122], they have reported that the risk of coronary heart disease in most of both men and women is rare in the first two decades of life, becoming more prevalent after the age of 30 and much more marked beyond the age of 60 years. Coronary heart disease in females beyond 60 years increases at an accelerated rate and after the seventh decade the rate approaches that in males (figure 4.1).

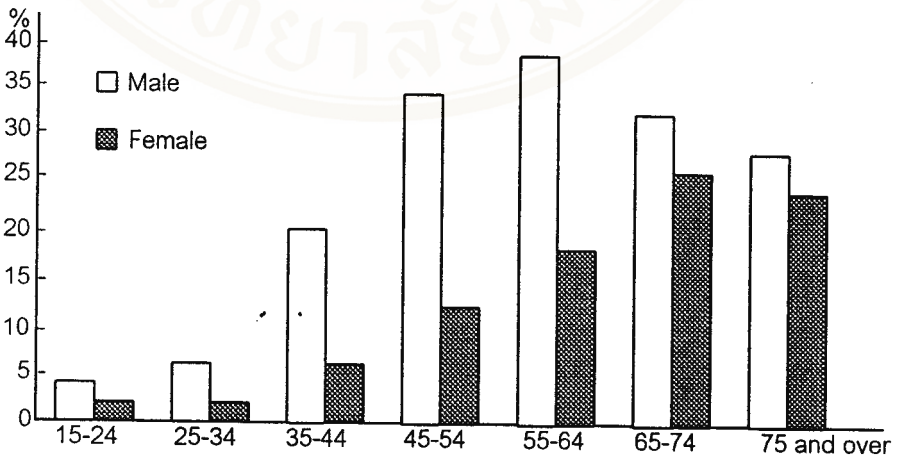


Figure 4.1 CHD death rates as a percentage of all deaths in men and women at different ages in Scotland, 1994 [122]

Copyright by Mahidol University

In addition, the exercise level required to improve a patient's risk factor profile has hot been fully established but an excess energy expenditure of approximately 2000 kilocalories per week is thought to provide the same protection as that observed in athletes. It is of interest that studies have shown are association between moderate alcohol concentration and a lower incidence of coronary heart disease. This may be due to elevation of HDL concentration, but it has been claimed that red wine is particularly beneficial, perhaps become of its contents of antioxidants.

Since the highly significant increases of LDL-cholesterol and triglyceride levels in hyperlipidaemia as significant risk factors of CHD were observed in this study, the genetic information of apo B and apo E which contained in LDL and VLDL particles should be further investigated to predict the causes of lipid elevation as another risk factors in Thai population. The genetic information are the potential risk factors to predict hyperlipidaemia better than other risk factors because it is not altered by the disease process, life style, or time and can be represented the information from biochemical and physiological risk factors, as shown in table 4.1. The presence of these polymorphisms may lead to the early development of CHD and this may adversely affect survival and longevity.

DNA extraction.

The genomic DNA (gDNA) was extraced from peripheral blood leucocytes of normal individuals and primary hyperlipidaemia subjects by guanidine-HCL extraction as described in Materials and Methods. Size fractionation and DNA quality was analyzed by electrophoresis on 0.7% agarose gel in 1X TBE buffer. The gDNA band

Copyright by Mahidol University

were verified under the UV transilluminator after ethidium bromide staining (Figure 4.2).

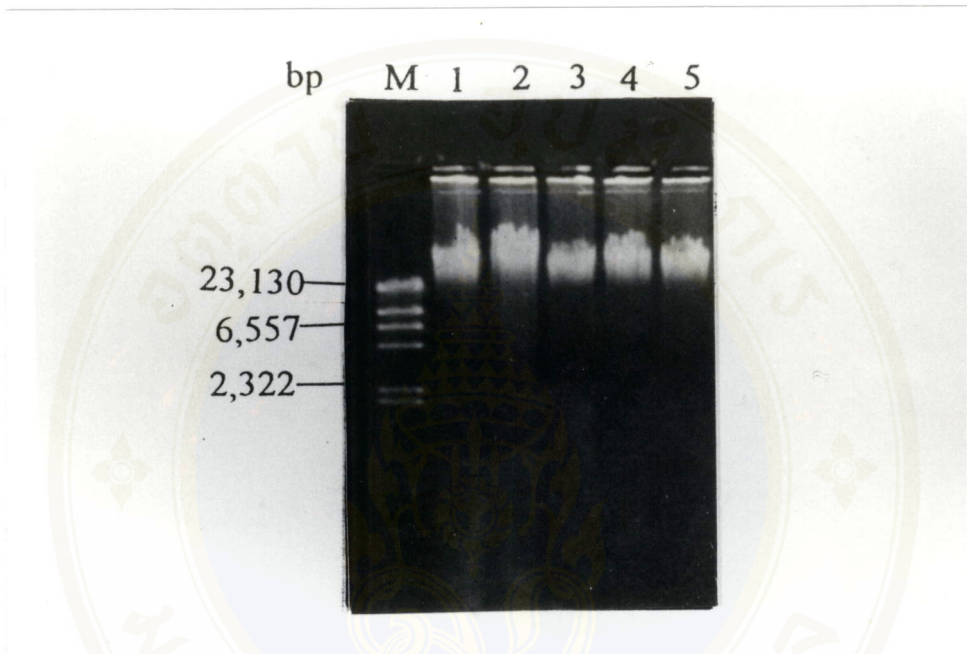


Figure 4.2 Ethidium bromide staining patterns of genomic DNA extracted from peripheral blood leukocytes by guanidine-HCl extraction. Lane M was HindIII-digested λDNA marker. Lane 1to 5 were gDNA from different individuals.

Apo B-100 insertion/deletion polymorphism

An insertion or deletion of three codons involving three amino acids, leucine-alanine-leucine, within exon 1 of the apoB gene has been investigated in this study. This amino terminal cleavable signal sequence directs the emerging protein to translocate through the endoplasmic reticulum membrane. Thus, this signal peptide polymorphism within the apoB gene may play a role in affecting the translocation of the apoB polypeptide

Copyright by Mahidol University

into the endoplasmic reticulum. Several previous studies have investigated the impact of this polymorphism on plasma lipid levels. Some studies have found the correlations between the ins/del polymorphism and the lipid levels.

The PCR product of the apo B signal peptide insertion/deletion genotypes after polyacrylamide gel electrophoresis was shown in Figure 4.3. The two alleles are easily visualized by UV transillumination, the larger (Ins) allele is 93 bp and the smaller allele (Del) is 84 bp. The upper band in heterozygote is the heteroduplex of 93 and 84 bp, which occurred during the PCR cycle. Genotype frequencies of the apo B signal peptide insertion/deletion polymorphism in 171 Thai population of both 68 normolipidaemia and 103 hyperlipidaemia subjects are shown in Table 4.2. The allele frequencies of the apo B signal peptide polymorphism obtained from table 4.3 in these samples were used to calculate the χ^2 value to test the deviation of genotype frequencies from the criteria of Hardy-Weinberg equilibrium. From χ^2 method, it was shown that the distribution of these three genotypes (II, ID and DD) was in Hardy-Weinberg equilibrium (χ^2= 1.95; p=0.325; df =2) (Table 4.2).

Table 4.2. The genotype frequencies distribution of apo B signal peptide insertion/deletion polymorphism (χ^2 = 1.95; p = 0.325; df = 2).

Genotype	Ins/Ins*	Ins/Del	Del/Del
Observation	103 (60%)	60 (35%)	8 (5%)
Hardy-Weinberg expectation	104 (61%)	59 (34%)	8 (5%)

* No. in parenthesis refers to relative genotype frequencies

Copyright by Mahidol University

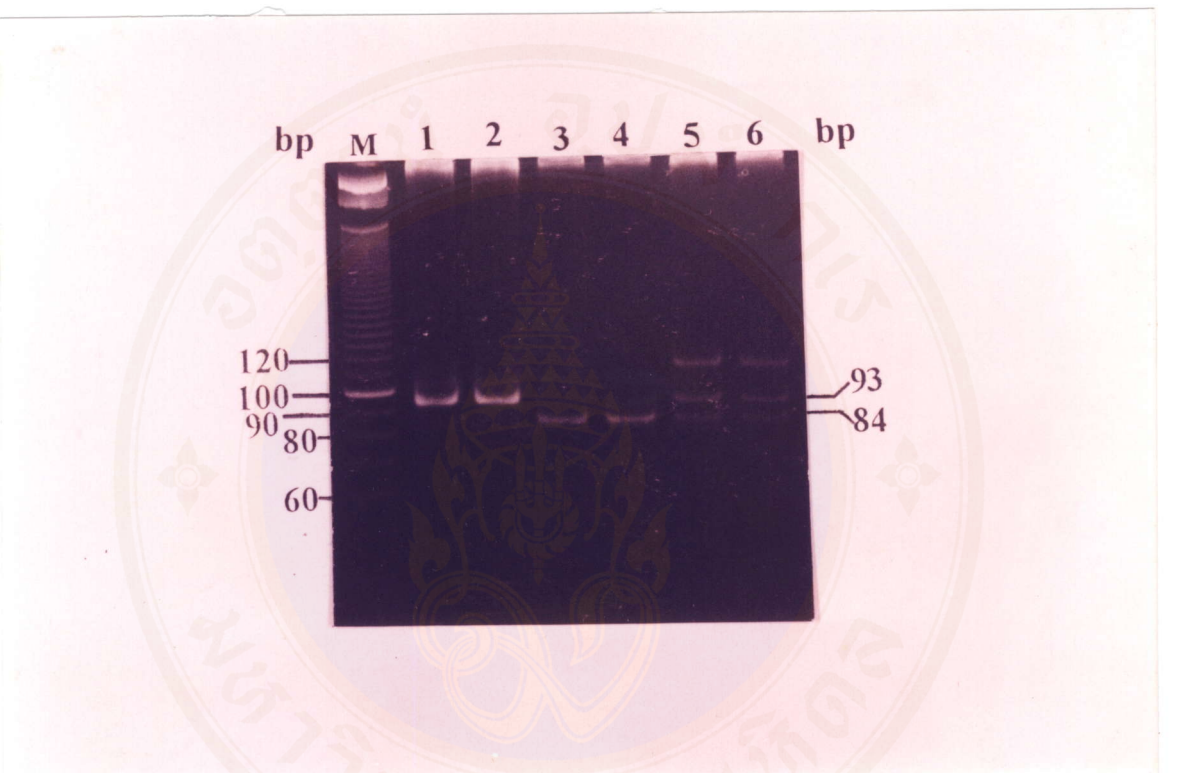


Figure 4.3 Polymerase chain reaction products of the apo B signal peptide insertion / deletion polymorphism genotypes. (lane M is marker, lane 1,2 are homozygous insertion, lane 3, 4 are homozygous deletion and lane 5,6 are heterozygous insertion/deletion).

The allele frequencies of insertion (Ins) and deletion (Del) alleles estimated by gene counting method in Thai population were found to be 0.78 and 0.22, respectively (Table 4.3). The distribution of apo B signal peptide Ins/Del polymorphism in relation to serum total cholesterol or triglyceride levels in hyperlipidaemia (>200 mg/dl) and

Copyright by Mahidol University

normolipidaemia (≤200 mg/dl) is also presented in Table 4.3. The frequency of the Del allele was significantly higher (0.26) in the hyperlipidaemic group than that (0.17) in subjects with normolipidaemic group (χ^2=3.82; p<0.05; df=1). However, the comparison of allele frequencies between Thai population and hyperlipidaemia and normolipidaemia showed no significant difference as shown in table 4.3 (χ^2 for Thai population vs hyperlipidemia =1.147; p=0.284; χ^2 for Thai population vs normolipidemia =1.49; p=0.22).

Table 4.3 Allele frequencies of apo B signal peptide insertion/deletion polymorphism in Thai population which subdivided into hyperlipidaemia, normolipideamia.

Type of allele	Thai population n =171	Hyperlipidaemia n =103	Normolipidaemia n = 68
Insertion (Ins)	0.78	0.74	0.83
Deletion (Del)	0.22	0.26	0.17

χ^2 for Thai population vs hyper =1.144; p=0.285

χ^2 for Thai population vs normal =1.49; p=0.222

χ^2 for hyper vs normal =3.82; p=<0.05

These genotype frequencies and allele frequencies between hyperlipidaemia and normolipidaemia were summarized in table 4.4. The result from this study was agreed with the previous report of Saha et al[50] who study in the Chinese in Singapore. The Del allele in Chinese was significantly higher (0.26) in hypercholesterolaemic group (> 250 mg/dl) than that (0.18) in subjects with normal serum cholesterolaemia (< 250 mg/dl). However, in the French series there was no significant association of the

Copyright by Mahidol University

polymorphism with hypercholesterolaemia (Del = 0.368) and normolipidaemia (Del = 0.345)[57]. This is due to the high Del allele frequency as compared with the Thai and Chinese populations.

Table 4.4 Distribution of apo B signal peptide insertion/deletion polymorphism in relation to serum total cholesterol and triglyceride levels.

Group of subjects	Genotypes				Allele frequencies	
	II	ID	DD	All	I	D
Hyperlipidemia	57	39	7	103	0.74	0.26
Normal	46	21	1	68	0.83	0.17

χ^2 =1.95; p=0.32; df = 2 for genotype frequencies

χ^2 = 3.82; p<0.05; df=1 for allele frequencies

The study of Ins/Del allele and the genotype distribution from this Thai population samples was also compared with other racial populations (Table 4.5). Significant differences were observed in the Ins/Del genotypes between the Thai and the various Caucasian and Indians in Singapore[52]. A chi-square test of heterogeneity indicated the statistically significant differences in apo B allele frequency distribution between the different populations. Two-sample χ^2 analysis showed that the allele frequencies of the Thai population differ highly significantly from those of the French [57], Norwegian[54], Indians in Singapore [52] (p<0.001), Finnish[48] and American[47] (p< 0.05). In Indians in Singapore, the significant difference of apo B allele frequency differ from the others, i.e., this population contained significantly high frequency of Ins allele and low of Del allele whereas other Caucasian populations contained significantly low

Copyright by Mahidol University

Ins allele and high Del allele. No significant differencefrom Thai population were found with the Chinese in Taiwan[56], South Asains in UK.[58], Nigerian[59], and Chinese in Singapore[50-51]. This study suggesting that the origins of this Del allele may be in central Europe because of the high prevalence of Del allele in Finland, France and Norway.

Table 4.5 Comparison of the frequency distributions of apo B signal peptide insertion/deletion polymorphism in different racial populations.

Population sample	No. of sample	Ins	Del	p value	Reference
Thailand	171	0.78	0.22	-	Present study
Singapore (Indians)	181	0.89	0.11	<0.001	Saha et al (1993)
UK (South Asians)	107	0.80	0.20	0.57	Renges et al (1991)
Singapore (Chinese)	269	0.80	0.20	0.475	Saha et al (1992a)
Singapore (Chinese)	221	0.80	0.20	0.49	Saha et al (1992b)
Finland	55	0.68	0.32	<0.05	Xu et al (1990)
French	194	0.66	0.34	<0.001	Visvikis et al 1990
Nigeria	1202	0.75	0.25	0.228	Anderson et al (1997)
Norway	547	0.63	0.37	<0.001	Bohon et al (1994)
Taiwan (Chinese)	319	0.81	0.19	0.263	Wu JH et al (1994)
American	71	0.68	0.32	<0.05	Boerwinkle et al (1992)

Copyright by Mahidol University

The observed apo B phenotype distribution and corresponding apo B allele frequencies between gender in 171 Thai population was presented in Table 4.6. The apo B signal peptide Insertion allele in Thai female might have a trend to be lower than that in Thai male (0.76 and 0.81, respectively). However, the Ins/Del genotypes distribution between both genders showed no significant difference.

Table 4.6 Distribution of apolipoprotein B genotypes and estimated allele frequencies between gender in Thai population (=171).

Genotype	Male No. observed	Relative frequencies(%)	Female No. observed	Relative frequencies(%)
II	40	66.7	62	55.9
ID	17	28.3	44	39.6
DD	3	5	5	4.5
Total	60	100	111	100

Gene (allele) frequencies		
Allele	Male	Female
I	0.81	0.76
D	0.19	0.24

Male vs Female $\chi^2 = 1.126$; p=0.289; df=1

Copyright by Mahidol University

Sample from hyperlipidaemic subjects, normolipidaemic subjects and this Thai populations were also analysed for differences in plasma lipid level in subjects with these genotypes of II, ID, and DD, respectively.

Mean lipid levels of these biochemical and physiological risk factors were not significantly different in this Thai population and normolipidaemia but showed significant difference in hyperlipidaemia as shown in table 8. Mean total cholesterol, LDL-C and triglyceride were significantly higher in heterozygote (I/D) of hyperlipidaemia but not in normolipidaemia and this Thai population. This variation may be due to the influence of age, sex, life style, and BMI as the parameter were regressed for these covariates. Nevertheless, no significant differences in plasma lipid levels were found in this Thai population and normolipidaemia suggesting that other risk factors rather than genetic risk factors may also play an important role in the fluctuation of plasma lipid levels.

Copyright by Mahidol University

Table 4.7 Mean ± SE of lipids in this Thai population, hyperlipidaemia and normolipidaemia of different genotypes in insertion/deletion (I/D) polymorphism in the signal peptide region of DNA at the apo B locus.

Mean ± SE levels in population with genotype					
Thai populations					
Parameter	DD (n=8)	ID (n=60)	II (n=103)	H	p-value
Total cholesterol (mg/dl)	243±10	245±8	232±6	2.6	0.28
LDL-C (mg/dl)	162±10	166±8	154±5	2.0	0.36
Triglycerides (mg/dl)	139±33	153±13	139±7	0.57	0.75
HDL-C (mg/dl)	53±1	48±2	50±1	2.1	0.34
VLDL-C (mg/dl)	28±6	30±3	28±2	0.56	0.75
Hyperlipidaemia					
Parameter	DD (n=7)	ID (n=39)	II (n=57)	H	p-value
Total cholesterol (mg/dl)	249±9	284±6	278±6	6.3	<0.05
LDL-C (mg/dl)	195±5	201±8	194±6	7.6	<0.05
Triglycerides (mg/dl)	142±38	182±18	171±11	1.6	0.46
HDL-C (mg/dl)	55±3	46±2	51±2	4.4	0.11
VLDL-C (mg/dl)	28±8	36±4	34±2	1.6	0.44
Normolipidaemia					
Parameter	DD (n=1)	ID (n=21)	II (n=46)	H	p-value
Total cholesterol (mg/dl)	198	175±5	176±3	1.3	0.53
LDL-C (mg/dl)	136	104±4	106±3	2.1	0.34
Triglycerides (mg/dl)	117	100±9	99±6	0.50	0.78
HDL-C (mg/dl)	39	51±3	50±2	1.0	0.6
VLDL-C (mg/dl)	23	20±2	20±1	0.50	0.78

Using non-parametric test of Kruskal-Wallis (H- test) to test lipid levels among II, ID and DD.

Copyright by Mahidol University

Table 4.8 represented the lipid profile of hyperlipidaemic patients before and after treatment with lipid-lowering drugs and with lipid-lowering dietary recommendations. The homozygous Ins and heterozygous Ins/Del can significantly reduce total cholesterol, LDL-cholesterol and triglycerides about 34-37 mg/dl, 29-31mg/dl, and 34-44 mg/dl, respectively after treatment for a long period of 3-5 years. The homozygous deletion genotype showed no significant differences in lipid levels after treatment for a period of 3-5 years but might show a trend to increase in total cholesterol and LDL cholesterol. Moreover, the HDL-cholesterol level decreased significantly. This result implied that patients contain homozygous deletion allele were not responsive to the lipid-lowering drug treatment.

Table 4.8 Effect of lipid-lowering drug@ on lipid levels in apo B signal peptide insertion/deletion polymorphism.

Lipid profile	Ins/Ins (n= 57)		Ins/Del (n=38)		Del/Del (n=6)	
	Before	After	Before	After	Before	After
Total cholesterol	278±6	244±5***	286±6	249±7***	255±8	257±9
LDL cholesterol	194±6	165±5**	204±8	173±8**	169±1	180±8
VLDL-cholesterol	34±2	33±6	36±4	27±3	31±8	28±3
HDL-cholesterol	51±2	52±2	46±2	50±2	55±4	45±4*
Triglycerides	171±11	137±9**	180±18	136±12*	156±41	142±15

@=lipid-lowering drugs used such as Lopid, Mevalotin, Questran, Lipantil, Simvastatin including lipid-lowering diet for 3-5 years in these patients. (*** =p <0.0001; ** =p <0.001; * =p <0.05).

Copyright by Mahidol University

LIBRARY AND INFORMATION CENTER MAHIDOL UNIVERSITY

Apo B-100 point mutation

Apo B-100 has two functional domain: a N-terminal domain that associates with lipids and a C-terminal domain that binds to LDL receptors on cell membranes. The genetic defect often form in the C-terminal, which contains two large exons (exon 26 and exon 29) (Figure 2.6) of apo B-100 that interferes with its capacity to bind to the LDL receptor. This defect was result from a G-to-A transition at nucleotide 10708 or at codon 3,500 in exon 26 of apo B gene. Because of this, the name of disorder was named familial defective apolipoprotein B-100 (FDB)[61]. However, the region of the apo B molecule interacting with the LDL receptor may be quite large, thus it seems probable that other apo B amino acid substitutions could interfere with its ability to bind to the LDL receptor. The other mutations in exon 26 of apo B-100 gene have been identified such as Arg 3531Cys and Arg3611Gln, which are associated with a minor decrease in LDL receptor binding[74, 80].

The 5' primer carries a substitution (C instead of A) one base before the 3' nucleotide that corresponds to the first base of codon 3500 has been modified as shown in Figure 4.4. The mismatched base is incorporated in all products resulting from PCR amplification with this primer. Thus, an Msp I cutting site (CCGG) is introduced in all wild-type allele products; by contrast, no such cutting site is generated in PCR fragments for which the mutant allele, coding for glutamine (CAG), has served as a template (Figure 4.4).

The 3' primer was placed downstream of a naturally occurring polymorphic Msp I site in codon 3611 of apo B. Incubation of the resulting 477-bp-long fragment with MspI and subsequent electrophoretic determination of fragment length gave

Copyright by Mahidol University

44852

simultaneous information on the polymorphisms in codons 3500 and 3611. Figure 4.4 shows the expected fragment lengths after cleavage and the deduced hapotypes.

Results from the analysis of the 171 Thai population with 103 hyperlipidaemic subjects and 68 normolipidaemic subjects were shown in Figure 4.6. In this study only two haplotypes of 3611 were observed from ten of expected haplotypes of 3500 and 3611. The homozygous for wild-type allele at codon 3500 and at codon 3611, contain 333-, 121- and 23-bp fragments after digestion (Figure 4.5 lane 1-5). The two heterozygosities at the 3611 site were only found in this study which is characterized by the presence of the 454-, 333-, 121- and 23-bp (Figure 4.5 lane 6, 7). In this haplotype no MspI cleavage sites are present at the one allele of codon 3611, the 454-bp fragments are formed. In addition, the small fragment 23-bp was too small to be clearly visible in all analyses.

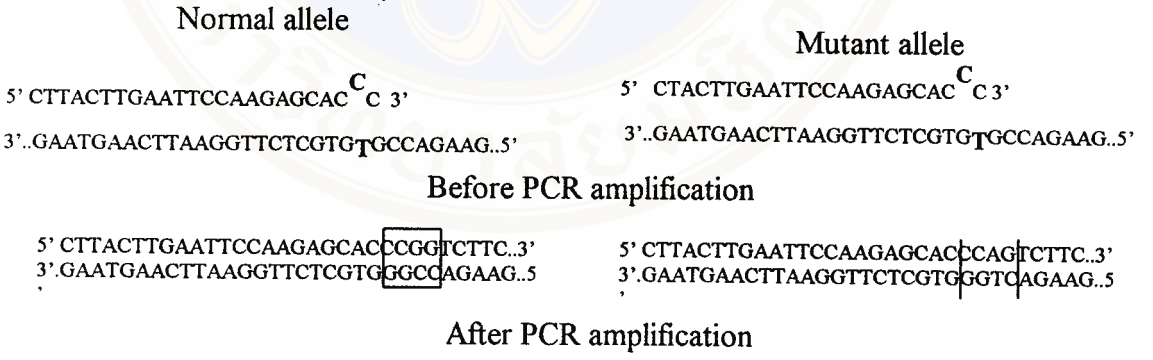
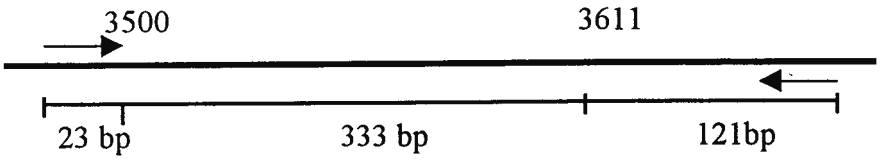


Figure 4.4 Principle of the use of sequence-modifying primers for the detection of apo B (3500:Arg) Upper part: the situation at the beginning of the PCR reaction; a 24-base-long synthetic oligonucleotide that is mismatched with the apo B wild-type sequence in one position is used as an upper-strand primer for amplificatin of the normal (left) as well as the mutation (right) allele. The two bases that distinguish between the normal and the mutant allele are boxed. Lower part: the situation after completion of PCR amplification; the vast majority of PCR product contains the sequence introduced by the mismatched primer. The difference between the normal and the mutant allele is conserved in the PCR product The base change introduced by the mismatching primer in conjunction with the wild-type sequence in codon 3500 produces and Msp I restriction site (boxed), whereas the amplification product of the mutant allele does not contain this site (bracketed)[66].

Copyright by Mahidol University



3500 3611	Haplotypes	477	454	356	333	121	23
	A,+ A,+				+	+	+
	A,+ A,-		+		+	+	+
	A,- A,-		+				+
	A,+ G,+			+	+	+	+
	A,+ G,-	+			+	+	+
	A,- G,+		+	+		+	+
	A,- G,-	+	+				+
	G,+ G,+			+		+	
	G,+ G,-	+		+		+	
	G,- G,-	+					

Figure 4.5 Principle of haplotype determination Top: schematic drawing of the region spanning codons 3500 and 3611 of the apo B gene; arrows indicate positions of the PCR primers. The length of the resulting PCR fragment is 477 bp. Below: fragments expected after cleavage at the polymorphic cutting sites and deduced haplotypes (at codon 3500; A, Arg and G, Gln, at codon 3611, + , frequent allele and - , rare allele). The two smaller restriction fragment of 23 bases are not regularly seen in the 5% polyacrylamide gel[66].

Copyright by Mahidol University

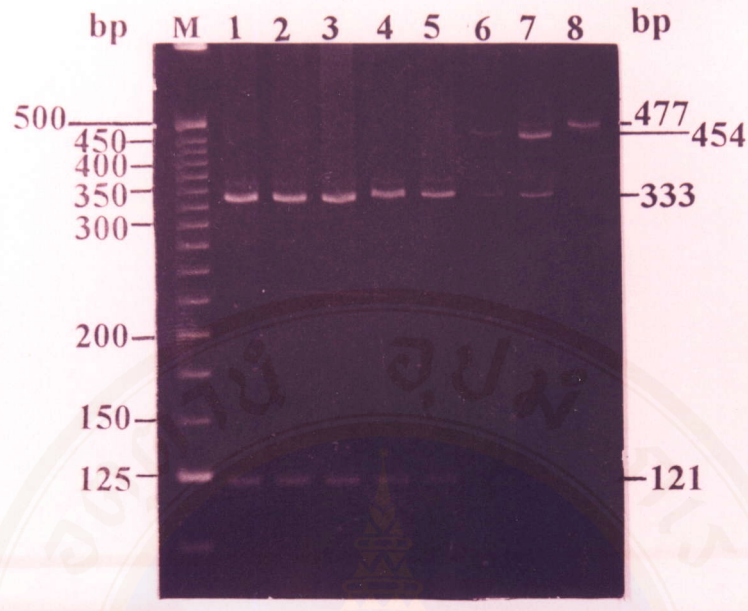


Figure 4.6 Haplotype determination: result of an electrophoretic fragment length determination after cleavage with MspI, lane M contains a fragment-size marker, lane 1-5 are result for wild-type, lane 6,7 show result for subject Arg3611 Gln mutation, the last lene is uncut PCR products.

The Arg3531Cys mutation is caused by the substitution of thymine for cytosine at position 10,791, in exon 26 of apo B gene. The presence of this mutation was determined in pooled samples by the polymerase chain reaction (PCR). The primers set BM31 (P5,P6 and P7) as shown in table 3.1 was used to amplify a common band of 334 bp and a mutation-specific band 111 bp in case of Arg3531Cys. The DNA segment located within nucleotide 10,551-10,884 for common band and nucleotide 10,774 -10,884 for a mutation at Arg3531Cys, in exon 26 of apo B gene. The polymerase chain reaction (PCR) to amplification of Arg3531Cys was modified from

Copyright by Mahidol University

the methods of Anne Tygjaer-Hensen, et al(1998)[20] (Figure 4.7). From this study, the Arg3531Cys mutation in exon 26 of apo B gene has not been found in all samples of population studied.

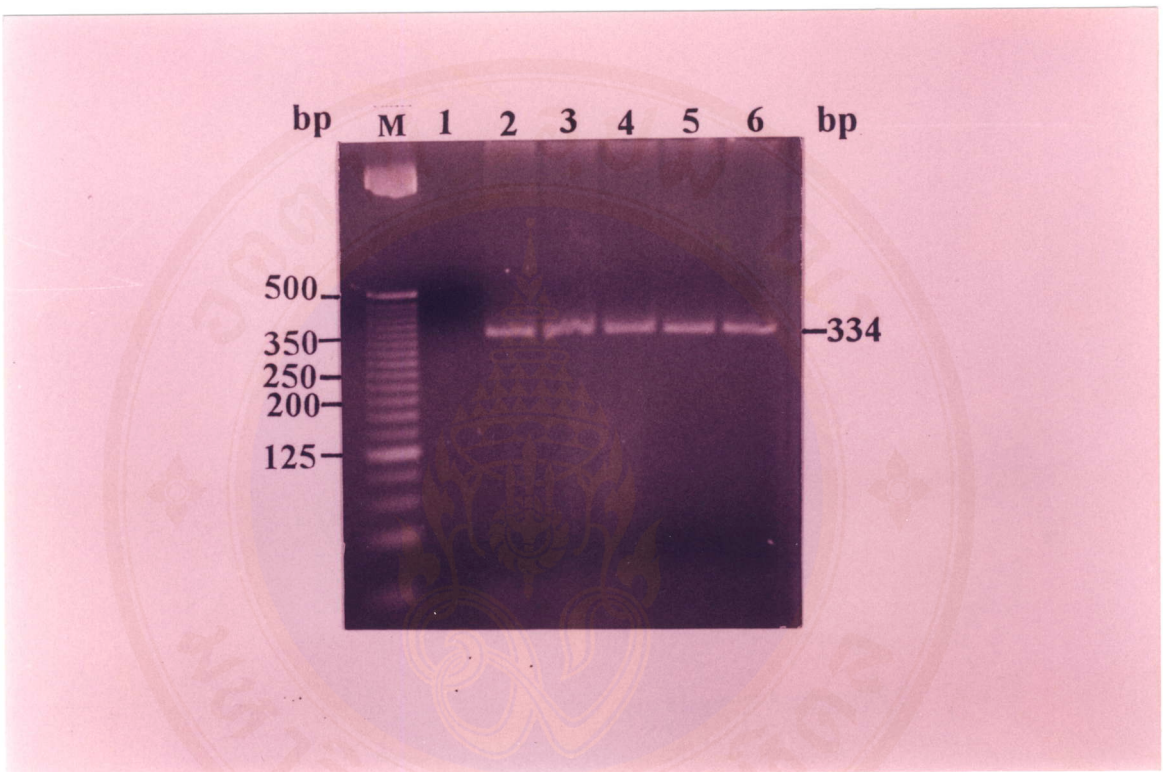


Figure 4.7 Polymerase chain reaction products of three primers amplification for apo B Arg3531Cys mutation .The only common band 334 bp PCR fragment was present and not found a mutation specific band 111 bp in the case of Arg3531Cys mutation in all of samples.

Copyright by Mahidol University

Frequencies of Apo B mutations

The Arg3500Gln and Arg3531Cys mutations were not identified among the 171 subjects in Thai population with 103 subjects of hyperlipidaemia and 68 subjects of normolipidaemia. The Arg3611Gln mutation was identified in 2 of the 171 subjects in Thai population. The distribution of the normal R3611 and Q3611 mutant apo B genotypes was shown in table 4.9. There were no significant differences in the frequencies of the normal and mutant alleles between these two groups (p=0.095). This may be due to the small sample size of normal control.

Table 4.9 Distribution of the Arg3611 (R3611) to Gln3611 (Q3611) mutation in the apo B-100 gene.

Genotype	Normolipidaemia		Hyperlipidaemia	
	No. observed	Frequency(%)	No. observed	Frequency(%)
apo B R3611/R3611	68	100	102	98
apo B R3611/Q3611	0	0	2	2
apo B Q3611/Q3611	0	0	0	0

Gene (allele) frequencies		
Allele	Normolipidaemia	Hyperlipidaemia
R3611	1.00	0.98
Q3611	0.00	0.02

χ^2 for normal and mutant alleles =2.793; p= 0.095; df=1.

All 2 subjects (1 male and 1 female) were heterozygous for the mutations and also contain the heterozygous insertion/deletion in signal peptide of apo B gene. For the apo E genotype, a male subject was E3/E4 phenotype, whereas female subject was E3/E3 genotype. All both subjects were in the hyperlipidaemic groups. The comparison of lipid levels between before and after treatment of lipid-lowering drugs was no significantly difference in these subjects (table 4.10).

Copyright by Mahidol University

Table 4.10 Lipid level of subjects contain Arg3611Gln mutation

Subjects	S. K (female)		P. P (male)		Average	
Genotype	Arg3611Gln, ID, E3/E3		Arg3611Gln, ID, E3/E4		Mean ± SE	
Lipid	Before	after	Before	after	Before	after
Cholesterol (mg/dl)	304	294	247	212	276±28	253±21
LDL-C (mg/dl)	227	229	158	132	192±34	180±48
Triglyceride (mg/dl)	167	122	321	202	244 ± 77	162 ± 40
HDL-C (mg/dl)	44	41	25	40	34 ± 10	40 ± 0.5
VLDL-C (mg/dl)	33	24	64	40	48 ± 16	32 ± 8

@=lipid-lowering drugs used such as Lopid, Mevalotin, Questran, Lipantil, Simvastatin including lipid-lowering diet for 3-5 years in these patients.

Apo E polymorphisms

The molecular basis for apo E polymorphism has been established by Mahley and coworkers[90]. The primary structure of apo E was determined and found that the isoforms E4, E3 and E2 of exon 4 differed from one another by single amino acid substitutions at two sites in the protein. The existence of the single amino acid substitutions confirmed that E2, E3 and E4 arose from separate alleles at a single gene locus. These substitutions also explained the single unit change differences among the three isoforms, since they involve the substitution of the neutral amino acid cysteine for the basic amino acid arginine.

Amino acid substitutions accounted for the differences among apo E4, E3, and E2. Apo E4 differs from apo E3 in that in apo E4 arginine is substituted for the normally occurring cysteine at amino acid residue 112. The most common form of apo

Copyright by Mahidol University

E2 differs form apo E3 at residue 158, where cysteine is substituted for the normally arginine.

Result of apo E genotype

The strategy of the apoE genotyping based on the HhaI restriction fragment lengths polymorphism of an amplified DNA fragment is illustrated in Figure 4.8. It relies on the analysis of two specific sequences of the apo E gene. During the PCR , the first pair of amplification primers (P1,P2) as shown in table 3.1 directs the generation of a DNA fragment 292 bp long (PCR1). This fragment contains seven constant HhaI sites, which yield 61, 18-, 16-, 7-, 11-, and 5-bp. The latter pair of amplification primer (P2, P3) directs the generation of a DNA fragment 115-bp longs (PCR2). This fragment contains five constant HhaI site which yield 9-, 9-, 7-, 11-, and 5-bp (Fig 4.8). The polymerase chain reaction products 292 bp and 115 bp for PCR1 and PCR 2 were shown in Figure 4.9. In addition, HhaI cleaves this fragment whenever a cleavage site (nucleotide sequence GCGC) occurs at codon 112 or 158, i.e., when this codon codes for arginine instead of cysteine. In restriction identification, each of the six genotypes yielded a characteristic restriction fragment pattern in addition to the constant fragments of 61-, 18-, 16-, 11-, 7-, and 5-bp. The small invariant 18-, 16-, 11, 7-, 15-, and 5- bp fragments were too small to be clearly visible in this analyses.

Copyright by Mahidol University

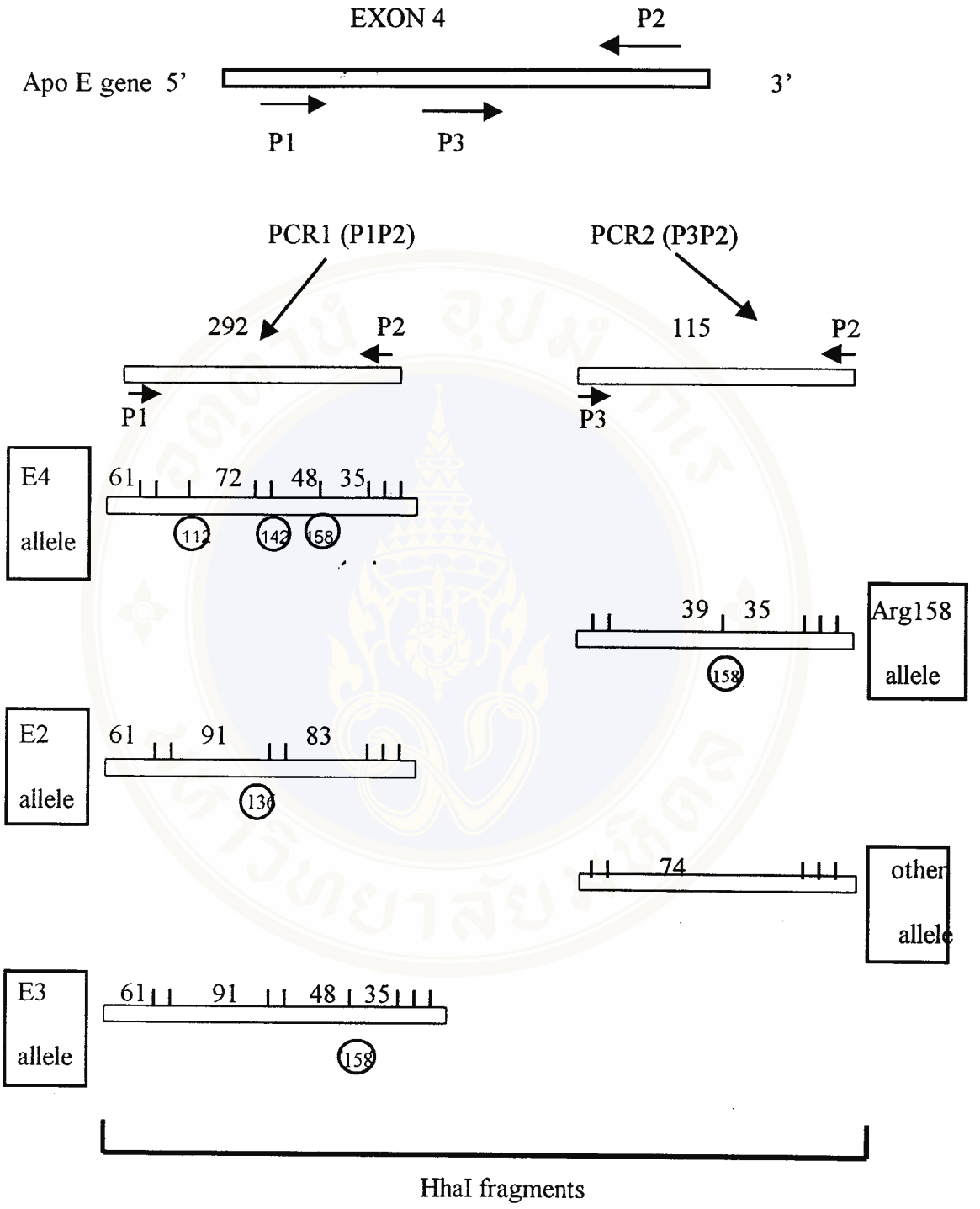


Figure 4.8. Strategy for differentiation of common apo E allele by restriction site analysis on PCR1 and PCR2 products. P1, P2, P3 are primer 1, primer 2, primer 3. HhaI cutting site is GCGC. Numbers in circles indicate vairable amino acids. numbers shown sizes in base pairs of visible restriction fragments formed[120-121].

Copyright© by Mahidol University

The ε2/ε2 genotype is characterized by the presence of the 91- ,83- and 61-bp fragments (Figure 4.10 lane 3, 5). Because in this genotype no HhaI cleavage are present at the two polymorphic (112 and 158) sites, the 72-, 48-, and 35-bp fragment are not formed. The genotype ε2/ε3 shows an additional 48-, and 35-bp fragment (Figure 4.11 lane 3, 4) whereas the genotype ε3/ε3 lacks the 83-, and 72-bp fragments (Figure 4.12 lane 3, 5). In the genotype ε4/ε2 a 83-, and 72-bp fragments are formed, owing to the presence of the HhaI cleavage site at position 112 (Figure 4.13 lane 2). In the genotype ε4/ε3 (Figure 4.14 lane 3, 5) the 83-bp fragment is absent because both alleles have an arginine residue and thus HhaI cleavage site at position 158; however, a 91-bp fragment present in the ε4/ε3 genotype is, in turn, lacking in the genotype ε4/ε4 (Figure 4.15 lane 3)

For the other DNA fragment, PCR2 was performed with P3P2 primers and HhaI digestion to confirmed the Arg 158 containing site. The corresponding nucleotide sequence is TGCGCC from one mismatch that contains at 3' end of primer P3, which contains only an HhaI site. The ε2/ε2 genotype is characterized by presence of only 74-bp, fragments (Figure 4.10 lane 4, 6). Because in this genotype no HhaI cleavage site, the 39- and 35-bp fragments are not formed. The genotype ε3/ε2, ε3/ε3 and ε4/ε2, shows an additional 74-, 39- and 35-bp fragments (Figure 4.11 lane5, Figure 4.12 lane 4, 6 and Figure 4.13 , lane 1, 3; respectively), whereas the genotype ε4/ε4 and ε4/ε3, lacks the 74-bp fragments (Figures 4.14 lane 4,and 4.15 lane 4, 6)

Copyright by Mahidol University

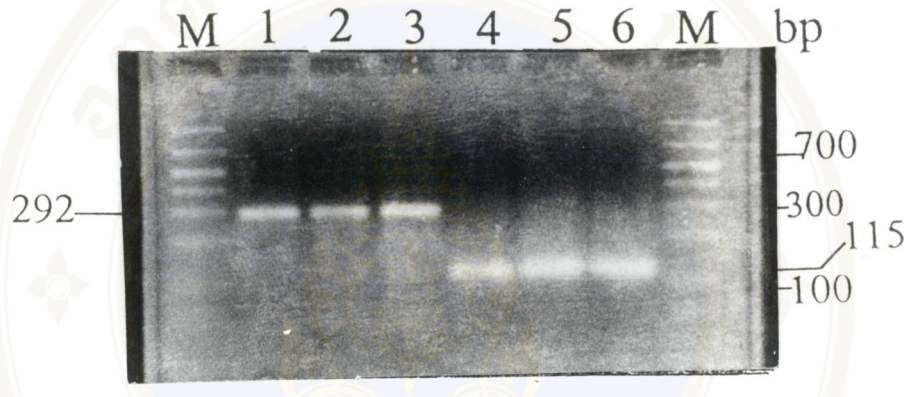


Figure 4.9 Polymerase chain reaction products of PCR 1 of 292 bp in lanes 1-3 and PCR 2 of 115 bp in lanes 4-6 respectively.

Copyright by Mahidol University

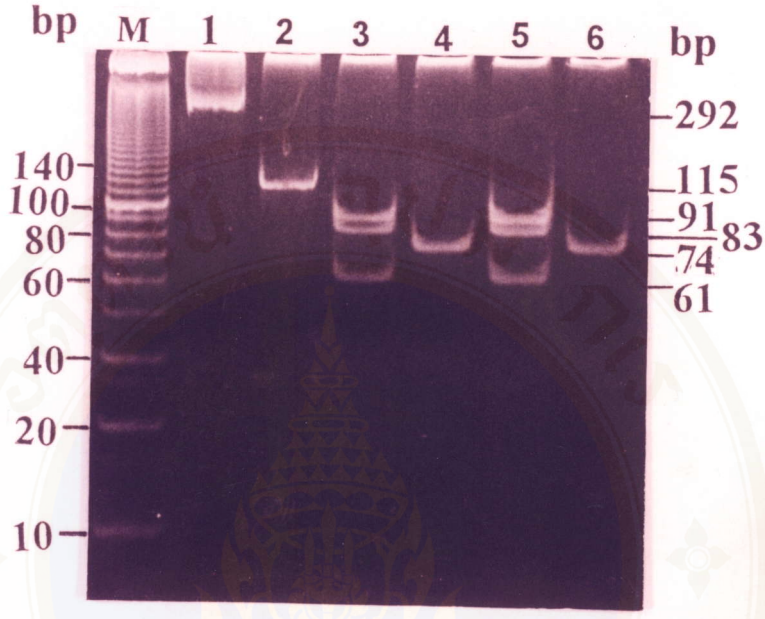


Figure 4.10 PCR products of E2/E2 genotypes: products were separated in 8% polyacrylamide gel stained with ethidium bromide. M, size marker 10 bp ladder; lane 1, undigested PCR1 product (292-bp); lane 2, undigested PCR2 product (115-bp); lanes 3 and 5 are PCR1 digestion with HhaI, which present 91-, 83-, and 61-bp fragments; lanes 4 and 6 are PCR2 digestion with HhaI, which present only 74-bp fragment.

Copyright by Mahidol University

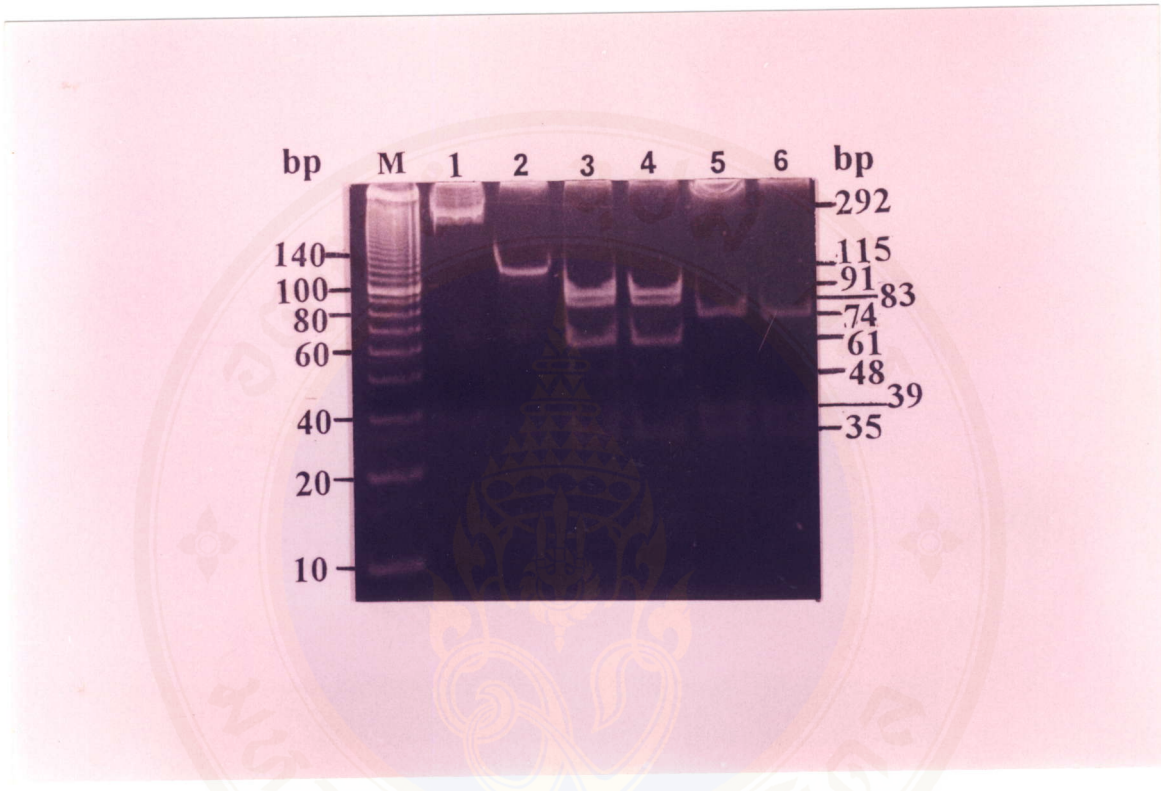


Figure 4.11 PCR products of E2/E3 genotypes: lanes 3 and 4 are PCR1 digestion with HhaI, which present 91-, 83-, 61-, 48-, and 35-bp fragments; lanes 5 and 6 are PCR2 digestion with HhaI, which present 74-, 39-, and 35-bp fragments.

Copyright by Mahidol University

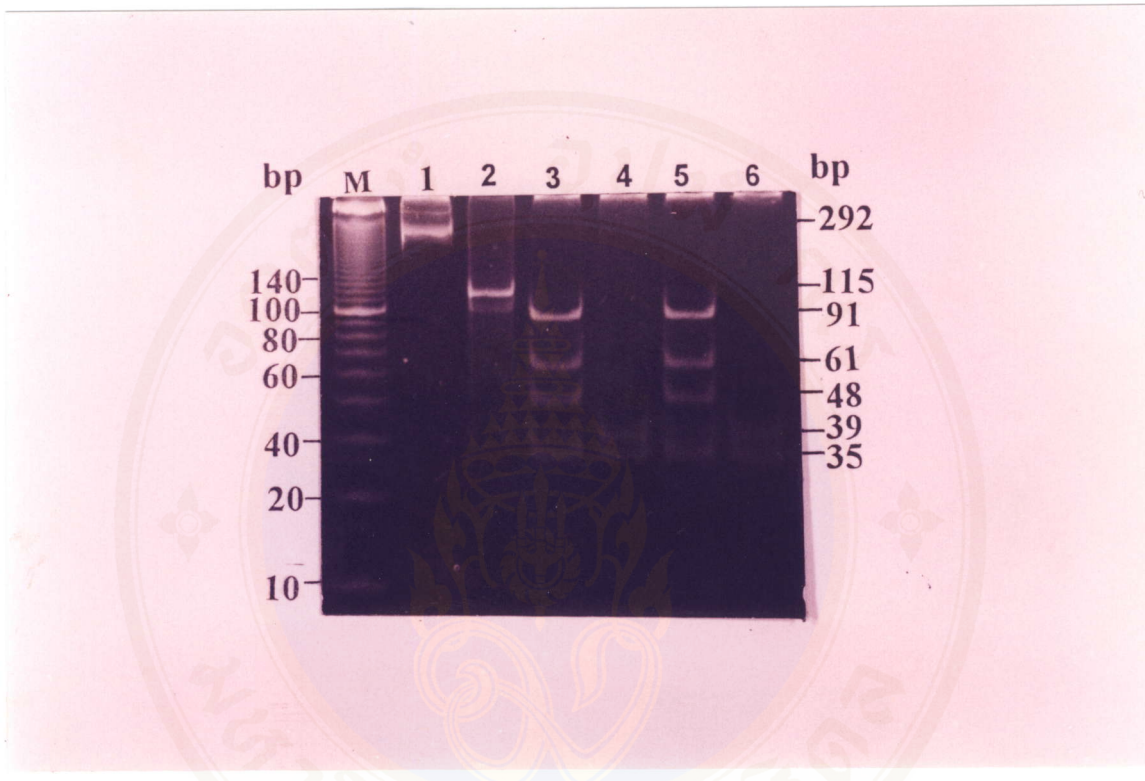


Figure .12 PCR products of E3/E3 genotypes: lanes 3 and 5 are PCR1 digestion with HhaI, which present 91-, 61-, 48-, and 35-bp fragments; lanes 4 and 6 are PCR2 digestion with HhaI, which present 39-, and 35-bp fragments.

Copyright by Mahidol University

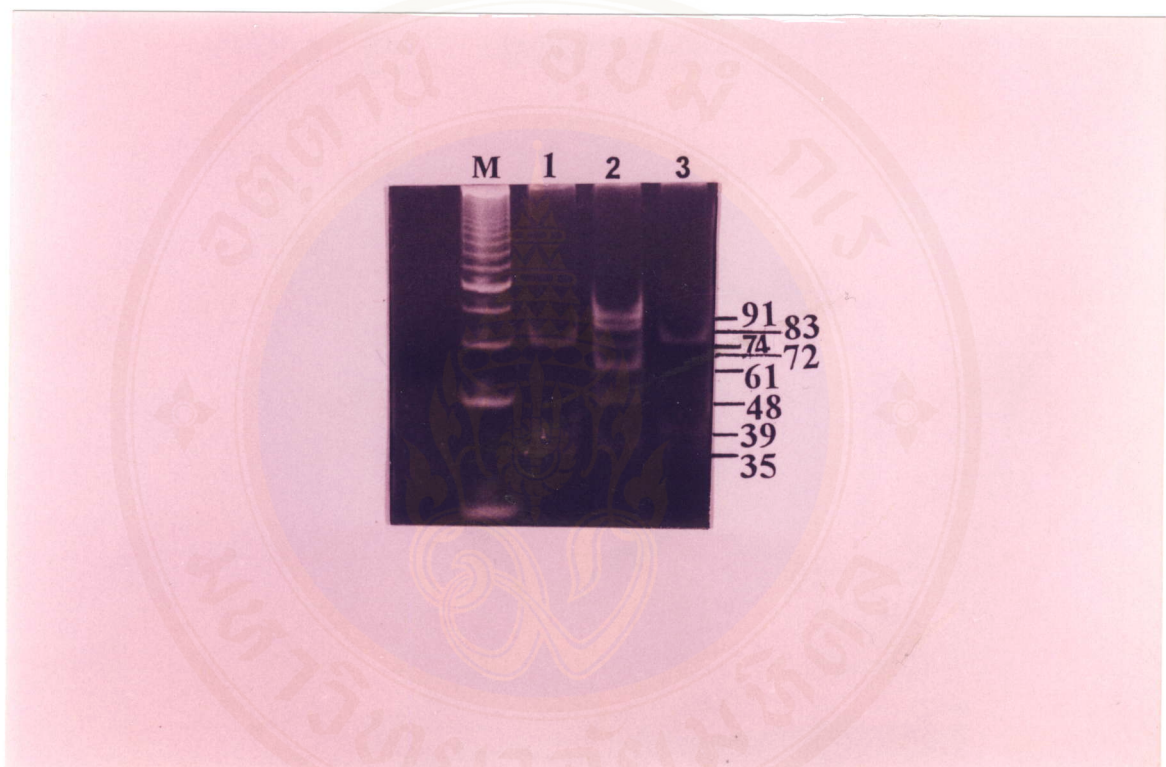


Figure 4.13 PCR products of E2/E4 genotypes: lanes 2 is PCR1 digestion with HhaI, which present 91-, 83-, 72-, 61-, 48-, and 35-bp fragments; lanes 1 and 3 are PCR2 digestion with HhaI, which present 74-,39-, and 35-bp fragments

Copyright by Mahidol University

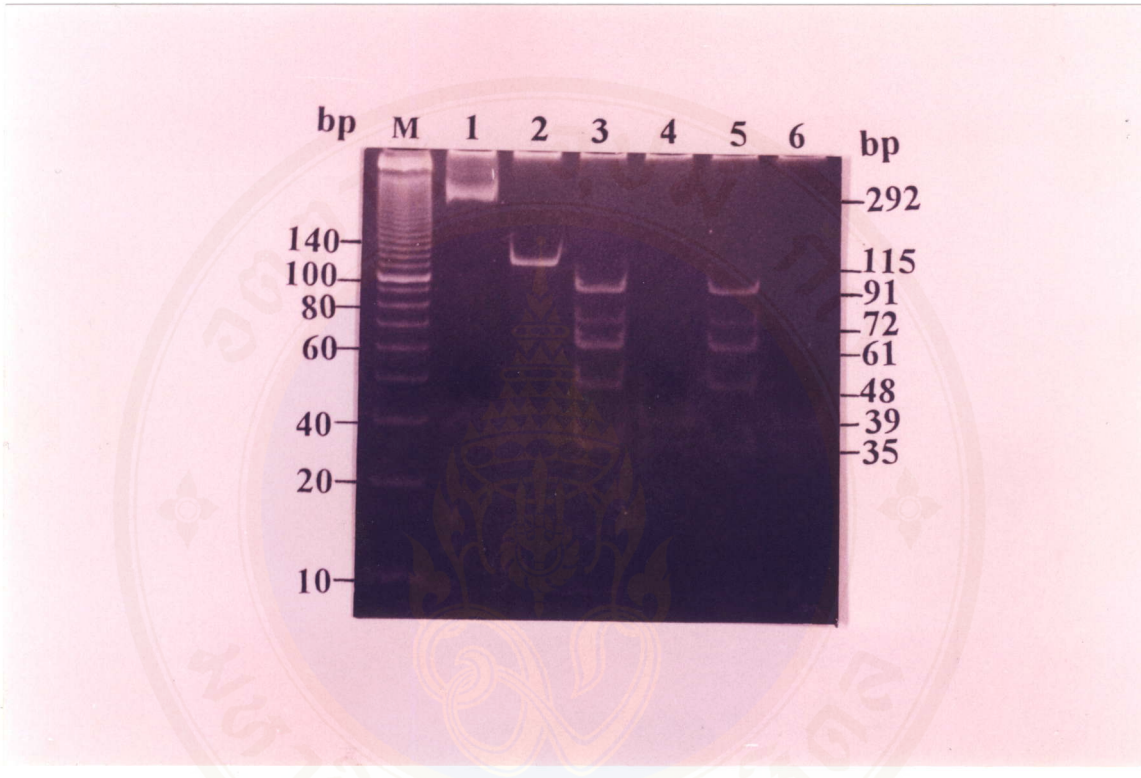


Figure 4.14 PCR products of E3/E4 genotypes: lanes 3 and 5 are PCR1 digestion with HhaI, which present 91-,72-, 61-, 48-,and 35-bp fragments; lanes 4 and 6 are PCR2 digestion with HhaI, which present 39-, and 35-bp fragments.

Copyright by Mahidol University

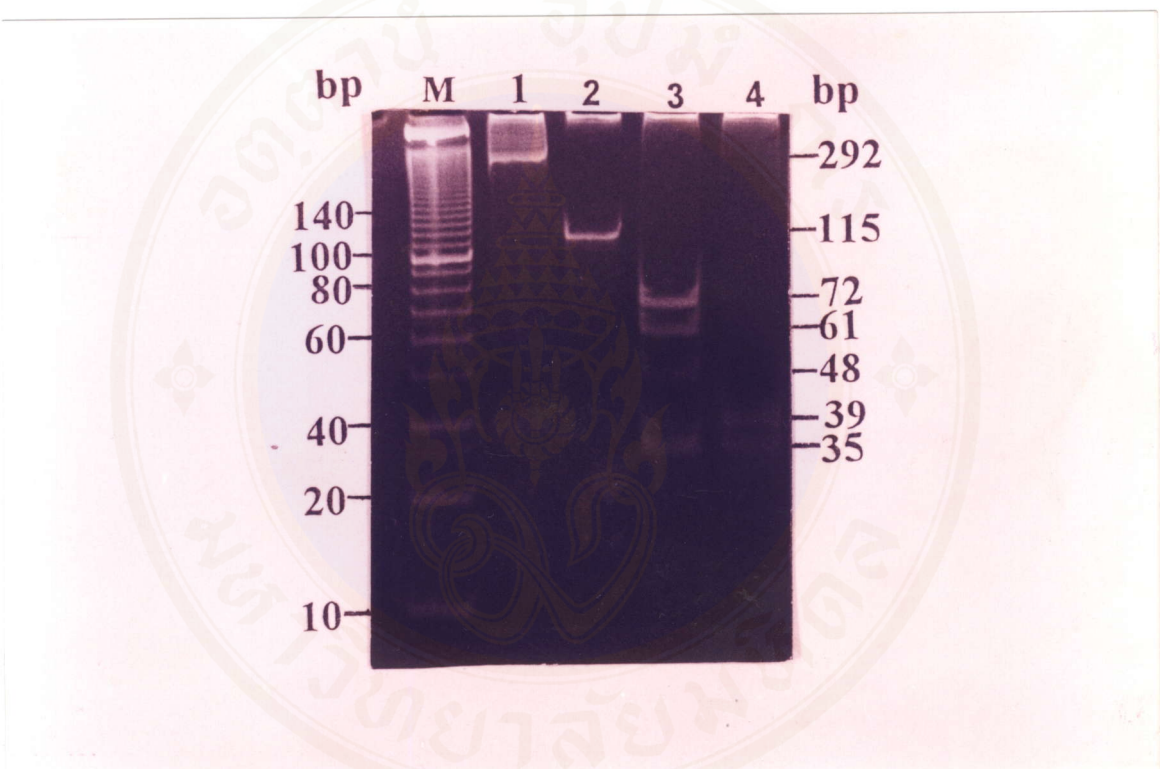


Figure 4.15 PCR products of E4/E4 genotypes: lanes 3 is PCR1 digestion with HhaI, which present 72-, 61-, 48-, and 35-bp fragments; lanes 4 is PCR2 digestion with HhaI, which present 39-, and 35-bp fragments.

Copyright by Mahidol University

The samples of 171 normal healthy subjects of both normolipidaemia and hyperlipidaemia, were randomly selected from department of Preventive and Social Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. For apo E identification. The apo E distribution and calculated apo E allele frequencies of general population, which subdivided into hyperlipidaemia and normolipidaemia, respectively are presented in tables 4.11, 4.12, 4.13.

The allele frequencies of apo E that were estimated by gene counting method using the criteria of Hardy-Weinberg equilibrium were also used for calculated the expected number. Differences in genotype distribution from that expected for Hardy-Weinberg equilibrium were estimated by the chi-square (χ^2) test. A chi-square test of heterogeneity in the apo E genotype distribution between this general study population and hyperlipidaemic population indicates statistically significant differences (df = 5, χ^2 =27.27; $p< 0.001$ and df =5, χ^2 =34.9 ; $p< 0.001$ (Tables 4.11 and 4.12, respectively). These implied that Thai general and hyperlipidaemic populations were deviated from Hardy-Weinberg equilibrium. This may be due to the high frequency of E2/E2 genotype. However, the apo E genotype distribution in normolipidaemic group was in Hardy-Weinberg equilibrium (χ^2 =2.99; p=0.700; df=5) (Table 4.13).

Copyright by Mahidol University

Table 4.11 Apolipoprotein E genotype distribution and calculated allele frequencies in Thai population studied. χ^2 of Hardy-Weinberg distribution is 27.27; $p<0.001$; (df=5).

Genotype	No. observed (n)	No. expected	Relative frequencies (%)
E3/E3	114	109	66.7
E3/E4	32	33	18.7
E3/E2	13	22	7.6
E2/E2	6	1	3.5
E4/E4	4	3	2.3
E4/E2	2	3	1.2
Total	171	171	100

Gene (allele) frequencies		
Allele	Observed	Expected
E3	0.80	0.80
E4	0.12	0.122
E2	0.08	0.078

Copyright by Mahidol University

Table 4.12. Apolipoprotein E phenotype distribution and calculated allele frequencies in Thai hyperlipidaemic population studied. χ^2 of Hardy-Weinberg distribution is 34.9; $p<0.001$ (df=5)

Genotype	No. observed (n)	No. expected	Relative frequencies (%)
E3/E3	64	59	62
E3/E4	25	25	24
E3/E2	5	1	5
E2/E2	4	12	4
E4/E4	3	3	3
E4/E2	2	3	2
Total	103	103	100

Gene (allele) frequencies		
Allele	Observed	Expected
E3	0.76	0.77
E4	0.16	0.16
E2	0.08	0.07

Copyright by Mahidol University

Table 4.13 Apolipoprotein E genotype distribution and calculated allele frequencies in Thai normolipidaemia. χ^2 of Hardy-Weinberg distribution is 2.99; p=0.70 (df=5)

Genotype	No. observed (n)	No. expected	Relative frequencies (%)
E3/E3	50	50	73.5
E3/E4	7	7	10.3
E2/E2	1	0.5	1.5
E2/E3	9	9	13.2
E4/E4	1	0.5	1.5
E4/E2	0	1	0
Total	68	68	100

Gene (allele) frequencies		
Allele	Observed	Expected
E3	0.85	0.85
E4	0.07	0.07
E2	0.08	0.09

The observed apo E genotype distribution and corresponding apo E allele frequencies between gender in Thai population, hyperlipidaemic, and nomorlipideamic population were also presented in Tables 4.14, 4.15, and 4.15. The apo E3 allele frequency was found to be the highest of both genders, whereas the allele frequency of apo E4 in male is higher and apo E2 is lower than that in female in all of these three groups. However, the chi-square test of heterogeneity in the apo E allele frequency of female and male in Thai population, normolipidaemia, and hyperlidaemia were significantly difference ($p<0.05$). this may be due to the high allele frequency of E3 and

Copyright by Mahidol University

low allele frequency of E4 in female. This suggested that degree of prevalence of hypercholesterolaemia in men is higher than that in woman.

Table 4.14 Distribution of apolipoprotein E genotypes and estimated allele frequencies between gender in Thai population (n=171).

	Male		Female	
Allele	No. observed (n)	Relative frequencies (%)	No. observed (n)	Relative frequencies (%)
E3/E3	37	62	80	69
E3/E4	15	25	17	15.6
E2/E2	1	1.6	5	4.5
E3/E2	2	3.3	8	11
E4/E4	3	4.9	1	1
E4/E2	2	3.3	0	0
Total	60	100	111	100

Gene (allele) frequencies		
Allele	Male	Female
E3	0.76	0.83
E4	0.19	0.09
E2	0.05	0.08

χ^2 for male vs female =7.71; $p<0.05$; df=2

Copyright by Mahidol University

Table 4.15 Distribution of apolipoprotein E genotypes and estimated allele frequencies between gender in Thai hyperlipidaemic population (n=103).

	Male		Female	
Genotype	No. observed (n)	Relative frequencies (%)	No. observed (n)	Relative frequencies (%)
E3/E3	22	64	42	61
E3/E4	9	25	16	24
E2/E2	0	0	5	7.5
E3/E2	0	0	4	6
E4/E4	2	5.5	1	1.5
E4/E2	2	5.5	0	0
Total	35	100	68	100

Gene (allele) frequencies		
Allele	Male	Female
E3	0.76	0.76
E4	0.21	0.13
E2	0.03	0.11

χ^2 for male vs female =6.4; $p<0.05$; df=2

Copyright by Mahidol University

Table 4.16 Distribution of apolipoprotein E genotypes and estimated allele frequencies between gender in Thai normolipidaemic population (n=68).

	Male		Female	
Genotype	No. observed (n)	Relative frequencies (%)	No. observed (n)	Relative frequencies (%)
E3/E3	15	60	38	89
E3/E4	6	24	1	2
E2/E2	1	4	0	0
E3/E2	2	8	4	9
E4/E4	1	4	0	0
E4/E2	0	0	0	0
Total	25	100	43	100

Gene (allele) frequencies

Allele	Male	Female
E3	0.76	0.94
E4	0.16	0.01
E2	0.08	0.05

χ^2 for male vs female =12.55; $p< 0.01$; df=2

In the study of Yamamura et al.[123], the heterogeneity of apo E in very low density lipoprotein from hyperlipidaemic subjects with or without atherosclerosis and patients with ischaemic heart disease and apparently healthy individuals was analysed. The distribution of six common apo E phenotype and allele frequencies in the groups of hyperlipidaemia and ischaemic heart disease was similar to that in the healthy group. In this present study the sample of hyperlipidaemia and normolipidaemia were randomly

Copyright by Mahidol University

selected from hyperlipidaemic clinic in Siriraj Hospital. The gene frequencies in Thai hyperlipidaemia (χ^2 = 1.79, df =2, p= 0.409) and normolipidaemia (χ^2 = 2.5, df=2, p= 0.27) are not significantly different as compared to the Thai population. However, the allele frequencies of hyperlipidaemic and normolipideamic subjects was significantly different (χ^2=6.18, df=2, p<0.05) (Table 4.17). From this table we also calculated the separate contribution of the total χ^2 value. It was shown that the E4 allele in hyperlipideamia significantly differed from normolipidaemia by an increased E4 86%.

Table 4.17 The allele frequencies of apo E in Thai population

Population sample	No. of subjects	Apo E allele frequency			Hardy-Weinberg distribution	
		E2	E3	E4	χ^2 df=5	p
Hyperlipidemia	103	0.08 (0%)	0.76 (14%↓)	0.16 (86%↑)	34.9	p<0.001
Normolipidaemia	68	0.08	0.85	0.07	2.99	p=0.70
Thai population	171	0.08	0.8	0.12	27.27	p<0.001

χ^2 = 6.18, df=2, p<0.05 for hyperlipidaemia vs normolipidaemia. No. in parenthesis showed the deviation of E2, E3, and E4 alleles from normolipidaemia.

χ^2 = 1.79, df=2, p= 0.409 for hyperlipidaemia vs Thai population

χ^2 = 2.5, df=2, p= 0.27 for Thai population vs normolipidaemia

Since apo E allele frequencies in Thai population were not agreement to the report of Japanese population, the apo E allele frequencies obtained in this present study were also compared with those observed in other racial population (Table 4.18). A chi-square test of heterogeneity indicates statistically significant differences in the apo E allele frequency distribution between the different populations (df = 44; χ^2 = 167; p<0.001). Two-sample χ^2 analysis showed that the allele frequencies of

Copyright by Mahidol University

the Thai population differ highly significantly from the populations of Sudan, Greenland, Sweden, and the two populations of Finland ($p<0.001$), and less significant difference was also observed in population of Trinidad ($p<0.05$). No significant differences from Thai population were found with the population of Japan, Germany, Netherland, Canada, the United States, Australia, Scotland, Spain, Morocco, and the Chinese, Indian, and Malay from Singapore.

Copyright by Mahidol University

Table 4.18 Apo E gene frequencies in several random population samples. χ^2 values at df = 2 and P values of 0.01 and 0.001 are, 9.21 and 13.95.

Population sample	No. subjects	E2	E3	E4	Hardy-Weinberg Distribution (χ^2);df=5 (p)		Different from the Thai population (χ^2);df=2 (p)		Reference
Thai	171	0.080	0.800	0.120	27.27	<0.005	-	-	This study
Australia	424	0.068	0.759	0.172	33.3	<0.005	3.0	NS	Wang et al (1995)
Austria	469	0.090	0.789	0.117	4.13	NS	0.34	NS	Hallmann et al (1991)
Canada	203	0.078	0.77	0.152	1.61	NS	1.61	NS	Davignon et al (1984)
Finland	615	0.041	0.733	0.227	7.09	NS	24.9	<0.001	Ehnholm et al (1986)
Finland	203	0.062	0.695	0.244	2.26	NS	18.92	<0.001	Menzel et al (1983)
Germany	1000	0.078	0.783	0.139	7.15	NS	0.90	NS	Menzel et al (1983)
Greenland	178	0.014	0.781	0.205	4.0	NS	24.2	< 0.001	Gerdes et al (1996)
Hungary	202	0.064	0.807	0.129	14.8	<0.05	0.8	NS	Hallmann et al (1991)
Iceland	185	0.068	0.768	0.165	2.14	NS	3.01	NS	Hallmann et al (1991)
Japan	319	0.081	0.849	0.067	3.51	NS	7.98	NS	Utermann (1987)
Morocco	100	0.065	0.850	0.085	4.5	NS	2.2	NS	Valveny et al (1997)
Netherlands	2018	0.082	0.751	0.167	2.84	NS	5.31	NS	Smit et al (1988)
New Zealand	426	0.119	0.739	0.141	14.4	<0.05	5.46	NS	Wardell et al (1982)
Scotland	400	0.083	0.77	0.145	3.7	NS	1.40	NS	Cumming et al (1984)
Singapore (Malay)	118	0.114	0.767	0.119	5.27	NS	1.92	NS	Hallmann et al (1991)
Singapore (Indian)	142	0.046	0.827	0.127	2.01	NS	2.98	NS	Hallmann et al (1991)
Singapore (Chinese)	190	0.097	0.828	0.074	2.24	NS	4.75	NS	Hallmann et al (1991)
Spanish	120	0.046	0.871	0.083	1.3	NS	5.1	NS	Valveny et al (1997)
Sudan	103	0.081	0.619	0.291	0.27	NS	26.3	<0.001	Hallmann et al (1991)
Sweden	407	0.078	0.719	0.23	62.5	<0.001	16.99	<0.001	Hallmann et al (1991)
Trinidad	268	0.147	0.694	0.159	12.3	< 0.05	13.2	< 0.05	Hegele et al (1999)
USA	1204	0.075	0.786	0.135	15.3	<0.01	0.68	NS	Ordovas et al. (1987)

Copyright by Mahidol University

From the tables generated from the respective two-sample χ^2 analyses (Table not shown), we were able to calculate the separate contribution of the total χ^2 value (Table 4.19). From the data presented in Table 4.19, it was obvious that 60% and 80% of the differences in apo E allele frequencies, measured as χ^2 value, between the Thai population and the two Finnish populations[99-108] were due to the relatively high E4 allele frequencies. The Sudanese[110] and Swedish[110] populations also differed from the Thai population in apo E allele frequencies by an increased E4 allele (80%). However, the Trinidad population was different from Thai population by an increased E2 allele (60%), but in contrast to Greenland this difference was occurred by a decreased E2 allele (68%).

Table 4.19 Relative contribution of the different apo E alleles to the χ^2 value as estimated for the difference in allele frequencies between the Thai and other populations. The contribution to the total χ^2 is due to an increased or decreased (↑,↓) allele frequency, respectively.

Apo E allele	Population (reference)					
	Finland (Ehnholm et al, 1986)	Finland (Menzel et al, 1984)	Sudan (Hallmann et al, 1991)	Sweden (Hallmann et al, 1991)	Trinidad (Robert et al, 1999)	Greenland (Gerdes et al, 1996)
E2	0.331 ↓	0.046 ↓	0.0001 ↑	0.003 ↓	0.59 ↑	0.677 ↓
E3	0.066 ↓	0.148 ↓	0.204 ↓	0.196 ↓	0.24 ↓	0.003 ↓
E4	0.602 ↑	0.806 ↑	0.796 ↑	0.800 ↑	0.168 ↑	0.319 ↑

Two-sample χ^2 analysis showed a significant difference in apo E allele frequencies among Thai and both of Finland, Sudan, Sweden, Trinidad, and Greenland population samples. Comparison between two Finnish populations showed no significant difference (χ^2 = 3.45; p= 0.178; df= 2).

Copyright by Mahidol University

Effect of allele substitution at the apo E gene locus and plasma lipid levels

To evaluate whether the allelic variation at the apo E gene locus significantly affects the serum lipid levels. Plasma total cholesterol, triglycerides, HDL-C, LDL-C and VLDL-C were determined. Table 4.20 presented the mean total cholesterol, triglyceride, LDL-C, HDL-C and VLDL-C levels in the different apo E genotype groups. The nonparametric test of Kruskal-Wallis (H-test) was used for examining the equality of the mean values among apo E phenotypes. The statistical analyses showed that plasma cholesterol, LDL-C, triglyceride and VLDL-C levels significantly differ among apo E genotype groups, but not HDL-C.

Table 4.20 Lipid levels in Thai population of different genotypes in apo E polymorphism (data expressed as mean ± SE).

Parameter	E2/E2 (n=6)	E2/E3 (n=14)	E2/E4 (n=2)	E3/E3 (n=114)	E3/E4 (n=32)	E4/E4 (n=4)	H	p
Cholesterol (mg/dl)	265±24	195±11	276±4	243±6	255±17	237±5	11.9	<0.05
LDL-C (mg/dl)	161±23	122±11	204±4	157±5	175±11	182±17	11.8	0.05
Triglyceride (mg/dl)	307±83	113±13	194±38	135±7	157±15	135±26	14.7	<0.05
HDL-C (mg/dl)	42±6	50±5	32±8	51±1	49±2	42±6	7.4	0.19
VLDL-C (mg/dl)	62±17	22±3	38±8	27±1	31±3	27±5	14.6	<0.05

Copyright by Mahidol University

Since the genotypes of apo E2 and E4 of this study were rather small, the combination of apo E genotypes was adjusted as indicated in table 4.21. This combination is only based on the influence of apo E allele on plasma cholesterol level as compared with the most common E3 allele, i.,e. the E4 allele leads to elevated plasma cholesterol and LDL-C levels, whereas the E2 allele is associated with an increased plasma triglyceride and decreased plasma cholesterol level as described in chapter 2. By using unpair student's t-test, it showed that apo E genotypes containing E2 allele or A in the table have only triglyceride significantly higher than apo E3/E3 genotypes or B in this table. As compared with apo E genotypes containing E4 allele or C in this table, the plasma total cholesterol and LDL-C are significantly higher. In comparison of E3/E3 genotype (B) with apo E genotypes containing E4 (C), only plasma total cholesterol and LDLC are significantly higher. From this study, we can conclude that the lipid levels in this study are agreement with the other reports as mentioned above. In addition, in group A and C as indicated in this table, the plasma lipid levels had a trend to increase whereas the HDL-C had a chance to decrease.

Copyright by Mahidol University

Table 4.21 Mean plasm lipid levels among different apo E phenotypes

Group of genotype	Total cholesterol (mg/dl)[a]	LDL-C (mg/dl)[b]	Triglyceride (mg/dl)[c]	HDL-C (mg/dl)[d]	VLDL-C (mg/dl)[e]
A (n=19)	218±13	136±11	175±34	47±4	35±7
B (n=115)	234±6	156±5	134±7	51±1	27±1
C (n=38)	256±10	179±9	156±13	47±2	31±3

A = combination of E2/E2 and E2/E3

B = only E3/E3

C = combination of E4/E4, E4/E3 and E4/E2

[a] A vs B; p=0.3 [a] A vs C; p<0.05 [a] B vs C; p<0.05

[b] A vs B; p=0.15 [b] A vs C; p<0.05 [b] B vs C; p<0.05

[c] A vs B; p<0.05 [c] A vs C; p=0.5 [c] B vs C; p=0.1

[d] A vs B; p= 0.2 [d] A vs C; p=0.9 [d] B vs C; p= 0.1

[e] A vs B; p= 0.5 [e] A vs C; p=0.5 [e] B vs C; p=0.1

Table 4.22 presents the mean plasma total cholesterol, triglyceride, LDL-C, HDL-C and VLDL-C levels of hyperlipidaemic subjects in before and after treatment with lipid-lowering drugs for a period of 3-5 years in the different apo E genotype groups. We used the pair student's t-test to compare these lipid levels before and after treatment in hyperlipidaemic patients. The statistical analyses showed that plasma total cholesterol, triglyceride and LDL-C significantly reduced in only homozygous E3/E3 genotype after treatment ($p<0.05$). In the other genotype (E2/E2, E2/E4, E2/E3, E3/E4, and E4/E4) only showed a trend to decrease the lipid levels after treatment, but no significant difference was observed.

Copyright by Mahidol University

Table 4.22 Effect of Lipid-lowering drug @ on lipid levels in apo E polymorphism

Apo E phenotypes	Total cholesterol (mg/dl)		LDL-C (mg/dl)		Triglyceride (mg/dl)		HDL-C (mg/dl)		VLDL-C (mg/dl)	
	Before	After	Before	After	Before	After	Before	After	Before	After
E2/E2 (n=5)	280±23	215±16	169±27	143±17	333±96	125±17	44±7	47±4	67±19	25±4
E2/E3 (n=4)	254±9	242±10	178±14	160±7	144±29	114±19	47±9	60±2	29±6	23±4
E2/E4 (n=2)	276±4	195±28	205±5	98±20	195±38	214±11	33±9	54±30	39±8	43±22
E3/E3 (n=62)	282±5	247±5***	199±6	171±5***	166±10	132±8**	50±2	50±2	33±2	26±2
E3/E 4(n=25)	278±10	258±6	195±11	177±7	166±18	149±17	50±3	53±3	33±4	42±13
E4/E4 (n=3)	269±12	244±21	198±10	164±25	141±26	141±50	43±9	57±9	28±7	28±10

@ = lipid-lowering drugs used in these patients were Lopid, Mevalotin, Questran, Lipantil , Simvastatin including diet recomendation for 3-5 years during genetic analysis.

*** = $p < 0.0001$, ** = $p < 0.01$

Due to the small number of apo E2 and E4 genotypes, the combination of apo E2 and E4 genotypes was performed as indicated in table 4.23. By using pair student's t-test, the statistical analysis showed that apo E genotypes containing E2 allele (or A) showed no significantly decrease total cholesterol, LDL-C, triglyceride, and VLDL-C. Whereas the E3/E3 genotype (B) was significantly decreased total cholesterol, LDL-C, and triglyceride. For the genotype containing E4 allele (or C), it was significantly decreased total cholesterol, LDL-C but triglycerides tended to decrease. The results showed that lipid-lowering drug and calorie-lowering dietary manipulation can also significantly decrease plasma total cholesterol, LDL-C and triglyceride, only in normal apo E and apo E containing E4 allele.

Copyright by Mahidol University

Table 4.23 Effcet of lipid-lowering drug on lipid levels in apo E polymorphism.

Apo E genotypes	Total cholesterol (mg/dl)		LDL-C (mg/dl)		Triglyceride (mg/dl)		HDL-C (mg/dl)		VLDL-C (mg/dl)	
	Before	After	Before	After	Before	After	Before	After	Before	After
A (n=9)	268±13	227±10	173±15	150±10	249±62	120±12	45±5	53±3	50±12	24±2
B (n=62)	282±5	247±5***	199±6	171±5***	166±10	132±8**	50±2	50±2	33±2	26±2
C (n=30)	277±9	253±6*	196±9	171±8*	166±16	152±16	48±3	52±3	33±3	40±11

A = combination of E2/E2 and E2/E3

B = only E3/E3

C = combination of E4/E4, E4/E3 and E4/E2

*** = $p < 0.0001$, ** = $p < 0.01$, * = $p < 0.05$

Effect of apo B and apo E polymorphisms on plasma lipid levels

To assess the influence of apo B and apo E polymorphisms that significantly affects on the serum lipid levels. Plasma total cholesterol, triglycerides. HDL-C, VLDL-C, and LDL-C in various types of apo B and E polymorphisms were determined. Table 4.24 showed the mean total cholesterol, triglycerides, LDL-C, VLDL-C, and LDL-C in the different combination of apo B and apo E genotype groups. The non-parametric Kruskal-Wallis test (H-test) was used for determining the equality of the mean value among these combination groups. The statistical analyses showed that no significant difference among apo B and apo E combination groups. However, from this table we observed that in apo B containing II genotype group, the apo E containing E2/E2 genotype has a higher triglyceride level whereas apo E containing E4/E4 genotype has higher cholesterol than that in other genotypes. In apo B containing ID genotype group, the apo E containing E2/E2 genotype has both total cholesterol and triglyceride higher than the other groups. For the combination of apo B

Copyright by Mahidol University

containing DD genotype and apo E genotypes, the amounts of this group was rather small. Therefore, we could not observe the difference of lipid levels.

Table 4.24 Mean plasma lipid levels among the apo B and apo E combinations.

Apo B and E genotypes	Total cholesterol (mg/dl)	LDL-C (mg/dl)	Triglyceride (mg/dl)	HDL-C (mg/dl)	VLDL-C (mg/dl)
II and E3/E3 (n=71)	228±7	168±11	135±9	51±1	27±2
II and E2/E2 (n=3)	278±51	182±37	262±54	43±4	53±10
II and E2/E3 (n=8)	176±10	103±10	107±21	51±7	21±4
II and E2/E4 (n=2)	276±4	204±4	194±38	32±8	38±8
II and E3/E4 (n=17)	258±17	177±16	145±19	52±4	29±4
II and E4/E4 (n=2)	279±9	206±10	121±51	49±11	24±10
ID and E3/E3 (n=39)	245±11	168±11	131±11	50±2	26±2
ID and E2/E2 (n=3)	252±4	140±30	353±171	41±13	71±34
ID and E2/E3 (n=4)	215±22	145±24	125±14	45±5	25±3
ID and E3/E4 (n=17)	252±59	172±17	180±25	44±4	36±5
ID and E4/E4 (n=1)	248	181	182	31	36
DD and E3/E3 (n=3)	235±14	143±3	191±83	54±1	38±17
DD and E2/E3 (n=1)	251	160	95	72	19
DD and E3/E4 (n=2)	250±4	177±1	105±46	52±4	21±9
DD andE4/E4(n=1)	198	136	117	39	23

As mentioned in the earlier data that some genotypes of apo B and apo E were rather small, these combinations were recombined as indicated in table 4.25. The results showed that no significant difference of these plasma lipid levels in this

Copyright by Mahidol University

combination of apo B and apo E genotype groups. Since hyperlipideamia is multifactorial (multiple risk factors) as mentioned in chapter 1, we could not conclude in this study that which risk factors between life style or genetics is prominent in leading to the high blood lipid levels. Reasons for these discrepancies are not well understood and are likely to be complex. However, this finding is confirmed to the fact that aging may influence the disturbance of plasma lipid levels causing the elevated plasma lipid in both the normal and variant type of these polymorphisms.

Table 4.25 Mean plasma lipid levels among the apo B and apo E combinations.

Apo B and E genotypes	Total cholesterol (mg/dl)	LDL-C (mg/dl)	Triglyceride (mg/dl)	HDL-C (mg/dl)	VLDL-C (mg/dl)
A (n=72)	228±7	150±6	134±9	51±1	23±2
B (n=34)	239±12	159±11	148±14	51±3	30±3
C (n=36)	249±12	174±11	133±12	48±2	27±2
D (b=21)	243±11	162±12	191±29	43±3	38±6
E (n=8)	243±10	162±10	139±3	53±4	28±6

A = II and E3/E3, B = II and non E3/E3, C = ID and E3/E3, D = ID and non E3/E3, E = DD and all apo E genotypes

Table 4.26 presents the mean plasma cholesterol, triglyceride, LDL-C, HDL-C and VLDL-C levels of hyperlipidaemic patients before and after treatment with lipid-lowering drugs in the combination of apo B and apo E phenotype groups. We used the pair student's t-test to compare these lipid levels before and after treatment in hyperlipidaemic patients. The statistical analyses showed that plasma total cholesterol, triglyceride, LDL-C and VLDL-C were decreased after treatment in the case of

Copyright by Mahidol University

homozygous Insertion for apo B signal peptide and homozygous E3/E3 phenotype. In the case of heterozygous Insertion/deletion in apo B signal peptide and homozygous E3/E3 or E2/E2 genotype were significantly reduced only cholesterol after treatment with lipid-lowering drugs. However, as mentioned earlier the genotypes of apo E2 and apo E4 were rather small, the combinations of these genotypes with II, ID, and DD were adjusted as indicated in the Table 4.27. From these combinations we found that the common genotypes (II and E3/E3 or A) were significantly decreased total plasma cholesterol, LDL-C, triglyceride, and VLDL-C whereas the other combinations of variant showed significant decrease only total plasma cholesterol.

Table 4.26 Effect of Lipid-lowering drug @ on lipid levels in apo E polymorhism

Apo B and E genotypes	TC (mg/dl)		LDL-C (mg/dl)		Triglyceride (mg/dl)		HDL-C (mg/dl)		VLDL-C (mg/dl)	
	Before	After	Before	After	Before	After	Before	After	Before	After
II and E3/E3 (n=36)	276±6	242±5***	192±6	165±5**	170±13	132±12*	51±2	51±2	34±3	26±2*
II and E2/E2 (n=2)	322±47	217±44	213±34	135±47	303±60	153±40	48±0.5	51±5	61±12	31±8
II and E2/E3 (n=1)	233	268	153	177	228	161	34	59	46	32
II and E2/E4 (n=2)	276±4	195±28	205±5	98±20	195±38	214±114	33±9	54±30	39±8	43±22
II and E3/E4 (n=14)	279±17	259±7	195±16	180±8	154±23	133±15	54±4	53±3	31±5	48±22
II and E4/E4 (n=2)	279±9	247±36	206±10	158±42	121±50	156±83	49±11	58±11	20±10	317
ID and E3/E3 (n=23)	295±8	215±10**	215±10	223±44	155±14	133±13	48±2	49±3	31±3	27±3
ID and E2/E2 (n=3)	252±4	213±13*	140±30	148±12	353±170	160±6	41±13	44±7	71±34	21±2
ID and E2/E3 (n=2)	256±10	229±10	199±15	151±10	126±10	89±23	41±3	61±5	25±2	18±5
ID and E3/E4 (n=9)	283±13	253±11*	200±16	168±18*	200±33	168±40	43±15	52±6	40±7	34±8
ID and E4/E4 (n=1)	248	237	181	178	182	111	31	37	36	22
DD and E3/E3 (n=3)	259±17	248±13	167±27	171±9	211±69	125±22	50±4	44±1	42±14	25±4
DD and E2/E3 (n=1)	251	243	160	160	95	118	72	60	19	24
DD and E3/E4 (n=2)	251±4	277±11	177±1	202±1	105±46	178±5	53±5	40±11	21±9	36±1

@ = lipid-lowering drugs used in these patients were Lopid, Mevalotin, Questran, Lipantil , Simvastatin including diet control for 3-5 years during genetic analysis. *** = $p < 0.0001$, ** = $p < 0.01$, * = $p < 0.05$

Copyright© by Chiang Mai University

Table 4.27 Effect of Lipid-lowering drug [@] on lipid levels in apo E polymorhism.

Apo B and E genotypes	Total cholesterol (mg/dl)		LDL-cholesterol (mg/dl)		Triglyceride (mg/dl)		HDL-choles-terol (mg/dl)		VLDL-choles-terol (mg/dl)	
	Before	After	Before	After	Before	After	Before	After	Before	After
A (n=36)	276± 6	242±5***	192±6	165±5**	170±13	132±12*	51±2	51±2	34±3	26±2*
B (n=21)	281±12	248±8*	196±1	165±9	172±19	146±15	50±3	54±4	34±4	44±14
C (n=23)	295±8	255±10**	215±10	223±14	155±14	133±13	43±2	49±3	30±3	26±3
D (b=15)	272±9	241±8**	187±12	162±10	219±40	141±25	42±4	50±4	44±8	28±5
E (n=6)	255±8	257±9	169±12	180±8	156±41	142±15	54±4	45±4*	31±8	28±3

A = II and E3/E3(n=36), B = II and non E3/E3 (n=21), C = ID and E3/E3, D = ID and non E3/E3,

E = DD and all of apo E genotypes *** = $p < 0.0001$, ** = $p < 0.01$, *= $p < 0.05$

Copyright by Mahidol University

CHAPTER V

Discussion

Dyslipidaemia and Atherosclerosis

Epidemiological and experimental evidence links the more common dyslipidaemia, especially high total and LDL-cholesterol including triglycerides levels and low levels of HDL-cholesterol, to an increase risk of developing coronary artery disease (CAD)[131]. Angiographic studies have also demonstrated a positive relationship between the presence of CAD and serum levels of apolipoprotein B and E [132]. The correlation between the genetic forms of hypercholesterolaemia, such as familial hypercholesterolaemia[13, 77], familial defective apo B-100[20,60, 67, 69,], signal peptide insertion/deletion polymorphisms of apo B[49,56], and apo E polymorphisms [87, 117], and the early onset of coronary events strongly supports a causal relation between hypercholesterolaemia and CAD. In addition, several clinical trials have firmly established that aggressive manipulation and normalization of elevated total and LDL cholesterol reduces the progression of atherosclerosis [133] and increases the disease-free intervals for overall mortality. Based on these finding, it appears logical that cholesterol and triglycerides reductions are warranted to be decreased the CAD morbidity and mortality in hyperlipidaemia patients[134].

The United States National Cholesterol Education Program (Adult Treatment Panel II) (NCEP, ATP-II) [135] and the European Atherosclersis Society (EAS) [136] have

Copyright by Mahidol University

published two documents on the prevention of CAD through the control of dyslipidaemia. These documents underline the great importance of LDL-cholesterol and HDL-cholesterol values which suggest that a plasma LDL-cholesterol level between 155 and 175 mg/dl will be satisfactory for those subjects with a moderately elevated risk, i.e., patients with total cholesterol levels below 300 mg/dl, with no other nonlipid risk factors (hypertension, cigarette smoking, diabetes, etc.), and a ratio of total cholesterol/ HDL cholesterol between 4.5 and 5, which indicates normal positive or high HDL levels. Since LDL-cholesterol is considered to be the real marker for the lipid profile, and thus is the primary factor to be taken into account for any dyslipidaemic therapy. In addition, these documents also suggest that HDL-cholesterol levels below 35 mg/dl in men and 42 mg/dl in women must be treated aggressively.

From the guidelines for the prevention of coronary artery disease of these two documents, the lipid profile as shown in table 4.1 suggested that dyslipidaemia in Thai population is increasing as the same rate as in developing population and may be the increasing cause of death in Thailand. However, in hyperlipidaemic group the ratio of total cholesterol /HDL-cholesterol is higher than 5 suggesting that plasma LDL-cholesterol levels are in high risk of developing coronary disease. At present, the genetic risk factors have been considered that the individual risk evaluation should start from the genetic information to predict the onset of hyperlipidaemia because genotypes are not altered by the disease process or time. Since hyperlipidaemia is a triggering event for atherogenesis thus, it's prevention in advance is one of the most efficacious means for halting or retarding the onset of CAD.

Copyright by Mahidol University

As apo B and apo E polymorphisms have been claimed to associate with dyslipidaemia and thus this study was concentrated on this polymorphisms. This genetic information may be useful for prediction and preventing the onset of dyslipidaemia in the future.

Apo B-100 insertion / deletion polymorphisms

The apo B signal peptides are transmitted in a manner consistent with an autosomal codominant mode of inheritance with two alleles. An insertion or deletion of three codons involving three amino acids, leucine-alanine-leucine[47], within exon 1 of apo B gene causes variation in the length of the signal peptide of the apo B protein. This amino terminal cleavable signal sequence directs the emerging protein to translocate through the endoplasmic reticulum membrane. This mutation may affect the three-dimentional structure or the hydrophobicity of the leader peptide, which has a crucial role in membrane translocation. Thus this signal peptide polymorphism within the apo B gene may play a role in affecting the translocation of the apo B polypeptide into the endoplasmic reticulum. However, further studies on the role of this variation on apo B synthesis and metabolism, and its effect on lipid levels will elucidate the role of signal peptides in protein function and metabolism.

Several previous studies have investigated the impact of this polymorphism on plasma lipid levels. Some studies have found correlations between the Ins/Del polymorphism and lipid levels [49, 52, 55-56, 59], and some other studies have not found this association[50]. Reasons for these discrepancies are not well understood.

The present study was determined the extent of distribution and genotypic effects of apo B signal peptide Ins/Del polymorphism on lipid levels in Thai

Copyright by Mahidol University

LIBRARY AND INFORMATION CENTER MAHIDOL UNIVERSITY

population of both hyperlipidaemia and normolipidaemia. The comparison of apo B Ins/Del allele frequencies between hyperlipidaemia and normolipidaemia was significantly different. The significant correlation of Ins/Del polymorphism with hyperlipidaemia in the present series confirms a similar association observed in the Indian and Chinese in Singpore[51-52]. This finding also showed that Deletion allele was higher in the hyperlipidaemia than that in normolipidaemia. This suggested that the deletion allele may affect the three dimentional structure or the rate of translocation of newly synthesized of apo B from the cytoplasm into the endoplasmic reticulum. This in turn might alter the transport of LDL-cholesterol as apo B is a major constituent of LDL particle.

In addition, the observed genotype frequencies presented in table 4.2 are not different from those expected assuming Hardy-Weinberg equilibrium. Comparison of the apo B insertion/ deletion allele frequencies estimated in this study with those reported for other population samples (table 4.5) showed that there are marked differences between Caucasian and Asian populations. The differences are mainly due to an increase in Del allele and a decrease in Ins allele in Caucasian populations. These indicated that the populations in Asia and Europe are different in ethnic background and geographical isolation. For the population in America, this difference may be due to a combination of population admixture and genetic drift.

Mean lipid levels were significantly different in total cholesterol and LDL-C between various Ins/Del genotypes in hyperlipidaemic group but not in

Copyright by Mahidol University

44852

normolipidaemic group (table 4.7). This finding suggests that the influence of the polymorphism of apo B on distributions of plasma lipid is complex and related to the other influential or nongenetic factors. However, these associations are still not well understood but it seems to be very complex. In this study the correlation between Ins/ Del genotypes and lipid levels are not pertinent to the genotypes observed. This may be due to the small sample sizes or genetic risk factors is particularly strong in early age as has been shown in family and twin studies[15]. Nonetheless, it seems likely to be that in Thai population individuals with the deletion allele might tend to have higher lipid levels than those insertion allele.

Since as much as two-thirds of total body cholesterol in individuals is of endogenous origin[137]. Meta-analysis of randomized trials including cholesterol-lowering drugs, e.g. resins, fibrates, statins, nicotinic acid or diet has shown a decrease in overall incidence of CAD with reduction of hypercholesterolaemia. The lipid lowering drugs used in this study were resins, fibrates and statins for observation the efficacy of these drugs in reduction the lipid levels of subjects containing various apo B and apo E polymorphisms. An attractive way to lower levels of plasma cholesterol is to inhibit its biosynthesis, which consists of several enzymatic conversion steps (Figure5.1)[138]. 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting step in cholesterol synthesis, thus providing an attractive target for pharmacological intervention. A number of cholesterol-lowering drugs are currently available for human use[63, 140, 144]. In the last decade, a new class of agents was

Copyright by Mahidol University

developed that specifically inhibits HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis[139]. Four HMG-CoA reductase inhibitors are available for clinical use: lovastatin[140], pravastatin[141], simvastatin[142], and fluvastatin[143]. In addition to statins, administratin of sequestrant resins (cholestyramine or Questran) which bind bile acids strongly results in a decrease in plasma cholesterol levels was also use in this study. The effects of these manoeuvers on plasma lipid levels

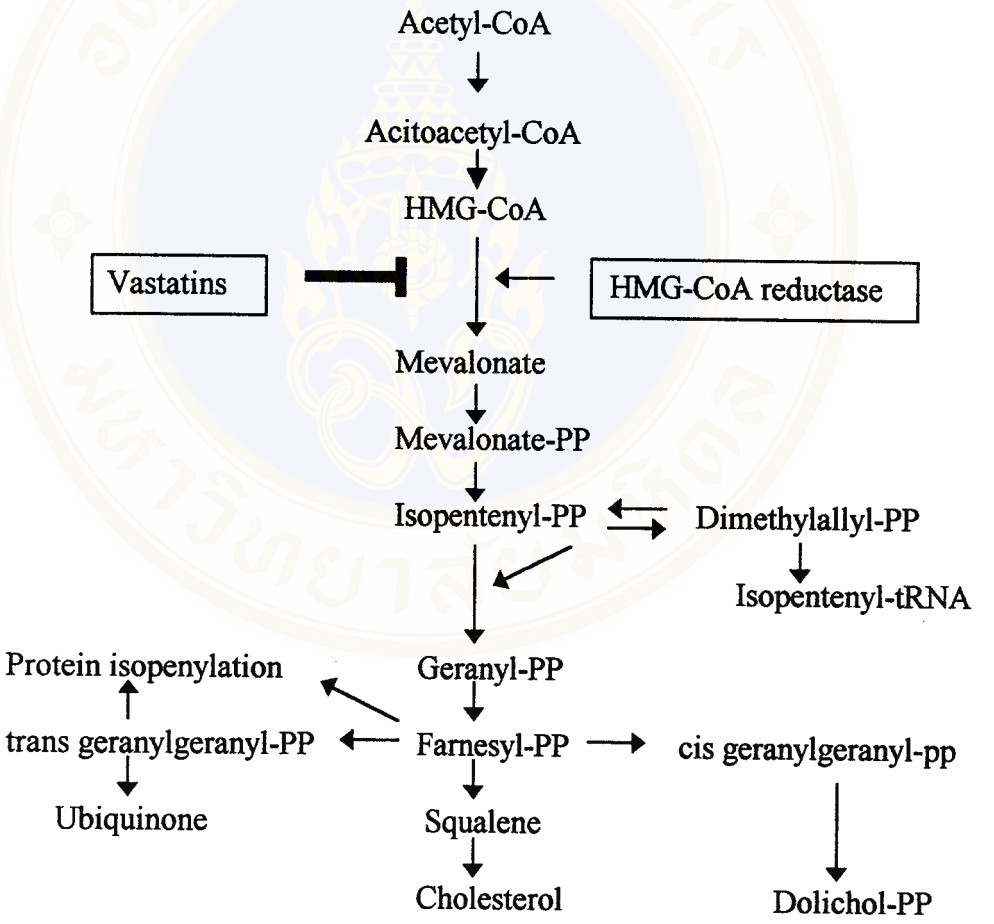


Figure5.1 Mevalonate pathway of cholesterol synthesis in mammalian cells[143].

are secondary to the liver's compensatory response of generating more bile acids to maintain efficient lipid absorption from the intestine. Loss of bile acids from the portal return to the liver leads to activation of cholesterol-7-alpha

Copyright by Mahidol University

7-alpha Acetyl-CoA hydroxylase to synthesize bile acids from cholesterol (Figure 5.2). Synthesis of bile acids goes up several folds and the result is a decrease in intracellular cholesterol levels[144]. Administration of fibrates (lopid or lipantil) results in a decrease of both plasma cholesterol and triglycerides. The mechanisms to explain the influence of fibrates on VLDL metabolism have been proposed. It is known that the supply of fatty acids to hepatocytes is a principal determinant of the rate of VLDL assembly and secretion. When cells are deprived of this lipid source, apo B, which

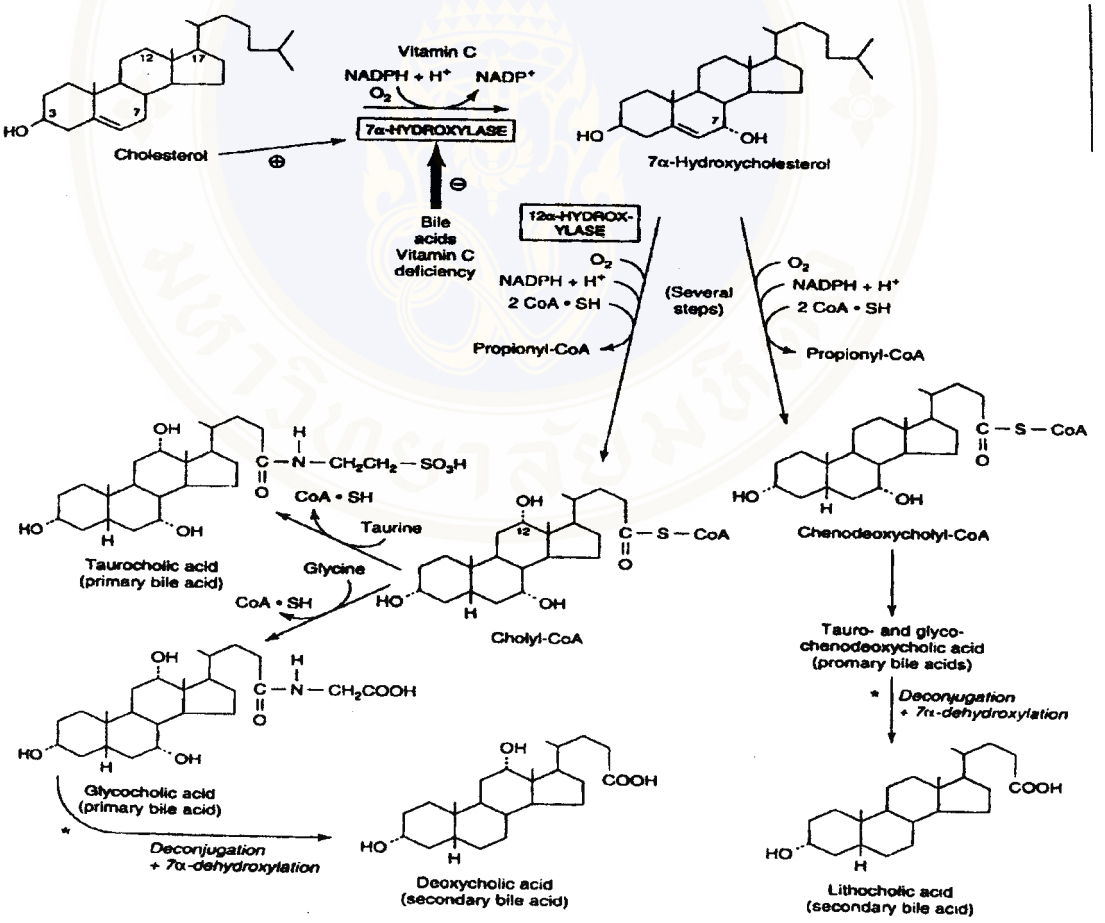


Figure 5.2 Biosynthesis and degradation of bile acids[8].

Copyright by Mahidol University

is made continuously, is degraded intracellularly; an abundant supply of fatty acids, on the other hand, stabilizes apo B and promotes the formation of large, triglyceride-rich particles. It has been shown that fibrates inhibit the formation of large VLDL and promotes the conversion of larger VLDL to smaller VLDL by activation the lipoprotein lipase activity. Fibrates also have a complex effect on the concentration, composition, and metabolism of LDL. It has become clear that LDL is composed of a number of distinct subfractions, namely:

LDL I - the least dense, most lipid rich

LDL II - the most abundant

LDL III - the smallest and densest subfraction.

A preponderance of LDL III in the LDL profile is associated with a three to seven fold increase in the risk of coronary heart disease. It has been shown that fibrates perturb the LDL subfraction pattern, shifting the distribution from smaller to larger particles probably as the result of the alteration in plasma triglycerides, since the concentration of this lipid has a profound influence on the LDL subfraction profile. Small, dense LDL bind poorly to receptors whereas the larger LDL species have a higher affinity; thus when fibrates shift the spectrum away from small, dense LDL the nature of the binding lipoprotein changes and, conceivably, it is this action that promotes clearance of the lipoprotien through the LDL receptor pathway[145]. The major effects of these lipid-lowering drugs used on the plasma lipoprotein profile have been shown in table 5.1

Copyright by Mahidol University

Table 5.1 Lipid-lowering drugs[122]

Class	Marketed agents	UK trade names	Indication	Range of response to therapy
Bile acid sequestrant resins	Cholestyramine colestipol	Questran (Questran-light) Colestid	Raised LDL	10-25% decrease in LDL
Statins	Fluvastatin	Lescol	Raised LDL	15-35% decrease in LDL
	Pravastatin	Lipostat, Mevalotin		
	Simvastatin	Zocor		
Fibrates	Bezafibrate	Bezalip Mono	Raised LDL	LDL change +40% to –35% TG decrease in 15-70%
	Clofibrate	Atromid-s	Raised TG	
	Ciprofibrate	Modalim		
	Fenofibrate	Lipantil Micro		
	Gemfibrozil	Lopid		
Nicotinic acid	Acipimox	Nicotinic acid	Raised LDL	LDL decrease 10% –20% TG decrease 15-70%
	Nicofuranose	(generic)	Raised TG	
		Olbetam, Bradilan		
Fish oil		Maxepa	Raised TG	TG decrease 10-50%
Probucol		Lurselle	Raised LDL	LDL decrease 0-20%

Copyright by Mahidol University

The recently reported Scandinavian Simvastatin Survival Study (4S) evaluate the effect of HMG-CoA reductase inhibitor on mortality and morbidity in patients with CAD. A total of 4,444 men and women from 35-70 years of age with angina or previous myocardial infarction and serum cholesterol of 212-230 mg/dl received simvastatin or placebo for 5.4 years. The results of this trial showed that a 25% reduction in plasma cholesterol concentrations results in 30% fewer deaths from all causes and 42% fewer coronary death[146].

In this study we found that homozygous Insertion and heterozygous Ins/Del can reduce total cholesterol, LDL-cholesterol and triglycerides after treatment with lipid-lowering drugs for 3-5 years. Homozygous deletion can not reduce lipid after treatment (table 4.8). This result signified that patients containing homozygous deletion allele were not responsive to the all lipid-lowering drugs treatment. This is the first report to show that homozygous Del allele has a potential to reduce plasma lipid levels less than the Ins allele. However, this study is consistent with several reports showing beneficial effects on coronary atherosclerosis within two years of effective lipid-lowering drug therapy. This result may suggest that the alteration of apo B three dimensional structure as a result of the deletion in signal peptide might alter apo B conformation and decrease the binding to its receptor or the inefficient translocation of deletion signal peptide might stimulate the excessive hepatic synthesis of apo B containing lipoproteins through the increase of VLDL or VLDL remnant leading to high level of LDL.

Copyright by Mahidol University

Apo B mutation

The interaction between LDL and the LDL receptor plays a major role in determining plasma cholesterol levels in humans[12]. Apo B-100 is the major protein component of LDL and is responsible both for maintaining the structural integrity of the particle and for the binding of these lipoproteins to the LDL receptor [77]. The relevance of this catabolic pathway is best illustrated by the genetic disorders familial hypercholesterolaemia (FH) and familial defective apo B-100 (FDB), in which high levels of LDL accumulate in the circulation because mutations in the LDL receptor (FH) or in the ligand (FDB) disrupt the binding of LDL to its receptor[79]. Many different mutations in the LDL receptor cause FH,[147] but FDB is associated with a single site mutation, the substitution of glutamine for the normally occurring arginine at residue 3,500 of apo B-100. With exception of an arginine to cysteine mutation at residue 3,531[74], the arginine for glutamine at residue 3,611[81] is associated with a minor decrease in LDL receptor binding.

The frequency of the Arg3500Gln mutation has been found to be approximately 1/500 to 1/700 in several Caucasian populations in North America and European[69]. The highest frequency was found in the Swiss population (approximately1/210)[75]. On the other hand, the frequency of this mutation was slightly lower in population of Denmark, (approximately 1/1250) and found no instance of this mutation in a large screening programme in Israel, Finland and Japan [70, 71].

Because of the binding of LDL to its receptor is important in the lipid transported pathway and the frequencies of this mutation in the ligand which disrupt

Copyright by Mahidol University

the binding of LDL to LDL receptors are different in many reports . In this study, we estimated the frequency of this ligand defective in Thai populations.

Arg 3500Gln , Arg3531Cys

The frequencies of Arg 3500 Gln and Arg 3531Cys mutations were not identified among all of the 171 subjects in Thai populations with 103 hyperlipidaemic and 68 normolipidaemic subjects. This indicates the lower frequency of these mutations in Thai population or the sample sizes used in this study are rather small to detect these mutations. From this study, we conclude that these mutations may be not a common cause of elevated plasma cholesterol in Thai population corresponding with the other Asian populations from Israel and Japan[70, 71]. This supports the hypothesis that these mutations may occur in the central Europe and are rare in Asia.

Arg 3611Gln

The Arg 3611 Gln mutation was found in 2 of the 103 hyperlipidaemic subjects but not identified in 68 normolipidaemic subjects (table 4.9). All 2 subjects were heterozygous for this mutation which was commonly found in other populations agreement with the report of Xu et al[80]. In addition, they also showed that no significant association was found between this RFLP and serum cholesterol and apo B level. They concluded that this mutation has no significant effect on apo B function. Nevertheless, the sample sizes of their population studied were rather small, i,e., 60 from London, 98 were Finnish and 101 were Italian, thus the correlation of lipid levels with this polymorphisms may not be found owing to the influence of other risk factors. In this study, the comparison of lipid levels before and after treatment of lipid-

Copyright© by Mahidol University

lowering drugs showed no significant differences (table 4.10). This implied that the three dimensional structure (conformation) of apo B may change and bind poorly to the LDL receptors leading to a slow clearance of lipid from the circulation after treated with lipid-lowering drugs. This finding was agreed with the other reports which demonstrated that the variant of apo B at position 3611 were associated with obesity, high blood cholesterol levels and increased risk of coronary artery disease[82, 84].

Apo E polymorphism

Apo E genotypes are transmitted in a manner consistent with an autosomal dominant mode of inheritance with two alleles. Apo E is a structural component of chylomicrons, very-low-density lipoproteine (VLDL), and high-density lipoprotein (HDL). It has a major regulatory role in the lipid metabolism of these lipoprotein particles via specific apo E receptors and receptors for low-density lipoprotein (LDL; containing apo B, E) in the liver and peripheral tissues.

The apo E gene locus on chromosome 19 is polymorphic, with three common protein isoforms, E2, E3, and E4, which are encoded by three different alleles, ε2, ε3 and ε4. The E2 and E4 variants differ from the more common E3 isoform by single amino acid substitution. Whereas apo E3 has cysteine at position 112 and arginine at position 158, apo E4 has arginine at position 112 and apo E2 has cysteine at both sites. These substitutions affect ligand binding of triglyceride rich lipoprotein to the chylomicron remnant and LDL receptors.

Copyright by Mahidol University

In this study the observed genotype frequencies in Thai population presented in table 4.11 were significantly deviated from those expected assuming Hardy-Weinberg equilibrium (χ^2 = 27.27, df =5, p<0.001). Nevertheless, the populations from Australia, Hungary, New Zealand, Sweden, Trinidad, and USA were also deviated from Hardy-Weinberg equilibrium (table 4.18). Without supporting evidence, we did not believe this deviation is due to selection. Initially, we suspected technical laboratory reasons. We have retyped all of these samples and corroborated their genotypes again. The observed genotypes also did not support this methodological hypothesis. Thus we have subdivided all of these subjects into hyperlipidaemic and normolipidaemic groups (tables 4.12 and 4.13). The genotype frequencies from hyperlipidaemic samples significantly deviated from the Hardy-Weinberg expectations (χ^2 = 34.9, df = 5, p<0.001). In nomolipidaemic samples the genotype frequencies were in accordance with Hardy-Weinberg expectation (χ^2 = 2.99, df = 5, p=0.70). It is for these reasons that we believed that in the sample of Thai population the deviation of the apo E genotype frequencies from Hardy-Weinberg expectations was due to chance. This was occurred from the high frequencies of E2/E2 genotypes. This finding supported the hypothesis that in subjects with E2/E2 genotype the environmental factors may be necessary for expression of the hyperlipidaemia after challenging the exacerbating factors. The reason for the higher prevelence of E2/E2 genotypes in hyperlipidaemic subjects is not clear, but it could be speculated that this allele may delay the onset of CHD at early age (under 40), and in turn possibly supporting the attainment of old age. Hence this genotype is mostly found in old age and may be associated with longevity[122].

Copyright by Mahidol University

Since apo E genotype frequencies calculated from Hardy-Weinberg distribution between hyperlipidaemia and normolipidaemia were significantly differences. The apo E allele frequencies between hyperlipidaemia and normolipidaemia were compared (table 4.17). The results showed that the apo E4 allele frequencies of hyperlipidaemia is significantly higher by an increase 86% than that of normolipidaemia corresponding to the high prevalence of hypercholesterolaemia in Thai population. This study confirmed that the prevalence of apo E4 in hyperlipidaemia lead to the higher levels of plasma cholesterol and LDL-C that recognizes as the significant risk factors in developing CAD. This finding was corresponded with present study of Canadian population by Nassar et al[23]. However, in both normolipidaemia and hyperlipidaemia, the apo E allele frequencies were not significantly differences from Thai population. The result from these three alleles, termed E2, E3, E4 in Thai population occurred with a frequency of about 8, 80, and 12 %, respectively, which agreed to the several population studied (E2 = 8%, E3 = 77%, and E4 = 15%)[109, 113,124-126].

Comparison of the apo E allele frequencies estimated in this study with those reported for other population samples (Table 4.18) showed that there are marked differences between the Thai population and that of two populations of Finland, Sweden, Greenland, Trinidad and Sudan. These differences are mainly due to differences in frequencies of the E2 allele (decreased in Greenland and increased in Trinidad) and E4 allele (increased in two populations of Finland ,Sudan and Sweden), whereas the frequencies of the E3 allele appear to be rather similar for all population considered.

Copyright by Mahidol University

The differences in apo E allele frequencies among the Thai, Finnish, Swedish, Trinidadian , Greenlander, and Sudanese populations may be due to the differences in ethnic background and geographical isolation. The similarities of these apo E allele frequencies were also observed between Thai population and other Asian populations. This may be due to the similarities in ethnic and social cultural background and geographical distribution. For the populations from Austria, Germany, Hungary, Iceland, Netherlands, and Scotland the similarities of apo E allele frequencies with Thai populations may be due to a genetic drift maintained by national-geographic or social-cultural isolation. The similarities of apo E allele frequencies from Australian, Canadian, New-Zealander, and American to the Thai population may be due to a combination of population admixture and genetic drift. It should, however, be noted that for the Australia, New Zealand, Sweden, Trinidad and USA population samples the observed apo E genotype distributions differ significantly from the expected Hardy-Weinberg distributions (table 4.18). These differences can be attributed to differences between the observed and expected number of genotypes exhibiting the E4 and E2 alleles (table 4.19). In particular, for the Finnish, Swedish, and Sudanese populations the apo E 4 allele frequency was significantly deviated from Thai population sample corresponding to the Thai hyperlipidaemic sample (table 4.17). From this study, it seems likely to be that apo E4 allele frequency has a chance to increase and apo E2 and E3 have a chance to decrease. For Trinidadian the apo E2 and E4 alleles frequencies have a trend to increase and apo E3 allele frequency has a trend to decrease. In contrast to the Greenlander the apo E2 has a trend to decrease whereas apo E4 has a trend to increase (table 4.19). Boerwinkle et al[105] also observed these

Copyright by Mahidol University

statistically significant differences in apo E allele frequencies among different ethnically and/or geographically distinct populations corresponding to this report.

Several population studies have shown that subjecst with the E2 allele usually have lower LDL-C or total cholesterol and apo B but higher triglyceride levels and those with E4 allele have higher LDL-C or total cholesterol and apo B levels compared with subjects with the E3. Reciprocally, the apo E4 allele is associated with a reduced plasma apo E level, whereas the apo E2 allele leads to a highly significant increase in plasma apo E concentrations [113, 116-117, 119]. The mechanisms underlying these associations are the result of (I) a more efficient catabolism of chylomicron and VLDL remnants by the liver in individuals with the apo E4 allele will lead to the enhanced uptake by the liver, thereby reducing the hepatic receptor activity and these elevating plasma LDL levels. (II) A less efficient catabolism of these lipoprotein particles in subjects exhibiting the apo E2 allele due to a defect in binding of apo E2 to hepatic lipoprotein (apo E2 has less than 2% binding activity) receptors leads to a diminished uptake of lipoprotein remnants by the liver. This will lead to an enhanced hepatic LDL receptor activity and eventually to lower plasma LDL concentration.

In this study we also confirmed the effect of allelic substitution at the apo E locus on total plasma cholesterol, LDL-C , VLDL-C, HDL-C and triglyceride levels in Thai population. Statistically significant differences was observed in mean levels of plasma cholesterol , LDL-C, Triglyceride and VLDL-C among several apo E phenotype groups. As compared with the most common E3 allele, the E4 allele leads to elevate plasma cholesterol and LDL-C levels, whereas the E2 allele is associated

Copyright by Mahidol University

with increased plasma triglyceride and decreased plasma cholesterol levels (Tables 4.20 and 4.21).

Statistically significant differences in apo E genotype distribution between hyperlipidaemic and normolipidaemic subjects were mentioned before in tables 4.12 and 4.13). These differences is mainly due to the differences in frequencies of the E4 allele. These differences in apo E4 allele frequencies may lead to the differences in the plasma lipid levels. This result is similar to the previous studies which showed the association of the apo E4 allele and elevated plasma cholesterol levels. In addition, the contribution of genetic variance in Thai population associated with the apo E locus to the total phenotypic variance of plasma lipid profile is different when compared with the results from Caucasian populations and similar when compared with the results from Asian populations [106, 110]. This difference can be ascribed almost exclusively to the pronounced total genotypic variance of these parameters in Thai population compared with Caucasian population samples. Consequently, we concluded that the total genetic variance of apo E locus associated with total lipid profile leading to coronary artery disease is low in Thai population compared with the Caucasian population. This result is consistent with the incidence of coronary heart disease which is the single most important cause of death and, more importantly, the single biggest cause of premature death in modern, industrialized countries[122].

The mean total plasma cholesterol, triglyceride, LDL-C, HDL-C and VLDL-C levels of hyperlipidaemic patients were evaluated before and after treatment with lipid-lowering drugs in the various apo E phenotype groups (table 4.22, 4.23). The statistical analyses showed that plasma cholesterol, triglyceride, LDL-C and VLDL-C

Copyright by Mahidol University

were significantly reduced after treatment in homozygous E3/E3 genotype, whereas other genotypes containing E4 allele were significantly reduced only cholesterol but triglycerides have a chance to decrease. The effect of lipid-lowering drugs on apo E4 - or E2- bearing individuals can be explained by the fact that the drug will either inhibit cholesterol synthesis or activate lipoprotein lipase depending on the kinds of drug received leading to the lower cholesterol levels in plasma. If the LDL-C maintained in plasma are only moderately elevated after treatment, the hepatic LDL-receptor activity was inrceased leading to a more efficient catabolism of chylomicrons and VLDL in individuals with apo E4 allele. By this way, the lipid-lowering drug may be useful to treat the patients with apo E4-bearing. In individuals with the apo E2 allele, the LDL-C levels are low due to an impaired VLDL and chylomicron remnant catabolism. These individuals are at lower risk as long as the levels of the atherogenic remnant particles remain below the level at which atherosclerotic risk increases. Once the atherosclerotic risks increase, the lipid-lowering drugs used may not be effective due to its defect in binding to LDL-receptors.

From this study we conclude that:

1. Individuals with variant alleles of apo B and apo E polymorphism have a trend to increase lipid profile in plasma faster than that with normal alleles in these polymorphisms.

2. The influence of environmental risk factors in individuals with variant alleles of apo B and apo E polymorphisms may enhance the plasma lipid levels faster than that with normal alleles.

Copyright by Mahidol University

3. The lipid-lowering drugs used to decrease plasma lipid levels may be less effective in individuals with variant alleles of apo B and apo E polymorphisms than that of normal alleles.

4. The best way to reduce plasma lipid levels in individuals with variant alleles is to avoid the environmental risk factors as long as possible because genetic risk factors shows a trend to elevate the plasma lipid levels after challenging with the exacerbating factors.

5. Genetic risk factors should be detected in the early age of life to predict the early onset of dyslipidaemia, especially, in one with a family history of dyslipidaemia.

Copyright by Mahidol University

REFERENCES

1. Scott MG. Cholesterol and Atherosclerosis Diagnosis and Treatment. J.B. Lippincott company, New York,1990.

2. Charles R. Scriver AL, Beaudet WS, Valle SD. The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill Inc., New York, 1995; 2:1871.

3.Kannel WB, Gordon T. Some characteristics related to the incidence of cardiovascular disease and death: Framingham Study, 16-year follow-up. Washington ,DC: US Gov. Print. off.,1970.

4. Sing CF, Zerba KE, Reilly SL. Tranversing the biological complexity in the hierarchy between genome and CAD endpoints in the population at large. Clin Genet 1994; 46:6-14.

5. Sing CF, Moll PP. Strategies for unraveling the genetic basis of coronary artery disease, In : Berg K, Retterstol N, Refsun S eds. From phenotype to gene in common disorders, Copenhagen: Munksgaard, 1990: 37-59.

6. Durrinton P N, Hyperlipidemia Diagnosis and Management, 2nd ed., Butterworth-Heinemann Ltd., Oxford, 1995.

7.Packard CJ, Chris J, Shepherd J. Lipoprotein heterogeneity and apolipoprotein B metabolism. Arterioscler Thromb and Vasc Biol 1997;17:3542-3556.

8. Montgomery R, Conway TW, Spector AA. Biochemistry a Case Oriented Approach; The C.V. Mosby Company, Baltimore, 1990.

9. Murray RK, Daryl KG, Mayes PA, Victor WR. Harper's Biochemistry; Prentice-Hall International Inc., Connecticut, 1996.

Copyright by Mahidol University

10. Noe DA, Rock RC. Laboratory Medicine: The Selection and Interpretation of Clinical Laboratory Studies. Williams&Wilkins, Baltimore 1994:p476-551.

11. Gaw A, Cowan RA, O'Reilly DJ, Stewart MJ, Shepherd J. Clinical Biochemistry, an Illustrated Colour Text, Churchill Livingstone, Edinburgh 1995: 120-123.

12. Parums DV. Essential Clinical Pathology. Blackwell Science, Massachusetts, 1996: 278-279

13. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986; 232: 34-47.

14. Nassar BA,Dunn J, Title LM, O'Neill BJ, Kirkland SA, Zayed E, Bata IR, Cantrill RC, Johnstone J, Dempsey GI, Tan M, Breckenridge WC, Johnstone DE. Relation of genetic polymorphisms of apoliipoprotein E, angiotensin conversting enzyme, apolipoprotein B-100, and glycoprotein IIIa and early-onset coronary heart disease. Clin Biochem 1999; 32: 275-282.

15. Kannel WB, Castelli WP, Gordon T. Serum cholesterol, lipoproteins and the risk of coronary heart disease : The Framingham study. Ann Intern Med 1971; 74: 1-12.

16. Meisenberg G, Simmons WH. Lipoprotein and Atherosclerosis. In : Principle of Medical Biochemistry, Missouri, Meshy, 1998: 407-18.

17. Kane JP, Hardman DA, Paulus HE. Heterogeneity of apolipoprotein B isolation of a new species from human chylomicrons. Proc Natl Acad Sci USA 1980;77: 2465-2469.

18. Knott TJ, Pease RJ, Powell LM, Wallis SC, Rall Jr SC, Innerarity TL, Blackhart, B, Taylor WH, MarcelY, Milne R, Johnson D, Fuller M, Lusis AJ, McCarthy BJ, Mahley RW, Levy-Wilson, B, Scott J. Complete protein sequence and identification of structural domains of human apolipoprotein B. Nature 1986; 323: 734-738.

Copyright by Mahidol University

19. Williams RJ, Knott T J, Wallis SC, Sweetnam P, Yarnell J, Cox N, Bell GI, Miller, N E, Scott J. Variation of apolipoprotein B gene is associated with obesity, high cholesterol levels, and increased risk of coronary heart disease. Lancet 1988:1442-1446.

20. Tybjaerg-Hansen A, Steffensen R, Meinertz H, Schnohr P, Nordestgaard BG, Association of mutations in the apolipoprotein B gene with hypercholesterolemia and the risk of ischemic heart desease. New Eng J Med 1998; 338: 1577-1584.

21. Young SG. Recent progress in understanding apolipoprotein B. Circulation 1990; 82: 1574-1590.

22. Cladaras C, Hadzopoulou-Cladaras M, Robert T. Atkinson ND, Zannis IV. The complete sequence and structural analysis of human apolipoprotein B-100: relationship between apo B-100 and apo B-48 forms. EMBO J 1986; 5: 3495-3507.

23. Visvikis S, Chan L, Sitest G, Drourin P, Boerwinkle E. An insertion deletion polymorphism in the signal peptide of the human apolipoprotein B gene. Hum Genet 1990; 84: 373-375

24. Weisgraber KH, Rall Jr SC. Human apolipoprotein B-100 heparin-binding sites. J Biochem 1987; 262: 11,097-11,103.

25. Olofsson SO, Bjursell G, Bostrom K, Carlsson P, Elovson J, Protter AA, Reuben MA, Bondhers G. Apolipoprotein B: Structure, biosynthesis and role in the lipoprotein assembly process. Atherosclerosis 1987; 68: 1-17.

Copyright by Mahidol University

26. De Loof H, Rosseneu M, Yang CY, Li WH, Gotto Jr AM, Chan L. Human apolipoprotein B : Analysis of internal repeats and homology with other lipoproteins. J Lipid Res 1987; 28: 1455-1465.

27. Chen GC, Hardman DA, Hamilton RL, Mendel CM, Schilling JW, Zhu S, Lau K, Wong JS, Kane JP. Distribution of lipid-binding regions in human apolipoprotein B-100. Biochemistry 1989; 28: 2477-2484.

28. Phillips ML, Schumaker VN. Conformation of apolipoprotein B after lipid extraction of low density lipoproteins attacked to an electron microscope grid. J Lipid Res 1989; 30: 415-422

29. Yang CY, Gu ZW, weng SA, Kim TW, Chen SH, Powenall HJ, Sharp PM, Liu SW, Li WH, Gotto AM Jr, et al. Structure of apolipoprotein B-100 of human low density lipoproteins. Arteriosclerosis 1989; 9: 96-108.

30. Milne R, Theolis Jr R, Maurice R, Pease RJ, Weech PK, Rassart E, Fruchart JC, Scott J, Marcel YL. The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. J Biol Chem 1989; 264: 19754-19760.

31. Deeb SS, Disteche C, Motulsky AG, Lebo RV, Kan YW. Chromosomal localization of the human apolipoprotein B gene and detection of homologous RNA in monkey intestine. Proc Natl Acad Sci USA 1986; 83: 419-422.

32. Rash JM, Rothblat GH, Sparks CE. Lipoprotein apolipoprotein synthesis by human hepatoma cells in culture. Bichim Biophis Acta 1981; 666: 294-298.

Copyright by Mahidol University

33. Glickman RM, Rogers M, Glickman JN: Apolipoprotein B synthesis by human liver and intestine *in vitro*. Proc Natl Acad Sci USA.1986; 83: 5269-5300.

34. Lusis AJ, West R, Mehrabian M, Reuben MA, LeBoeuf RC, Kaptein JS, Johnson DF, Schumaker VN, Yuhasz MP, Schotz MC, Elovson J. Cloning and expression of apolipoprotein B, the major protein of low and very low density lipoproteins. Proc Natl Acad Sci USA 1985; 82: 4597-4601.

35. Mehrabian M, Sparkes RS, Mohandas T, Klisak IJ, Schumaker VN, Heinzmann C, Zollman S, Ma Y, Lusis AJ. Human apolipoprotein B: chromosomal mapping and DNA polymorphisms of hepatic and intestinal species. Somat Cell Molec Genet 1986;12: 245-254.

36. Lodish H, Baltimore D, Berk A, Zipursky SL, Matsudaira P, Darnell J. Molecular Cell Biology. Third edition W.H Freeman and Company, New York 1995.

37.Chen SH, Habib G, Yang CY, Gu ZW, Lee BR, Weng SA, Silberman SR, Cai SJ, Deslypere JP, Rosseneu M. Apo B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon, Science 1987; 238: 363-366.

38. Powell LM, Wallis SC, Pease RJ, Edwards YH, Knott TJ, Scott J. A novel form of tissue-specific RNA processing produces apolipoprotein B-48 in intestine. Cell 1987; 50: 831-840.

39. Hospattankar AV, Hifuchi K, Law SW, Meglin N, Brewer Jr HB. Identification of a novel in-frame translational stop codon in human intestine apo B mRNA. Biochem Biophys Res Commun 1987;1 48: 279-285.

Copyright by Mahidol University

40. Hardman DA, Protter AA, Schilling JW, Kane JP. Carboxylterminal analysis of human B-48 protein confirms the novel mechanism proposed for chain termination. Biochem Biophys Res Commun1987; 149: 1214-1219.

41. Bostrom K, Boren J, Wettesten M, Sjoverg A, Bondjers G, Wiklund O, Carlsson P, Olofsson SO. Studies on the assembly of apo B-100 containing lipoproteins in HepG2 cells. J Biol Chem 1988; 263: 4434-4442.

42. Davies MS, Walles SC, Driscoll DM, Wynne JK, Williams GW, Powell LM, Scott J. Sequence requirements for apolipoprotein B RNA editing in transfected rat hepatoma cells. J Biol Chem 1989; 264: 13395-13398

43. Driscoll DM, Wynn JK, Wallis SC, Sott J, An *in vitro* system for the editing of apolipoprotein B mRNA. Cell 1989; 158: 519-525

44. Scott J, Wallis SC, Dacies MS, Wynne JK, Powell LM, Driscoll DM. RNA editing: A novel mechanism for regulating lipid transport from the intestine. Gut Festschrift 1989; 30: 35-43.

45.Chen SH, Li X, Liao WSL, Wu JH, Chan L. RNA editing of apolipoprotein B mRNA: Sequence specificity determined by *in vitro* coupled transcription editing. J Biol Chem 1990; 265; 6811-6816.

46 McCarthy BJ. Polymorphisms and markers associated with apolipoprotein B Curr Opin Lipidol 1991; 2: 81-85.

47. Boerwinkle E, Chen S-H, Visvikis S. Signal peptide-length variation in human apolipoprotein B gene : Molecular characteristics and association with

plasma glucose levels. Diabetes 1991; 40: 1539-1544.

Copyright© by Mahidol University

48. Chun-fang Xu, Matti J. Tikkanen, Jussi K, Huttunen P, Butler R, Humphries SE, Talmud PJ. Apolipoprotein B signal peptide insertion/deletion polymorphism is associated with Ag epitopes and involed in the determination of serum triglyceride levels. J Lipid Res 1990; 31: 1255-1261.

49. Hansen PS, Gerdes LU, Klausen IC. Polymorphisms in the apolipoprotein B-100 gene contributes to normal variation in plasma lipids in 464 Danish men born in 1948. Human Genet 1993; 91: 45-50.

50. Saha N, Tay JSH, Chew LS. Influence of apolipoprotein B signal peptide insertion/deletion polymorphism on serum lipids and apolipoproteins in a Chinese population. Clin Genet 1992a; 41: 152-156.

51. Saha N, Tong MC, Tay JSH, Jeyaseelan K, Humphrews SE. DNA polymorphisms of the apolipoprotein B gene in Chinese coronary artery disease patients. Clin Genet 1992b; 42:164-170.

52. Saha N, Tay JSH, Heng CK. DNA polymorphisms of the apolipoprotien B gene are associated with obesity and serum lipids in healthy Indians in Singapore. Clin Gent 1993; 44: 113-120.

53. Humphries SE, Talmud PJ. Hyperlipidaemia associated with genetic variation in the apolipoprotein B gene. Curr Opin Lipidol 1995; 6: 215-222.

54. Bohn M, Bakken A, Erikssen J, Berg K. The apolipoprotein B signal peptide insertion/deletion polymorphism is not associated with myocardial infarction in Norway. Clin Genet 1994; 45: 255-259.

Copyright by Mahidol University

55. Visvikis S., Cambou JP, Arveiler D. Apolipoprotein B signal peptide polymorphism in patients with myocardial infarction and controls: The ECTIM study. Human Genet 1993; 90: 561-565.

56. Wu JH, Wen MS, Lo SK, Chen MS. Increased frequency of apolipoprotein B signal peptide sp24/ 27 in patients with coronary artery disease. General allele survey in the population of Taiwan and comparison with Caucasians. Clin Genet 1994; 45: 250-254.

57. Monsaly Mv, Youg r, Jobsis J, Wiseman SA, Dharmu S, Powell JT, Greenhalgh Rm, Humphries SE. DNA polymorphism of the gene for apolipoprotein B in patients with peripheral and coronary artery disease. Athersclerosis 1988; 70: 123-129.

58. Renges HH, Wile DB, Mcgeig PM, Marmot MG, Humphries SE. Apo B gene polymorphisms are associated with lipid levels in men of South Asian descent. Atherosclerosis.1991; 91: 267-275.

59. Anderson JL, Bunker CH, Aston CE, Kamboh IM. Relationship of two apolipoprotein B polymorphism with serum lipoprotein and lipid levels in African Blacks. Hum Biol 1997; 69: 793-808.

60. Vega GL, Grundy SM. *In vivo* evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. J Clin Invest 1986; 78: 1410-1414.

Copyright by Mahidol University

61. Innerarity TL, Weisgraber KH, Arnold KS, Mahley RW, Krauss RM, Vega GL, Grundy SM. Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding. Proc Natl Acad Sci USA1987; 84: 6919-6923.

62. Weisgraber, KH, Innerarity TL, Newhouse YM, Young SG Arnold KS, Krauss RM, Vega GL, Grundy SM, Mahley RW. Familial defective apolipoprotien B-100 enhanced binding of monoclonal antibody MB47 to abnormal low density lipoproteins. Proc Natl Acad Sci USA 1988; 85: 9758-9762.

63. Illingworth DR, Vakar F, Mahley RW, Weisgraber KH. Hypochloesterolaemic effects of lovastatin in familial defective apolipoprotein B-100. Lancet 1992; 339; 598-600.

64. Soria LF, Ludwig EH, Clarke HRG, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. Proc Natl Acad Sci USA 1989: 86; 587-591.

65. Ludwig EH, McCarthy BJ. Haplotype analysis of the human apolipoprotein B mutation associated with familial defective apoliprotein B-100. Am J Hum Genet 1990; 47; 712-720.

66. Motti C, Funke H, Rust S, Dergunov A, Assmann G. Using mutagenic polymerase chain reaction primers to detect carriers of familial defective apolipoprotein B-100. Clin Chem 1991; 37: 1762-1766.

Copyright by Mahidol University

67. Loux, N, Saint-Jore B, Collod G, Benlian P, Cambou JP, Senat M, Junien C, Boileau C. Identification of the haplotype associated with the apo B 3500 mutation in a French hypercholesterolemic subject: further support for a unique European ancestral mutation. Hum Mutat 1993; 2: 145-147.

68. Bryony G, Henderson, Philip R, Wenham J, Peter Ashby, Blundell G. Detecting familial defective apolipoprotein B-100: three molecular scanning methods compared. Clin Chem. 1997; 43: 1630-1634.

69. Rauh, G, Keller C, Schuster H, Wolfram G, Zollner N. Familial defective apolipoprotein B-100: a common cause of primary hypercholesterolemia. Clin Invest 1992; 70: 77-84.

70. Friedlander Y, Dann EJ, Leitersdorf E. Absence of familial defective apolipoprotein B-100 in Israeli patients with dominantly inherited hypercholesterolemia and in offspring with parental history of myocardial infarction. Hum Genet 1993; 91: 299-300.

71. Hamalainen T, Palotie A, Aalto-Setala K, Kontula K, Rikkanen MJ. Absence of familial defective apolipoprotein B-100 in Finnish patients with elevated serum cholesterol. Atherosclerosis 1990; 82: 177-183.

72. Tybjaerg-Hansen A. Humphries SE. Familial defective apolipoprotein B-100: a single mutation that causes hypercholesterolemia and premature coronary artery disease. Atherosclerosis 1992; 96: 91-107.

Copyright by Mahidol University

73. Lund-Katz S, Innerarity TL, Arnold KS, Curtiss LK, Phillips MC. ^{13}C NMR evidence that substitution of glutamine for arginine 3500 in familial defective apolipoprotein B-100 disrupts the conformation of the receptor-binding domain. J Biol Chem 1991;266:2701-2704.

74. Pullinger CR, Hennessy LK, Chatterton JE, Liu W, Love JA, Mendel CM, Frost PH, Mallloy MJ, Schumaker VN, Kane JP. Familial ligand-defective apolipoprotein B: identification of a new mutation that decreases LDL receptor binding affinity. Clin Invest 1995; 95: 1225-1234.

75. Miserez AR, Laoger R, Chiodetti N, Keller U. High prevalence of familial defective apolipoprotein B-100 in Switzerland. J Lipid Res 1994; 35: 574-583.

76. Tybjaerg-Hansen A, Gallagher J, Vinvent J, Houlston R, Talmud P, Dunning AM, Seed M, Hamsten A, Humphries SE, Myant NB. Familial defective apolipoprotein B-100: detection on the United Kingdom and Scandinavia and clinical characteristics of ten cases. Atherosclerosis 1990; 80: 235-242.

77. Miserez AR, Keller U. Differences in phenotypic characteristics of subjects with familial defective apolipoprotein B-100 and familial hypercholesterolemia. Arrterioscler Thromb Vasc Biol 1995; 15: 1719-1729.

78. Schaefer JR, Scharnagl H, Baumstark MW, Schweer H, Zech LA, Seyberth, H, Winkler K, Steinmetz A, Marz W. Homozygous familial defective apolipoprotein B-100: enhanced removal of apolipoprotein E-containing VLDLs and decreased production of LDLs. Arterioscler Thromb Vasc Biol 1997; 117: 348-353,.

Copyright by Mahidol University

79. Innerarity TL, Mahley RW, Weisgraber KH, Bersot Tp, Krauss RM, Vega GL, Grundy SM, Friedl W, Davignon J, McCarthy BJ. Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia. J Lipid Res 1990; 31: 1337-1350.

80. Xu C, Nanjee N, Matti J, Tikkanen JK, Pietinen HP, Butler R. Apolipoprotein B amino acid 3611 substitution from arginine to glutamine creates the Ag 9h/I) epitope: the polymorphism is not associated with differences in serum cholesterol and apolipoprotein B levels. Hum Genet 1989; 82: 322-326.

81. Huang LH, Graaf J, Breslow JL. Apo B gene MspI RFLP in exon 26 changes amino acid 3611 from Arg to Glu. J Lipid Res 1988; 239: 63-67.

82. Demant T, Houlston RS, Caslake MJ, Series JJ, Shepherd J, Packard CJ, Humphries SE. Catabolic rate of low density lipoprotein is influenced by variation in the apolipoprotein B gene. J Clin Invest 1988; 82: 797-802.

83. Rajput-Williams J, Knott TJ, Wallis SC, Sweetnam P, Yarnell J, Cox N, Bell GI, Miller NE, Scott J. Variation of apolipoprotein-B gene is associated with obesity, high blood cholesterol levels, and increased risk of coronary heart disease. Lancet 1988: 1442-1446.

84. Gavish D, Brinton EA, Breslow JL. Heritable allele-specific differences in amounts of apoB and low-density lipoproteins in plasma. Science 1989; 244: 72-76.

85. Chappell DA, Medh JD Receptor-mediated mechanisms of lipoprotein remnant catabolism. Prog Lipid Res 1998 ; 37: 393-422

Copyright by Mahidol University

86. Wenham PR, Newton CR, Price WH, Analysis of apolipoprotein E genotypes by the amplification refractory mutation system. Clin Chem 1991; 37: 241-244

87. Moore JH, Reilly SL, Ferrell RE, Sing CF. The role of apolipoprotein E polymorphism in the prediction of coronary artery disease age of onset. Clin Genet 1997; 51: 22-25.

88. Wilson PWF, Schaefer EJ, Larson MG, Ordovas JM. Apolipoprotein E Alleles and risk of coronary disease a meta-analysis. Arterioscler Thrombo Vasc Biol 1996; 16: 1250-1255.

89. Stengard JH, Rekkanen J, Ehnholm C, Nissinen C, Sing CF. Genotypes with the apolipoprotein E4 allele are predictors of coronary heart disease mortality in a longitudinal study of elderly Finnish men. Hum Genet 1996; 97: 677-684

90. Rall SC, Weisgraber Jr KH, Mahley RW Apolipoprotein E the complete amino acid sequence. J Biol Chem1982; 257: 4171-4178.

91. Brown M.S, Kovanen PT, Goldstein JL. Regulation of plasma cholesterol by lipoprotein receptors. Science 1981; 212: 628-625.

92. Brown MS, Goldstein JL. Lipoprotein receptors in the liver: control signal for plasma cholesterol traffic. Clin Invest 1983; 72: 743-747.

93. Mahley RW. Apolipoprotein E cholesterol transport protein with expanding role in cell biology. Science 1988; 240: 622-30.

94. Wilson C, Wardell MR, Weisgraber KH, Mahley RW, Agard DA. Three dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. Science 1991; 252: 1817-1822.

Copyright by Mahidol University

95. Innerarity TL, Weisgraber KH, Arnold KS, Rall Jr SC, Mahley RW. Normalization of receptor binding of apolipoprotein E2. Evidence for modulation of the binding site conformation. J Biol Chem 1984; 259: 7261-7267.

96. Wilson C, Mau T, Weigraber KH, Mahley RW, Agard DA. Salt bridge relay triggers defective LDL receptor binding by mutant apolipoprotein. Structure 1994; 2: 713-718.

97. Steinmitz A, Jakobs C, Motzny S, Kaffarnik H. Differential distribution of apolipoprotein E isoforms in human plasma lipoproteins. Arteriosclerosis 1989; 9: 405-411.

98. Weisgraber KH. Apolipoprotein E distribution among human plasma lipoproteins: Role of the cysteine-arginine interchange at residue 112. J Lipid Res 1990; 31: 1503-1511.

99. Menzel HJ, Kladetzky RG, Assmann G. Apolipoprotein E polymorphism and coronary atery disease. Atheriosclerosis 1983; 3: 310-315.

100. Cattin L. Fisicaro M, Valenti M, Danek GM, Fonda M, DaCol PG. Polymorphism of the apolipoprotein E gene and early carotid atherosclerosis defined by ultrasonography in asymptomatic adults. Arterioscler Thromb Vasc Biol. 1997; 17: 91-94.

101. Utermann G, Langenbeck U, Beisiegel U, Weber W: Genetics of the apolipoprotein E system in man. Am J Hum Genet 1980; 32: 339.

102. Zannis VI, Breslow JL. Human very low-density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification. Biochemistry 1981; 20: 1033-1040.

Copyright by Mahidol University

103. Zannis VI, Just PW, Breslow JL. Human apolipoprotein E isoprotein subclasses are genetically determined. Am J Hum Genet 1981; 33: 11-24.

104. Mahley RW. Atherogenic lipoproteins and coronary artery disease. Concepts derived from recent advances in cellular and molecular biology. Circulation 1985; 72: 943-948.

105. Boerwinkle E, Visvikis S, Welsh D, Steinmetz J, Hanash SM, Sing CF. The use of measured genotype information in the analysis of quantitative phenotypes in man. II The role of the apolipoprotein E polymorphism in determining levels, variability and covariability of cholesterol, beta-lipoprotein, and triglycerides in a sample of unrelated individuals. Am J Med Genet 1987; 27: 567-582.

106. Eto M, Watanabe K, Ishii K. A racial difference in apolipopretein E allele frequencies between the Japanese and Caucasian populations. Clin Genet 1986; 30: 422-427.

107. Gerdes LU, Gerdes C, Hansen PS, Klausen C, Faergemen D, Dyerberg J. The apolipoprotein E polymorphism in Greenland Inuit in its global perspective. Hum Genet 1996; 98: 546-550.

108. Ehbholm C, Lukka M, Kuusi T, Nikkila E, Utermann G. Apolipoprotein E polymorphism in the Finnish population gene frequencies and relation to lipoprotein concentrations. J Lipid Res 1986 ; 27: 227-235.

109. Utermann G. Apolipoprotein E polymorphism in health and disease. Am J Heart 1987; 113: 433-440.

Copyright by Mahidol University

110. Hallman DM, Boerwinkle E, Saha N, Sandholzer C, Menzel HJ, Utermann G. The apolipoprotein E polymorphism a comparison of allele frequencies and effects in nine populations. Am J Hum Genet 1991; 49: 338-591.

111. Sepehrnia B, Kamboh MI, Adams-Campbell LL, Bunker CH, Nwankwo M, Jajumder PP, Ferrell RE. Genetic studies of human apolipoproteins. X: The effect of the apolipoprotein E polymorphism on quantitative levels of lipoproteins in Nigerian blacks. Am J Genet 1989; 45: 586-591.

112. Ordovas JM, Litwack-Klein L, Wilson PWF, Schaefer MM, Schaeffer EJ. Apolipoprotein E isoform phenotyping methodology and population frequency with identification of apo E1 and apo E5 isofroms. J Lipid Res 1987; 28 : 371-380.

113. Hicson JE. Pathobiological determinants of atherosclerosis in youth (PDAY) research group. Apolipoprotein E polymorphisms affect atherosclerosis in young males. Arterioscler Thromb Vasc Biol 1991; 11: 1237.

114. Lucotte G, Loirat F, Hazout S. Pattern of gradient of apolipoprotein E allele *4 frequencies in Western Europe. Hum Biol 1997; 69: 253-262.

115. Kamboh MI, Bhatia KK, Ferrell RE. Genetic studies of human apolipoproteins: XII Population genetics of apolipoproteins in Papua New Guinea. Am J Hum Biol 1990; 2: 17.

116. Sing CF, Davignon J. Role of the apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. Am J Hum Genet 1985; 37: 268-285.

Copyright by Mahidol University

117. Davignon J, Gregg RE, Sing CF. Apolipoprotein E polymorphism and atherosclerosis. Arteriosclerosis 1988; 8: 1-21.

118. Gregg RE, Zech LA, Schaefer EJ, Stark D, Wilson D, Brewer Jr HB. Abnormal *in vivo* metabolism of apolipoprotein E4 in human. J Clin Invest 1986; 78: 815-821.

119. Dallongeville J, Lussier-Cacan S, Davignon J. Modulation of plasma triglyceride levels by apo E phenotype: A meta analysis. J Lipid Res 1992; 33: 447-454.

120. Kontula K, Aalto-setala K, Kuusi T, Hamalainen L, Syvanen C. Apolipoprotein E polymorphism determined by restriction enzyme analysis of DNA amplified by polymerase chain reaction: convenient alternative to phenotyping by isoelectric fucusing. Clin Chem 1990; 36: 2087-2092.

121. Pascaie R, Ginette T, de Zulueta MP, De Fennes JL, Michei T, Cassaigne A, Bereziat G, Iron A. Common and rare genotype of human apolipoprotein E determined by specific restriction profiles of polymerase chain reaction-amplified DNA. Clin Chem 1994; 40: 24-29.

122. Grace M. Lindsay, Allan G. Coronary Heart Disease Prevention a Handbook for the Health Care Team. Churchill Livingstone, New york.1997.

123. Yamamura T, Yamamoto A, Sumiyoshi T, Hiramori K, Nishioeda Y, Numbu S. New mutants of apolipoprotein E associated with atherosclerotic disease but not to type III hyperlipoproteinmia. J Clin Invest 1984; 74: 1229-1237.

124. Wang XL, McCredie RM, Wilcken DEL. Polymorphisms of the apolipoprotein E gene and severity of coronary artery disease defined by angiography. Arterioscler Thomb Vasc Biol 1995; 15: 1030-1034.

Copyright by Mahidol University

125. Davignon JCF, Sing S, Lussier C, Bouthillier S. Xanthelasma, Latent dyslipoproteinaemia and atherosclerosis contribution of apo E polymorphism. In Latent Dyslipoproteinemia and Atherosclerosis. Dee Gennes JL, Polonowsky J, Paoletti R. New York, Raven Press,1984: 1213-223.

126. Valveny N, Esteban E, Kandil M, Moral P. Apo E polymorphism in Spanish and Moroccan populations. Clin Genet 1997; 51: 354-356.

127. Smitt M, Knijff P, Rosseneu M, Bury J, Klasen E, Frants R, Havekes L. Apolipoprotein E polymorphism in the Netherlands and its effect on plasma lipid and apolipoprotein levels. Hum Genet 1988; 80: 287-292.

128. Wardell MR, Suckling PA, Janus ED. Genetic variation in human apolipoprotein E. J Lipid Res 1982; 23: 1174-1182.

129. Cumming AM, Rebertson FW. Polymorphism at the apo E locus in relation to risk of coronay disease. Clin Genet 1984; 25: 310-313.

130. Hegele RA, Ban MR, Busch CP, Pamsewak S, Ramdath DD. Lipoprotein-genotype associations in Trinidadian neonates. Clin Biochem 1999; 32: 429-437.

131. Neaton JD, Wentworth DF. Serum cholesterol, blood pressure, cigarette smoking, and death from coronary heart disease. Overall findings and differences by age for 316,099 white men. Arch Intern Med 1992; 152: 56-63.

Copyright by Mahidol University

132. Levine GN, Keaney JF, Vita JA. Cholesterol reduction in cardiovascular disease. Clinical benefits and possible mechanisms. New Engl J Med 1995; 332: 512-522.

133. Jukema JW, Bruschke AVG, Boven AJ. Efffects of lipid lowering by pravastatin on progression of coronary artery disease in symptomatic men with normal to moderately elevated serum cholesterol levels: The regression growth evaluation statin study (REGRESS). Circulation 1995; 91:2528-2540.

134. Buchwald H, Campos CT, Boen JR, Nguten PA, Williams SE. Disease-free intervals after partial ideal bypass in patients with coronary heart disease and hypercholesterolemia. J Amer Coll Cardiol 1995; 26: 351-357.

135. National Cholesterol Education Program. Second report of the expert panel on detection, evaluation and treatment of high blood cholesterol in adults. Circulation 1994; 89: 1333-1448.

136. Pyorala K, De Backer G, Graham I, Poole-Wilson P, Wood D. Prevention of coronary heart disease in clinical practice: Recommendations of the task force of the European Society for Cardiology, European Society of Hypertension. Atherosclerosis 1994; 110: 121-161.

137. Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholestrol and low density lipoprotein homeostasis in different animal species, including humans. J Lipid Res 1993; 34: 1637-1659.

138. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990; 343: 425-430.

Copyright by Mahidol University

139. Endo A. The discovery and development of HMG-CoA reductase inhibitors. J Lipid Res 1992; 33: 1569-1582.

140. Alberts AW, Chen JS, Kuron G. Mevinolin: A highly potent competitive inhibitor of HMG-CoA reductase and a cholesterol-lowering agent. Proc Natl Acad Sci USA 1980; 77: 3957-3961.

141. Tsujita Y, Kuroda M, Shimada Y. CS-514, A competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase: tissue selective inhibition of sterol synthesis and hypolipidemic effect on various animal species. Biochim Biophys Acta 1986; 877: 50-60.

142. Stokker GE, Hoffman WF, Alberts AW. 3-Hydrozy-3-methylgluraryl coenzyme A reductase inhibitors. 1. Structural modifications of 5-substituted 3,5-dihydroxypentanoic acids and their lactone derivatives. J Med Chem 1985; 28: 347-358.

143. Kathawala FG. HMG-CoA reductase inhibitors: An exciting development in the treatment of hyperlipoproteinemia. Med Res Rev 1991; 11: 121-146.

144. Danielson H, Sjoval J. Bile acid metabolism. Ann Rev Biochem 1975; 44: 233-253.

145. Griffin BA, Freeman DJ, Tait GW, Thomson J Caslake MJ, Packard CJ, Shepherd J. Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. Atherosclerosis 1994 ;106: 241-253.

Copyright by Mahidol University

146. Scandinavian Simvastatin Survival Study Group. Randomised trial of cholesterol lowering in 4444 patients with coronary heart fisease. New Engl J Med 1987; 17: 1237-1245.

147. Hobbs, H.H., M.S Brown, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum Mutat 1992; 1: 445-466

Copyright by Mahidol University

APPENDIX 1

DNA sequence of Apo E

3601 TCTGCCTCTG CCCTCTGCAT CTGCTCTCTG CATCTGTCTC TGTCTCCTTC TCTCGGCCTC

3661 TGCCCCGTTC CTTCTCTCCC TCTTGGGTCT CTCTGGCTCA TCCCCATCTC GCCCGCCCCA

3721 TCCCAGCCCT TCTCCCCCGC CTCCCCACTG TGCGACACCC TCCCGCCCTC TCGGCCGCAG

3781 GGCGCTGATG GACGAGACCA TGAAGGAGTT GAAGGCCTAC AAATCGGAAC TGGAGGAACA

3841 ACTGACCCCG GTGGCGGAGG AGACGCGGGC ACGGCTGTCC AAGGAGCTGC AGGCGGCGCA

3901 GGCCCGGCTG GGCGCGGACA TGGAGGACGT GCGCGGCCGC CTGGTGCAGT ACCGCGGCGA

3961 GGTGCAGGCC ATGCTCGGCC AGAGCACCGA GGAGCTGCGG GTGCGCCTCG CCTCCCACCT

4021 GCGCAAGCTG CGTAAGCGGC TCCTCCGCGA TGCCGATGAC CTGCAGAAGC GCCTGGCAGT

4081 GTACCAGGCC GGGGCCCGCG AGGGCGCCGA GCGCGGCCTC AGCGCCATCC GCGAGCGCCT

4141 GGGGCCCCTG GTGGAACAGG GCCGCGTGCG GGCCGCCACT GTGGGCTCCC TGGCCGGCCA

4201 GCCGCTACAG GAGCGGGCCC AGGCCTGGGG CGAGCGGCTG CGCGCGCGGA TGGAGGAGAT

4261 GGGCAGCCGG ACCCGCGACC GCCTGGACGA GGTGAAGGAG CAGGTGGCGG AGGTGCGCGC

4321 CAAGCTGGAG GAGCAGGCCC AGCAGATACG CCTGCAGGCC GAGGCCTTCC AGGCCCGCCT

4381 CAAGAGCTGG TTCGAGCCCC TGGTGGAAGA CATGCAGCGC CAGTGGGCCG GGCTGGTGGA

Copyright by Mahidol University

APPENDIX 2

DNA sequence of apo B

3481 GAA TAT TCA GGA ACT ATT GCT AGT GAG GCC AAC ACT 10676

3493 TAC TTG AAT TCC AAG AGC ACA CGG TCT TCA GTG AAG 10712

3505 CTG CAG GGC ACT TCC AAA ATT GAT GAT ATC TGG AAC 10748

3517 CTT GAA GTA AAA GAA AAT TTT GCT GGA GAA GCC ACA 10784

3529 CTC CAA CGC ATA TAT TCC CTC TGG GAG CAC AGT ACG 10820

3541 AAA AAC CAC TTA CAG CTA GAG GGC CTC TTT TTC ACC 10846

3553 AAC GGA GAA CAT ACA AGC AAA GCC ACC CTG GAA CTC 10882

3565 TCT CCA TGG CAA ATG TCA GCT CTT GTT CAG GTC CAT 10918

3577 GCA AGT CAG CCC AGT TCC TTC CAT GAT TTC CCT GAC 10954

3589 CTT GGC CAG GAA GTG GCC CTG AAT GCT AAC ACT AAG 10990

3601 AAC CAC AAG ATC AGA TGG AAA AAT GAA GTC CGG ATT 11026

3613 CAT TCT GGG TCT TTC CAG AGC CAG GTC GAG CTT TCC 11062

3625 AAT GAC CAA AAG GCA CAC CTT GAC ATT GCA GGA TCC 11098

3637 TTA GAA GGA CAC CTA AGG TTC CTC AAA AAT ATC ATC 11134

3649 CTA CCA GTC TAT GAC AAG AGC TTA TGG GAT TTC 11167

Copyright by Mahidol University

Hyperlipidaemic subjects

Name	Age (year)	Apo B Polymor- phism	Apo B mutation	Apo E genotype	Lipid levels before treatment (mg/dl)					Lipid levels after treatment (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C	Cho	LDL-C	T G	HDL-C	VLDL-C
1. นางวรรณพร บรรจงเกียรติ	51	ID	-	E2/E2	259	169	116	67	23	238	168	103	49	21
2. นางพรพรรณ นาคามดี	52	ID	-	E2/E2	247	171	258	24	52	197	125	97	53	19
3. นางพลอยพรรณ การุณยชร	64	ID	-	E2/E2	249	80	684	32	137	205	150	118	31	24
4. นางจรรยา อัศวสุนทรกุล	49	II	-	E2/E2	275	179	243	47	49	261	182	113	56	23
5. นส.สมพร แซ่เตี๋ย	40	II	-	E2/E2	368	247	363	48	73	173	88	193	46	39
6. นางสมบูรณ์ ศรียะพันธ์	53	DD	-	E2/E3	251	160	95	72	19	243	160	118	60	24
7. นางพูลศรี สุนทรพงศ์	60	ID	-	E2/E3	275	214	115	38	23	219	141	111	56	22
8. นางอุดม เจริญไชยศรี	61	ID	-	E2/E3	255	184	136	44	27	239	161	66	65	13
9. นางชมภูนุช ขวัญจิตร	50	II	-	E2/E3	233	153	228	34	46	268	177	161	59	32
10. นายพะเนียง มีนาภา	62	II	-	E2/E4	272	200	157	41	31	223	118	103	84	21
11. นายจตุรงค์ รัตนโชติ	54	II	-	E2/E4	279	209	232	24	46	167	78	325	24	65
12. นางอัจฉรา ศัพท์หงษ์	41	DD	-	E3/E3	286	220	117	43	23	274	188	82	44	16
13. นางภาวิณี ไพเราะเสถียร	39	DD	-	E3/E3	228	141	169	53	34	232	157	157	44	31
14. นายสมพงษ์ ลิ่ววิริยะ	64	DD	-	E3/E3	262	139	346	54	69	239	169	137	43	27
15. นางนกเล็กบุญมาดี	59	ID	-	E3/E3	273	205	54	57	11	200	126	66	61	13
16. นางอรพิน นัดกระโทก	48	ID	-	E3/E3	292	223	73	54	15	288	222	70	52	14
17. นางปราณี วิสุทธิ	45	ID	-	E3/E3	247	180	87	50	17	189	120	80	53	16
18. นายรัชพร อุตตรานนท์	35	ID	-	E3/E3	307	232	90	57	18	208	150	127	33	25

Hyperlipidaemic subjects

Name	Age (year)	Apo B Polymorphism	Apo B mutaion	Apo E genotype	Lipid levels before treatment (mg/dl)					Lipid levels after treatment (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C	Cho	LDL-C	T G	HDL-C	VLDL-C
19. นางละเมียด สุนากร	56	ID	-	E3/E3	303	237	100	46	20	287	203	157	53	31
20. นายไพฑูรย์ ธนะวัฒฒโก	48	ID	-	E3/E3	360	306	109	32	22	320	260	58	49	12
21. นางเสริมสุข แสวงกิจ	51	ID	3611	E3/E3	263	196	122	43	24	288	217	119	47	24
22. นางจำเนียร ไชยปาล	49	ID	-	E3/E3	274	214	126	35	25	275	193	178	46	36
23. นส.วิไล พนาพฤกาชาติ	53	ID	-	E3/E3	248	176	134	45	27	222	162	87	43	17
24. นางเชี่ยมเง็ก แซ่เล้า	59	ID	-	E3/E3	266	171	134	68	27	278	202	134	49	27
25. นายโสรัจ เปี่ยมวิไล	45	ID	-	E3/E3	326	257	147	40	29	386	325	163	28	33
26. นางวราภา มีศรี	41	ID	-	E3/E3	312	224	150	58	30	261	156	210	63	42
27. นางปราณี เทพยสุวรรณ	64	ID	-	E3/E3	295	218	151	47	30	222	157	179	29	36
28. นส. สุพิน แซ่เฮง	47	ID	-	E3/E3	288	200	153	57	31	238	160	99	58	20
29. นางเงียดหงอ เจริญพานิช	53	ID	-	E3/E3	330	260	156	39	31	167	78	325	24	65
30. นางศรีสง่า มงคล	61	ID	-	E3/E3	357	282	161	43	32	263	193	156	39	31
31. นางอำไพ คงรุ่งเรือง	50	ID	-	E3/E3	265	168	167	44	33	237	158	178	43	36
32. นายเสริมศักดิ์ คงสุวรรณ	65	ID	-	E3/E3	304	227	167	44	33	294	229	122	41	24
33. นายประพันธ์ เทียนขวัญ	40	ID	-	E3/E3	294	224	198	30	40	247	172	91	57	18
34. นายบุญส่ง กังวาฬ	48	ID	-	E3/E3	313	211	212	60	42	240	1153	104	66	21
35. นางสุภาภรณ์ ศิลปสุนทร	53	ID	-	E3/E3	286	193	223	48	45	314	233	79	65	16
36. นส.สุชะรี ถูกจิตร	52	ID	-	E3/E3	372	267	323	40	65	229	139	93	71	19

Hyperlipidaemic subjects

Name	Age (year)	Apo B Polymor- phism	Apo B mutation	Apo E genotype	Plasma lipid levels before treatment (mg/dl)					Plasma lipid levels after treatment (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C	Cho	LDL-C	T G	HDL-C	VLDL-C
37. นส.นวลจันทร์ มาลัยศรี	33	ID	-	E3/E3	200	65	325	76	65	208	116	185	55	37
38. นางบัวทอง แสงจันทร์	58	II		E3/E3	281	203	75	63	15	242	172	123	45	25
39. นายสมาน อรุณโอษฐ์	47	II	-	E3/E3	272	197	83	58	17	242	150	55	81	11
40. นายสุดใจ ประดับศิลป์	58	II	-	E3/E3	247	176	87	54	17	248	185	104	42	21
41. นายกอบเกียรติ กรินทสุทธ์	63	II	-	E3/E3	261	169	92	74	18	264	191	77	58	15
42. นางละม่อม อุกฤษณ์	60	II	-	E3/E3	246	173	98	53	20	251	166	79	69	16
43. นางสำรวย ประดับศิลป์	57	II	-	E3/E3	278	195	99	63	20	240	147	69	79	14
44. นางสุภาณี หัสดิน	48	II	-	E3/E3	295	217	101	58	20	319	245	138	46	28
45.นส.เกื้อกูล เวชพานิช	42	II	-	E3/E3	247	165	105	61	21	289	215	121	50	24
46. นายจารึก ภูเผือกรัตน์	64	II	-	E3/E3	292	221	106	50	21	261	180	137	54	27
47. นส.ปองสุข แม่นธนู	65	II	-	E3/E3	311	224	107	66	21	232	157	89	57	18
48. นางปราณี พินิจไชย	57	II	-	E3/E3	369	304	114	42	23	206	153	61	41	12
49. นส.พิกุล จารุพงษ์	39	II	-	E3/E3	270	190	116	57	23	237	167	72	56	14
50. นางยุพา ศรีสกุล	58	II	-	E3/E3	403	295	124	83	25	275	152	245	74	49
51. นส.จำเริญ แม่นธนู	60	II	-	E3/E3	272	175	130	71	26	272	207	105	44	21
52. นางจุลลัดดา ถาวรสุข	40	II	-	E3/E3	274	179	135	68	27	320	249	127	46	25
53. นายกอ ทรงกำพล	60	II	-	E3/E3	266	200	140	38	28	214	181	90	42	18
54. นางมะลิ กลิ่นเฟื่องฟู	51	II	-	E3/E3	343	256	146	58	29	229	133	221	52	44

Hyperlipidaemic subjects

Name	Age (year)	Apo B Polymorphism	Apo B mutation	Apo E genotype	Plasma lipid levels before treatment (mg/dl)					Plasma lipid levels after treatment (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C	Cho	LDL-C	T G	HDL-C	VLDL-C
55. นางวาสนา อองละออ	45	II	-	E3/E3	244	170	157	43	31	189	133	73	41	15
56. นายธรรมนูญ เสือแย้ม	59	II	-	E3/E3	267	181	159	54	32	216	130	128	60	26
57. นส.ชโลม ตรีครุชพันธ์	60	II	-	E3/E3	278	205	160	41	32	194	130	69	50	14
58 นายสุวิทย์ กลิ่นเอม	42	II	-	E3/E3	269	172	161	65	32	244	153	174	56	35
59. นางศุภวรรณ สุกใส	45	II	-	E3/E3	236	166	162	38	32	246	169	218	51	44
60. นายพงษ์ประพาฬ ขันธ์ปะภา	45	II	-	E3/E3	258	184	162	42	32	258	184	162	42	32
61. นา ยธวัช อมรศักดากุล	60	II	-	E3/E3	262	190	167	39	33	264	166	285	41	57
62.นางรัตนา ช้างสัมฤทธิ์	44	II	-	E3/E3	249	201	173	41	35	275	192	86	66	17
63. นส.พูลศรี คุลีเมฆิน	47	II	-	E3/E3	283	197	181	50	36	243	155	160	56	32
64. นางลำภา กนิษฐสุด	56	II	-	E3/E3	225	146	199	39	40	225	146	199	39	40
65. นายเทียนทอง สะวรรณอินทร์	59	II	-	E3/E3	274	190	210	42	42	264	141	363	50	73
66. นางเฉลิมศรี พรหมทองนุ้ย	55	II	-	E3/E3	314	226	233	41	47	220	155	106	44	21
67. นายชาญยุทธ อินอรุณ	36	II	-	E3/E3	299	191	241	60	48	200	145	83	38	17
68.นายธนาคม พุทธงชัย	51	II	-	E3/E3	269	174	263	42	53	268	202	102	46	20
69. นางวันเพ็ญ ไวยครุธทา	57	II	-	E3/E3	291	209	265	29	53	224	164	71	46	14
70. นางบุญกอบ ชมโท	60	II	-	E3/E3	224	115	315	46	63	185	104	51	71	10

Hyperlipidaemic subjects

Name	Age (year)	Apo B Polymor-phism	Apo B mutation	Apo E genotype	Plasma lipid levels before treatment (mg/dl)					Plasma lipid levels after treatment (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C	Cho	LDL-C	T G	HDL-C	VLDL-C
71. นายเดช คงรุ่งเรือง	57	II	-	E3/E3	240	143	328	31	66	206	117	244	40	49
72.นางผิน แซ่เฮง	63	II	-	E3/E3	248	141	346	38	69	224	164	100	40	20
73. นายบรรณากร เทพยสุวรรณ	65	II	-	E3/E3	295	170	388	47	78	213	143	153	39	31
74. นางสมจิตร แซ่บ่าง	36	DD	-	E3/E4	247	178	59	57	12	288	201	182	50	36
75. นางพรพินันท์ พิมลอักษร	58	DD	-	E3/E4	254	176	151	48	30	266	202	173	29	35
76. นางอารยา อุไรรัตน์	50	ID	-	E3/E4	283	203	92	62	18	252	175	101	57	20
77. นางพวงวัลย์ เนตรหิน	52	ID	-	E3/E4	246	163	95	64	19	279	189	101	70	20
78. นส. จิราพร ยุติศาสตร์	64	ID	-	E3/E4	344	267	128	51	26	273	166	198	67	40
79. นายอดินันท์ แสงหิรัญ	63	ID	-	E3/E4	312	252	152	30	30	316	263	72	39	14
80. นางบัวไข หงษ์ศรี	46	ID	-	E3/E4	277	206	160	39	32	251	132	222	75	44
81. นาง วิไล บุญเสริม	53	ID	-	E3/E4	332	235	217	54	43	258	204	88	36	18
82. นายกฤชชัย เจนพานิชยการ	44	ID	-	E3/E4	281	201	257	29	51	208	140	80	52	16
83. นายไพบูลย์ ไพเราะเสถียร	46	ID	3611	E3/E4	247	158	321	25	64	212	132	202	40	40
84. นายอนุพงศ์ เจริญพานิช	41	ID	-	E3/E4	228	118	374	35	75	227	107	450	30	90
85. นส ปภาพิทย์ ยุวสุด	33	II	-	E3/E4	284	196	64	75	13	322	250	108	50	22
86. นายโกศล ธาราพัฒน์	57	II	-	E3/E4	479	374	93	86	19	243	137	72	92	14

Hyperlipidaemic subjects

Name	Age (year)	Apo B Polymorphism	Apo B mutation	Apo E genotype	Plasma lipid levels before treatment (mg/dl)					Plasma lipid levels after treatment (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C	Cho	LDL-C	T G	HDL-C	VLDL-C
87. นางลัดดา ศิริวรรณ	57	II	-	E3/E4	272	177	99	75	20	261	157	135	77	27
88. นายชะอื่น อินบุญนะ	58	II	-	E3/E4	275	187	104	67	21	275	170	134	78	27
89. นางมาริษา อัศวไพศาล	59	II	-	E3/E4	246	170	105	55	21	249	170	127	54	25
90. นางทัศนีย์ บุญเพิ่ม	76	II	-	E3/E4	290	223	123	42	25	234	166	67	55	13
91. นางทองหยิบ บุญมาดี	65	II	-	E3/E4	265	188	129	51	26	258	184	114	51	23
92. นายจุมพจน์ จันทร์โอ	35	II	-	E3/E4	278	202	142	48	28	265	189	187	39	337
93. นายเดือก เงียนวัน	60	II	-	E3/E4	278	194	149	54	30	276	210	124	41	25
94. นายบรรจง ศรีสุทธาภรณ์	54	II	-	E3/E4	243	170	154	42	31	268	203	87	47	17
95. นางพนิดา โกยเจริญ	51	II	-	E3/E4	286	211	169	41	34	262	191	104	50	21
96. นางสุมาลย์ ธรรมประเสริฐ	50	II	-	E3/E4	266	189	171	43	34	269	200	104	48	21
97. นางวารุณี โลหะปินะพรรรณ	60	II	-	E3/E4	251	162	245	40	49	241	167	237	27	47
98. นส.ชลัยพร จิตตะทัต	35	II	-	E3/E4	195	83	406	31	81	205	123	255	31	51
99. นายสง่า เนตรหิน	66	ID	-	E4/E4	248	181	182	31	36	237	178	111	37	22
100. นายเรืองวิทย์ เจนพานิชการ	47	II	-	E4/E4	270	196	70	60	14	211	116	239	47	48
101. นางธัญญา อาชีรพรรณ	54	II	-	E4/E4	288	216	171	38	34	283	199	73	69	15
102. นายศุภรัตน์ ประยูรหงษ์	51	ID	-	E3E3	203	111	258	40	52					
103.นายก่อเกียรติ วิวัฒน์เจริญกิจ	34	DD	-	E3E3	216	148	59	56	12					

Normolipidemic subjects

Name	Age (year)	Apo B Polymor- phism	Apo B mutation	Apo E genotype	Plasma lipid levels (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C
1. นายพงษ์ศักดิ์ ลำพอง	24	II	-	E2E3	154	93	87	44	17
2. นายศักกา มูลมา	24	II	-	E4E3	152	80	124	47	25
3. นายสันติ สท้าน	24	II	-	E3E3	198	107	132	65	26
4. นายไพฑูรย์ บัวลอย	24	II	-	E3E3	156	89	119	43	24
5. นายสุทัต คงทน	22	II	-	E3E3	164	91	139	45	28
6. นายไพร สุขพลัม	22	II	-	E4E3	165	103	102	42	20
7. นายทองโฮม บุญแต่ง	22	II	-	E3E3	170	100	100	50	20
8. นายวิชิต สุขตาม	27	II	-	E3E3	155	78	110	55	22
9. นายธวัชชัย ศาลางาม	22	II	-	E4E3	161	97	94	45	19
10. นายอนันต์ จันทร์ดี	23	II	-	E3E3	162	104	108	36	22
11. นายสมพงษ์ ทองมาก	24	II	-	E3E3	135	78	92	39	18
12. นายนพรัตน์ วิญญาณ	24	II	-	E3E3	193	117	77	61	15
13. นายสมพงษ์ เหลี่ยมมงคลกุล	36	II	-	E3E3	178	109	68	55	14
14. นส. ปาริชาติ เทอญชูชีพ	31	II	-	E3E3	193	123	83	53	17
15. นส. อรอนงค์ วรรัตน์	29	II	-	E3E3	181	116	36	58	7.2
16.นางอรนุช วิวัฒเจริญกิจ	34	II	-	E3E3	177	105	58	60	12
17. นส. สาวินีย์	21	II	-	E3E3	210	131	96	60	19

Normolipidaemic subjects

Name	Age (year)	Apo B Polymor- phism	Apo B mutation	Apo E genotype	Plasma lipid levels (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C
18. นส.กาญจนา	21	II	-	E3E3	138	75	83	46	17
19. นางพัชรี ผลดี	30	II	-	E3E3	190	105	189	47	38
20. นางแสงเดือน เย็นอ่อน	40	II	-	E3E3	200	141	50	49	10
21. นส. จุฬาลักณ์	23	II	-	E3E3	181	109	115	49	23
22. นส.ลินดา วรวีกุลวัฒนา	26	II	-	E3E3	183	111	88	54	18
23. นายสนอง แพใหญ่	32	II	-	E3E3	199	138	82	45	16
24. น.ส. เฮียง อังอ	60	II	-	E3E3	171	97	55	63	11
25.นางรัตนา บุญสอง	34	II	-	E3E3	196	117	68	65	14
26. นางบุปผา เขี้ย	43	II	-	E2E3	136	63	90	55	18
27. นางปริศนา ฐิติวร	50	II	-	E3E3	179	104	68	61	14
28. นางรัตนทิพย์	43	II	-	E3E3	166	86	56	69	11
29. นางทองเจือ เกียร	48	II	-	E3E3	149	85	44	55	8.8
30. นางนภาภรณ์ อ่อน	56	II	-	E2E3	171	88	90	65	18
31. น.ส. ดวงเดือน มณี	46	II	-	E2E3	173	86	46	78	9.2
32. นางชุติมา ส่งสุวรรณ	40	II	-	E3E3	174	101	62	61	12
33. นายวัฒนา พุฒใจบุญ	24	ID	-	E3E3	194	124	128	44	26
34. นายสุนทร โคตรสุ	46	ID	-	E4E3	190	120	76	55	15

Normolipidaemic subjects

Name	Age (year)	Apo B Polymor- phism	Apo b mutation	Apo E genotype	Pasma lipid levels (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C
35. นายสายฝน ผลดี	29	ID	-	E3E3	173	105	98	48	20
36. นายอธิป ลิขิตลิลิต	44	ID	-	E3/E4	200	134	109	44	22
37.นส. นพมาศ	28	ID	-	E2E3	187	106	149	51	30
38. นส.วัลยา สุทธิขำ	26	ID	-	E3E3	178	100	90	60	18
39. นางพัชรินทร์ สุร	38	ID	-	E3E3	184	111	79	57	16
40. นางประภา วงษ์	44	ID	-	E3E3	139	82	94	38	19
41. นายสุชาติ โชค	50	ID	-	E4E3	184	98	155	55	31
42. นางเสงี่ยม เจริญพัน	44	ID	-	E3E2	159	85	74	59	15
43. นางสุวรรณี สิ่ง	47	ID	-	E3E3	190	131	86	42	17
44. นาง วิไล หิรัญ	35	ID	-	E3E3	183	111	48	62	9.6
45. นางวันทนา สร้อย	36	ID	-	E3E3	147	87	40	52	8
46. นางมณฑา นิล	42	ID	-	E3E3	107	32	52	65	10
47. นางพยอม อยู่	46	ID	-	E3E3	169	102	58	55	12
48. นายกฤษณะ อรัญ	23	DD	-	E4E4	198	136	117	39	23
49. นางทองอยู่ ยิ้มจั่น	58	II	-	E3/E3	182	117	146	36	29
50. นส.ธนพร	31	II	-	E2/E3	200	116	48	74	9.6
51. นายภูธร ภู่กลั่น	24	ID	-	E3/E3	197	118	176	44	35

Normolipidaemic subjects

Name	Age (year)	Apo B Polymor- phism	Apo B mutation	Apo E genotype	Plasma lipid levels (mg/dl)				
					Cho	LDL-C	T G	HDL-C	VLDL-C
52. นางกาญจนา รุจิรภูมิ	39	II	-	E3/E3	200	116	142	56	28
53. นางบุญมา นิจกุล	65	II	-	E3/E3	200	137	106	42	21
54. นายประยูร อ่อนศิริ	51	II	-	E3/E3	200	121	209	37	42
55. นางฮุงกวง แซ่ตั้ง	61	II	-	E3/E3	200	128	68	58	14
56นางอุไร ภูมิพานิชพงษ์	63	ID	-	E3/E3	200	91	93	90	19
57. นางจุฑามาศ คูหาเรืองรอง	46	ID	-	E3/E3	200	108	65	79	13
58. นางปราณี มณีฉาย	51	II	-	E3/E3	139	76	120	39	24
59. นางวิชุดา สีสุข	47	II	-	E3/E3	188	125	147	34	29
60. นายไพบูรณ์ เรืองญาติ	53	II	-	E2/E2	191	120	179	35	36
61. นางสมหมาย แก้ววรรณ	48	ID	-	E3/E3	159	99	101	40	20
62. นายสวัสดิ์ สิทธิสนธ์	51	II	-	E3/E3	153	98	109	33	22
63. นายสนอง ภู่นาค	50	II	-	E3/E2	172	116	134	29	27
64. นางมยุรี พุ่มรุกขา	53	II	-	E3/E2	168	111	134	30	27
65. นางชลีพันธ์ ชิดชานาค	45	ID	-	E4/E3	153	83	200	30	40
66. นส.วรรณา ลิขิตลิลิต	48	II	-	E3/E3	195	142	85	36	17
67. นางกาญจนา รัตนสุวราหะ	47	ID	-	E3/E3	160	114	83	29	17
68. นางมารินทร์ รวยทัพย์	47	II	-	E3/E3	177	106	133	44	27

BIOGRAPHY

LIBRARY AND INFORMATION CENTER
MAHIDOL UNIVERSITY

NAME SAIPHON POLDEE

DATE OF BIRTH 7 August 1971

PLACE OF BIRTH Srisaket, Thailand

INSTITUTIONS ATTENDED

Rajabhat Institute Bansomdejchaopraya, 1993-1996:

Bachelor of Science (Health Education)

Mahidol University, 1997-2000:

Master of Science (Biochemistry)

POSITION & OFFICE

1993-Present, Department of Biochemistry,

Faculty of Medicine Siriraj Hospital,

Mahidol University Bangkok,Thailand.

Position: Medical Science Technician

Copyright by Mahidol University

44852