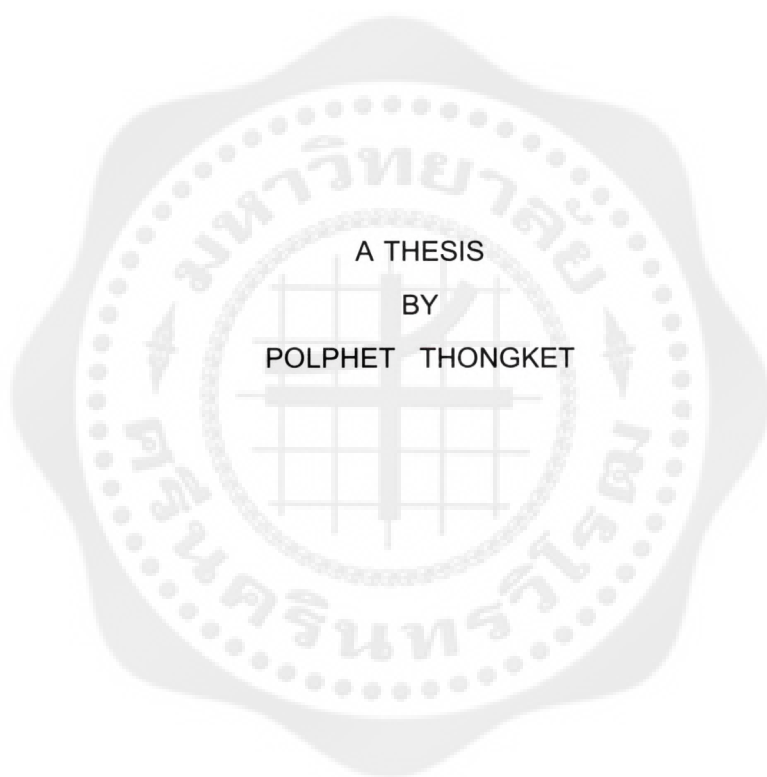


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ASSOCIATION STUDY ON SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) OF APOE
RECEPTOR 2 GENE IN DYSLIPIDEMIA AMONG THAI POPULATION



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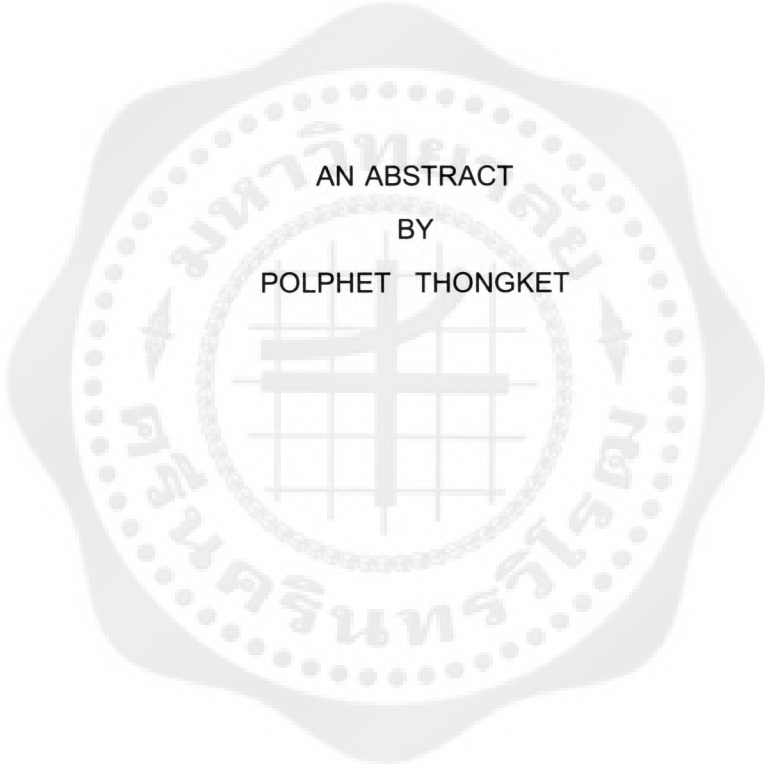
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Master of Science degree in Molecular Biology
at Srinakharinwirot University

May 2013

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ASSOCIATION STUDY ON SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) OF *APOE*
RECEPTOR 2 GENE IN DYSLIPIDEMIA AMONG THAI POPULATION



AN ABSTRACT
BY
POLPHET THONGKET

Presented in Partial Fulfillment of the Requirements for the
Master of Science degree in Molecular Biology
at Srinakharinwirot University
May 2013

Polphet Thongket. (2013). *Association study on single nucleotide polymorphisms (SNPs) of ApoE receptor 2 gene in dyslipidemia among Thai population*. Master thesis, M.Sc. (Molecular Biology). Bangkok: Graduate School, Srinakharinwirot University. Advisor Committee: Assoc. Prof. Dr. Wasana Sukhumsirichart, Dr. Sirinun Nilwarangkoon.

Dyslipidemia is an abnormal amount of lipid in the blood which may cause by environment (food consumption) and genetic factors. It is a major risk factor of coronary heart disease and metabolic disease. A genome-wide association study (GWAS) of blood lipoprotein and lipid revealed that the SNPs at apolipoprotein E gene (*ApoE*) associated with the high level of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglyceride. In the present study, the SNPs in exon of the *ApoE* receptor 2 gene (*ApoER2*) were analyzed for association with dyslipidemia in a Thai population. A total of 200 dyslipidemia patients and 200 unrelated normal controls with mean age of 47.01 ± 0.5 and 47.91 ± 0.5 years, respectively, were selected to include in the study using lipid level such as triglyceride, total cholesterol, LDL-cholesterol, and HDL-cholesterol as criteria. The DNAs were extracted from blood using Flexigene DNA kit. Genotyping was performed using polymerase chain reaction (PCR) and high-resolution melting (HRM) analysis. The difference in genotype distribution between patient and control was assessed by Chi-square test. The result revealed that the SNPs in the *ApoER2* gene, rs3737983, rs3737984 and rs2297660 showed significant association with risk of dyslipidemia in the Thai population (P -value = 0.011, 0.001 and 0.001, respectively). Further study is required for clarification the role of genetic variation in expression pattern of the *ApoE* receptor 2 gene which may be involved in dyslipidemia.

การศึกษาความสัมพันธ์ระหว่างสปีส์ของยีนตัวรับ *ApoE* ชนิดที่2 กับภาวะไขมันในเลือดผิดปกติใน
ประชากรไทย



บทคัดย่อ
ของ
พลเพชร ทองเกตุ

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาวิชาอนุชีววิทยา
พฤษภาคม 2556

พลเพชร ทองเกตุ. (2556). การศึกษาความสัมพันธ์ระหว่างสลิปส์ของยีนตัวรับ ApoE ชนิดที่2 กับ ภาวะไขมันในเลือดผิดปกติในประชากรไทย. ปรินูญานพนธ์ วท.ม. (อณูชีววิทยา). กรุงเทพฯ: บัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ. คณะกรรมการควบคุม: รองศาสตราจารย์ ดร.วาสนา สุขุมศิริชาติ, อ.ดร. สิรินันท์ นิลวรารุง.

โรคไขมันในเลือดผิดปกติคือภาวะที่มีระดับไขมันในเลือดสูงหรือต่ำกว่าปกติซึ่งมีสาเหตุมาจากปัจจัยด้านสิ่งแวดล้อม (พฤติกรรมการบริโภคอาหาร) และปัจจัยทางพันธุกรรม โรคนี้ยังเป็นปัจจัยหลักของความเสี่ยงต่อการเป็นโรคหลอดเลือดหัวใจและโรคที่เกิดจากความผิดปกติของเมตาบอลิซึมอีกด้วย จากรายงานการศึกษาจีโนม (Genome Wide Association Study; GWAS) ของไลโปโปรตีนในเลือดและไขมันในประชากรแมกซิกโก พบว่าการเกิด สลิปส์ หรือการเปลี่ยนแปลงของเบสเพียงเบสเดียวในยีนอะโปไลโปโปรตีนอี (ApoE) มีส่วนเกี่ยวข้องกับระดับที่เพิ่มขึ้นของ low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) และไตรกลีเซอไรด์ ในการศึกษาครั้งนี้ได้วิเคราะห์หาความสัมพันธ์ระหว่างการเกิดโรคไขมันในเลือดผิดปกติกับสลิปส์ในส่วนของ exon ของยีนตัวรับอะโปไลโปโปรตีนอี 2 (ApoER2) ในประชากรไทย ซึ่งยังไม่มีรายงานวิจัย ในการศึกษาได้คัดเลือกคนไข้โรคไขมันในเลือดผิดปกติ จำนวน 200 คน และคนปกติจำนวน 200 คน อายุเฉลี่ยอยู่ที่ 47.01 ± 0.5 และ 47.91 ± 0.5 ปี ตามลำดับ โดยแบ่งกลุ่มตามระดับของไตรกลีเซอไรด์ คอเลสเตอรอลทั้งหมด, LDL-C และ HDL-C ที่ได้ ทำการสกัดดีเอ็นเอจากการหาสลิปส์ใช้วิธีปฏิกิริยาลูกโซ่โพลีเมอร์ ร่วมกับ High-resolution melting (HRM) analysis ความแตกต่างของจีโนไทป์ระหว่างคนไข้กับคนปกติ วิเคราะห์โดยใช้ Chi-square test ผลการศึกษาพบว่าสลิปส์ในยีนตัวรับอะโปไลโปโปรตีนอี 2 หมายเลข rs3737983, rs3737984 และ rs2297660 มีความสัมพันธ์กับความเสี่ยงในการเป็นโรคไขมันในเลือดผิดปกติในประชากรไทยอย่างมีนัยสำคัญทางสถิติ (P -value = 0.011, 0.001 และ 0.001 ตามลำดับ) การศึกษาถึงบทบาทของตัวรับอะโปไลโปโปรตีนอี 2 หลังมีการเปลี่ยนแปลงทางพันธุกรรมในรูปแบบของการแสดงออกของยีน น่าจะมีการศึกษาต่อไปเพื่อดูว่ามีผลต่อการเกิดภาวะไขมันในเลือดผิดปกติหรือไม่

The dissertation titled
"Association study on single nucleotide polymorphisms (SNPs) of *ApoE* receptor 2 gene in
dyslipidemia among Thai population"

by
Polphet Thongket

has been approved by the Graduate School as partial fulfillment of the requirements for the
Master of Science in Molecular Biology of Srinakharinwirot University.



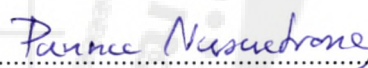
..... Dean of Graduate School
(Assoc. Prof. Dr. Somchai Santiwatanakul)

May 14, 2013

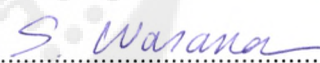
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Oral Defense Committee

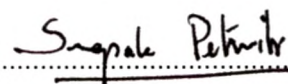

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude and deep appreciation to Associate Professor Dr. Wasana Sukhumsirichart, my advisor for her guidance, invaluable advice, helpfulness, understanding and encouragement throughout this study. I am very grateful to Dr. Sirinun Nilwarangkoon, my co-advisor for, guidance, comment and suggestion on thesis improvement. My special thanks to my committees, Assistant Professor Dr. Punnee Nusuetrong and Professor Dr. Songsak Petmitr for their constructive criticisms on and corrections of my thesis. Without them, this thesis would never have been completed successfully.

I am deeply grateful to Mrs. Arda Pakpichareon and Mr. Paisarn Khawsak, scientists of the Department of Biochemistry, Faculty of medicine, Srinakharinwirot University, and also special thank for Miss. Warin Deesukon, Miss. Benjaporn Panichareon and Miss. Siriporn Chatsuriyawong for their helpfulness, cheerfulness, and friendship and passionate guidance. Many thanks to my friends, the postgraduate students in Molecular Biology Program, Faculty of Medicine, Srinakharinwirot University for their cheerfulness, helpfulness and friendship.

I am deeply grateful to Graduates School, Srinakharinwirot University. I would like to thank the Biochemistry department, Srinakharinwirot University, for supporting the instruments. Special thanks for all my friends in this laboratory for their kind helpful assistance, friendship and always kindness to me.

Finally, I am deeply grateful to my dear family for their infinite love, support, valuable insights and giving me the courage to make it. Without them, this work could not have been completed. Thank You.

Polphet Thongket

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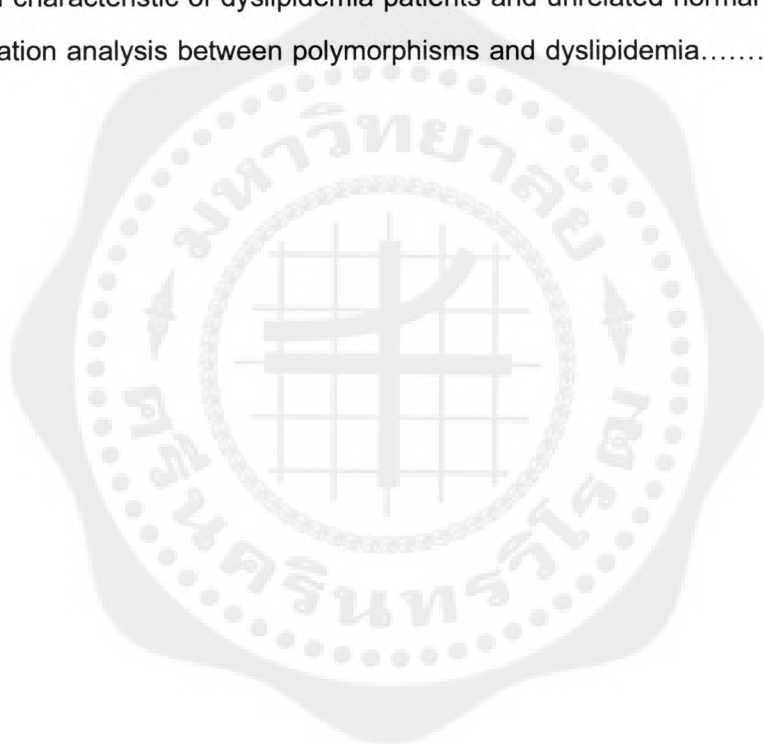
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CHAPTER I

INTRODUCTION

Dyslipidemia is an abnormal amount of lipids such as cholesterol and/or triglyceride in the blood. It may be manifested by elevation of the total cholesterol (TC), low – density lipoprotein cholesterol (LDL-C) and triglyceride (TG) concentrations, and a decrease in the high – density lipoprotein cholesterol (HDL-C) concentration in the blood. Dyslipidemia normally causes no symptoms but it is a major risk factor for coronary heart disease and other vascular diseases. The prevalence of dyslipidemia in China was 35.4% and associated with age, and a family history of dyslipidemia.⁽¹⁾ In 2005, World Health Organization (WHO) reported the deaths from coronary heart disease and atherosclerosis worldwide was 1.72 million in 2005 and increase as the intensity rises, which is expected to be approximately 25 million per year.⁽²⁾

In the previous studies, the genetic variability of several genes were observed to be associated with dyslipidemia such as the Thr56Met missense mutation of the autosomal recessive hypercholesterolemia (*ARH*) gene.⁽³⁾ The P143L polymorphism may play a role in causing decreased HDL-C levels, leading to increased risk of dyslipidemia.⁽⁴⁾ A genome-wide association study (GWAS) examined the concentrations of HDL-C and triglycerides in Europeans⁽⁵⁻⁷⁾ and identified the SNPs at 15 loci which associated with HDL-C levels (such as, *APOA1/C3/A4/A5* gene cluster) and SNPs at 12 loci which associated with triglycerides (such as, *APOB*, *APOE* genes). These known risk variants were re-examined in Mexican and found that they involved in the susceptibility to dyslipidemia.⁽⁸⁾ A meta-analysis of GWAS of blood lipoprotein and lipid phenotypes in European population showed SNPs at 30 loci were convincingly associated with LDL cholesterol, HDL cholesterol and triglyceride.⁽⁵⁾ Polymorphisms at the Apolipoprotein E (ApoE) locus have showed a significant association with total and LDL-cholesterol.

Because of there is no report an association between polymorphism of apolipoprotein E receptor with dyslipidemia. Therefore, this study aims to investigate the single nucleotide polymorphisms (SNPs) of *ApoE* receptor 2 gene in healthy controls and dyslipidemia patients and determine for the association with the risk of dyslipidemia in Thai population.

CHAPTER II

LITERATURE REVIEW

1. Dyslipidemia

Dyslipidemia is the condition of abnormally elevated levels of any or all lipid and/or lipoproteins in the blood. Lipids (fat-soluble molecules) are transported in a protein capsule, and the size of that capsule, or lipoprotein determines its density. As a result, the risk of atherosclerosis, cardiovascular disease, coronary heart disease, cerebrovascular disease and peripheral arterial disease. In developed countries, most dyslipidemias are hyperlipidemias; that is, an elevation of lipids in the blood, often due to diet and lifestyle. The prolonged elevation of insulin levels can lead to dyslipidemia. Increased levels of O-GlcNAc transferase (OGT) are known to cause dyslipidemia.⁽⁹⁾ In Thai population is the condition elevated LDL cholesterol was the most lipid abnormality in the report. Although 55% of the patients were taking lipid lowering agents, another 42% of the patient needed the medication. More than half of the patients treated needed more intensive lipid lowering in order to achieve the LDL. The prevalence of dyslipidemia increased with advancing age and increasing BMI.

Classification of dyslipidemia

Table 1 The classification of dyslipidemia.⁽⁹⁾

Type	Increase	Decrease
Lipid	Hyperlipidemia - Hypercholesterolemia: cholesterol. Familial hypercholesterolemia is a specific form of hypercholesterolemia due to a defect on chromosome 19 (19p13.1-13.3) - Hyperglyceridemia: glycerides - Hypertriglyceridemia: triglycerides	Hypolipidemia - Hypocholesterolemia: cholesterol
Lipoprotein	Hyperlipoproteinemia: lipoproteins (usually LDL unless otherwise specified) - Hyperchylomicronemia: chylomicrons	Hypolipoproteinemia: lipoproteins - Abetalipoproteinemia: betalipoproteins - Tangier disease: high density lipoprotein
Both	Combined hyperlipidemia: both LDL and triglycerides	

Table 2 The criteria of dyslipidemia by National Cholesterol Education Program (NCEP) ⁽¹⁰⁾

Lipid levels (mg/ml)	
Total cholesterol (TC)	
<200	Desirable
200-239	Borderline high
≥240	High
LDL cholesterol	
<100	Optimal
100-129	Near optimal/above optimal
130-159	Borderline high
160-189	High
≥190	Very high
HDL cholesterol	
<40	Low
≥60	High
Triglyceride (TG)	
<150	Desirable
150-199	Borderline high
200-499	High
≥500	Very high

Dyslipidemia was defined participants were taking oral hypolipemiant or those with total cholesterol above 200 mg/dL, HDL-C below 40 mg/dL, LDL-C above 130 mg/dL and TG above 150 mg/dL.

Etiology

Primary (genetic) and secondary (lifestyle and other) causes contribute to dyslipidemia in varying degrees. For example, in familial combined hyperlipidemia, the gene expression may occur only in the presence of significant secondary causes.^(11, 12)

The primary causes are single or multiple gene mutations that result in either overproduction or defective clearance of triglyceride and LDL cholesterol, or in underproduction or excessive clearance of HDL. Primary disorders, the most common cause of dyslipidemia in children, do not cause a large percentage of cases in adults.

The secondary causes contribute to most cases of dyslipidemia in adults. The most important secondary cause in developed countries is a lifestyle with excessive dietary intake of saturated fat, cholesterol, and trans fats. Trans fats are polyunsaturated or monounsaturated fatty acids to which hydrogen atoms have been added; they are commonly used in many processed foods and are as atherogenic as saturated fat. Other common secondary causes include diabetes mellitus, alcohol overuse, chronic kidney disease, hypothyroidism, primary biliary cirrhosis and other cholestatic liver diseases.⁽¹³⁾

In the previous studies, lipid participate in early stages of alcoholic cirrhosis. Therefore in the plasma lipid profile due to primary (genetic) or secondary (environmental) dyslipidemia could affect the development of liver disease. This study reported the association of hypertriglyceridemia and ApoE allele with the early onset of alcoholic liver cirrhosis, and the interaction between environmental factors, such as duration of alcohol abuse and amount of alcohol intake, and genetic factor (ApoE2 allele) on the hypertriglyceridemic process.⁽¹⁴⁾

Symptoms and signs

Dyslipidemia itself usually causes no symptoms but can lead to symptomatic vascular disease, including coronary artery disease (CAD) and peripheral arterial disease. High levels of triglycerides (> 1000 mg/dL or > 11.3 mmol/L) can cause acute pancreatitis. High levels of LDL can cause eyelid xanthelasmas; arcus corneae; and tendinous xanthomas at the Achilles, elbow, and knee tendons and over metacarpophalangeal joints. Patients with the homozygous form of familial hypercholesterolemia may have the above findings plus planar or cutaneous xanthomas. Patients with severe elevations of triglycerides can have eruptive xanthomas over the trunk, back, elbows,

buttocks, knees, hands, and feet. Patients with the rare dysbetalipoproteinemia can have palmar and tuberous xanthomas.

Diagnosis

Dyslipidemia is suspected in patients with characteristic physical findings (results of a physical examination. Observations made visually, by auscultation, palpation, smell, percussion, succussion and ballottement) or complications of dyslipidemia (eg, atherosclerotic disease). Primary lipid disorders are suspected when patients have physical signs (various signs that are elicited by auscultation, percussion, or palpation) of dyslipidemia, onset of premature atherosclerotic disease (at <60 yr), a family history of atherosclerotic disease, or serum cholesterol > 240 mg/dL (> 6.2 mmol/L). Dyslipidemia is diagnosed by measuring serum lipids. Routine measurements (lipid profile) include total cholesterol (TC), triglyceride, HDL cholesterol, and LDL cholesterol.

Lipid profile measurement: TC, triglyceride, and HDL cholesterol are measured directly; TC and TG values reflect cholesterol and triglyceride in all circulating lipoproteins, including chylomicrons, VLDL, intermediate-density lipoprotein (IDL), LDL, and HDL. TC values vary by 10% and triglyceride by up to 25% day-to-day even in the absence of a disorder. TC and HDL cholesterol can be measured in the nonfasting state, but most patients should have all lipids measured while fasting for maximum accuracy and consistency. Testing should be postponed until after resolution of acute illness, because triglyceride increase and cholesterol levels decrease in inflammatory states. Lipid profiles can vary for about 30 days after an acute myocardial infarction (MI); however, results obtained within 24 h after MI are usually reliable enough to guide initial lipid-lowering therapy.⁽¹²⁾

LDL cholesterol values are most often calculated as the amount of cholesterol not contained in HDL and VLDL. VLDL is estimated by triglyceride $\div$ 5 because the cholesterol concentration in VLDL particles is usually $\frac{1}{5}$ of the total lipid in the particle. Thus, LDL cholesterol = TC - [HDL cholesterol + (triglyceride $\div$ 5)] (Friedewald formula). This calculation is valid only when triglyceride are less than 400 mg/dL and patients are fasting, because eating increases triglyceride. The calculated LDL cholesterol value incorporates measures of all non-HDL, nonchylomicron cholesterol, including that in IDL and lipoprotein (a) [Lp(a)]. LDL can also be measured directly using plasma ultracentrifugation, which separates chylomicrons and VLDL

fractions from HDL and LDL, and by an immunoassay method. Direct measurement may be useful in some patients with elevated TGs, but these direct measurements are not routinely necessary. The role of Apo B testing is under study because values reflect all non-HDL cholesterol (in VLDL, VLDL remnants, IDL, and LDL) and may be more predictive of CAD risk than LDL alone.

Other tests: Patients with premature atherosclerotic cardiovascular disease, cardiovascular disease with normal or near-normal lipid levels, or high LDL levels refractory to drug therapy should probably have Lp(a) levels measured. Lp(a) levels may also be directly measured in patients with borderline high LDL cholesterol levels to determine whether drug therapy is warranted. C-reactive protein and homocysteine measurement may be considered in the same populations.

Screening: A fasting lipid profile (TC, triglyceride, HDL cholesterol, and calculated LDL cholesterol) should be obtained in all adults ≥ 20 yr and should be repeated every 5 yr. Lipid measurement should be accompanied by assessment of other cardiovascular risk factors, defined as

- Diabetes mellitus
- Cigarette use
- Hypertension
- Family history of CAD in a male 1st-degree relative before age 55 or a female 1st-degree

relative before age 65

A definite age after which patients no longer require screening has not been established, but evidence supports screening of patients into their 80s, especially in the presence of atherosclerotic cardiovascular disease. Indications for screening patients < 20 yr are atherosclerotic risk factors, such as diabetes, hypertension, cigarette smoking, and obesity; premature CAD in a parent, grandparent, or sibling; or a cholesterol level > 240 mg/dL (> 6.2 mmol/L) or known dyslipidemia in a parent. If information on relatives is unavailable, as in the case of adopted children, screening is at the discretion of the health care practitioner. Patients with an extensive family history of heart disease should also be screened by measuring Lp(a) levels.

Treatment

Lifestyle changes can involve diet and exercise. Dietary changes include decreasing intake of saturated fats and cholesterol; increasing the proportion of dietary fiber, and complex carbohydrates; and maintaining ideal body weight. Referral to a dietitian is often useful, especially

for older people. The length of time for which lifestyle changes should be attempted before beginning lipid-lowering drugs is controversial. In patients at average or low cardiovascular risk, 3 to 6 months is reasonable. Generally, 2 to 3 visits with a patient over 2 to 3 months are sufficient to assess motivation and adherence.⁽¹⁰⁾

Drug : Statins, fibrates and nicotinic acid (Table 3)

Statins

Statins (or HMG-CoA reductase inhibitors) are a class of drugs used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol in the liver. Increased cholesterol levels have been associated with cardiovascular diseases.

Statins act by competitively inhibiting HMG-CoA reductase, the first committed enzyme of the HMG-CoA reductase pathway. Because statins are similar to HMG-CoA on a molecular level they take the place of HMG-CoA in the enzyme and reduce the rate by which it is able to produce mevalonate, the next molecule in the cascade that eventually produces cholesterol, as well as a number of other compounds. This ultimately reduces cholesterol via several mechanisms.

Fibrates

Fibrates are a class of amphipathic carboxylic acids. They are used for a range of metabolic disorders, mainly hypercholesterolemia (high cholesterol), and are therefore hypolipidemic agents.

Evidence from studies in rodents and in humans is available to indicate five major mechanisms underlying the above-mentioned modulation of lipoprotein phenotypes by fibrates:

1. Induction of lipoprotein lipolysis: Increased Triglyceride Rich Lipoproteins (TRL) lipolysis could be a reflection of changes in intrinsic lipoprotein lipase (LPL) activity or increased accessibility of TRLs for lipolysis by LPL owing to a reduction of TRL apoC-III content.
2. Induction of hepatic fatty acid (FA) uptake and reduction of hepatic triglyceride production: In rodents, fibrates increase FA uptake and conversion to acyl-CoA by the liver owing to the induction of FA transporter protein (FATP) and acyl-CoA synthetase (ACS) activity.
3. Induction of the β -oxidation pathway with a concomitant decrease in FA synthesis by fibrates results in a lower availability of FAs for triglyceride synthesis, a process that is amplified by the inhibition of hormone-sensitive lipase in adipose tissue by fibrates.

4. Increased removal of LDL particles: Fibrate treatment results in the formation of LDL with a higher affinity for the LDL receptor, which are thus catabolized more rapidly. Reduction in neutral lipid (cholesteryl ester and triglyceride) exchange between VLDL and HDL may result from decreased plasma levels of TRL.

5. Increase in HDL production and stimulation of reverse cholesterol transport: Fibrates increase the production of apoA-I and apoA-II in liver, which may contribute to the increase of plasma HDL concentrations and a more efficient reverse cholesterol transport.

Niacin

Niacin is one of five vitamins (when lacking in human diet) associated with an epidemic deficiency disease. Niacin has been used for over 50 years to increase levels of HDL in the blood and has been found to modestly decrease the risk of cardiovascular events in a number of controlled human trials.

Niacin binds to and stimulates a G-protein-coupled receptor, which causes the inhibition of fat breakdown in adipose tissue. Nicotinamide does not bind this receptor which explains why it does not affect blood lipid levels. Lipids that are liberated from adipose tissue are normally used to build very-low-density lipoproteins (VLDL) in the liver, which are precursors of low-density lipoprotein (LDL) or "bad" cholesterol. Because niacin blocks the breakdown of fats, it causes a decrease in free fatty acids in the blood and, as a consequence, decreases the secretion of VLDL and cholesterol by the liver. By lowering VLDL levels, niacin also increases the level of high-density lipoprotein (HDL) or "good" cholesterol in blood, and therefore it is sometimes prescribed for people with low HDL, who are also at high risk of a heart attack.

Table 3 Dose providing at least a 30-40% decrease in LDL-cholesterol.⁽¹⁵⁾

Drugs	Adult doses	Comments
Statins		Lower LDL-C (primary), increase HDL-C, lower TGs (secondary)
Atorvastatin	10-80 mg po once/day	No renal excretion, long half-life Dose necessary to achieve recommended 30-40% reduction of LDL-C: 10 mg

Table 3 (continued)

Drugs	Adult doses	Comments
Fluvastatin	Immediate release: 20-80 mg po once/day at bedtime Extended release: 80 mg once/day	Least potent, no renal excretion Dose necessary to achieve recommended 30-40% reduction of LDL-C: 40-80 mg
Lovastatin	Immediate release: 20-80 mg po once/day at dinner Extended release: 20-60 mg po once/day	Dose necessary to achieve recommended 30-40% reduction of LDL-C: 40 mg
Pravastatin	10-80 mg po once/day	Dose necessary to achieve recommended 30-40% reduction of LDL-C: 40 mg
Rosuvastatin	5-40 mg po once/day	Most potent, long half-life Dose necessary to achieve recommended 30-40% reduction of LDL-C: 5-10 mg
Simvastatin	5-80 mg po once/day the evening	Dose necessary to achieve recommended 30-40% reduction of LDL-C: 20-40 mg

Table 3 (continued)

Drugs	Adult doses	Comments
Nicotinic acid (niacin)		
	Immediate release: 500 mg bid-1000 mg po tid	Increases HDL; lower TGs (low doses), LDL-C (higher doses), and Lp(a) (secondary)
	Extended release: 500-2000 mg po once/day at bedtime	Frequent adverse effect: Flushing, impaired glucose tolerance, increased uric acid
		Aspirin and administration with food minimize flushing
Fibrates		
		Lower TGs and VLDL, increase HDL, may increase LDL-C (in patients with high TGs)
Benzafibrate	200 mg po tid or 400 mg po once/day	Decreased dose required in renal insufficiency
		Not available in US
Ciprofibrate	100-200 mg po once/day	Not available in US
Fenofibrate	67-201 mg po once/day	Decreased dose required in renal insufficiency
		May be safest fibrate for use with statins
Gemfibrozil	600 mg po bid	Decreased dose required in renal insufficiency

*po = by mouth, orally (from "per os")

tid = three times a day (from "ter in die")

bid = twice a day (from "bis in die")

2. Lipoprotein

Lipoproteins are molecular complexes consisting of lipids and proteins that provide means for lipid transport between different organs and tissue. The lipids, by virtue of their immiscibility with aqueous solutions, depend on protein carriers for transport within bloodstream and extracellular fluids. A lipoprotein particle is a spherical structure having a hydrophobic core wrapped in a hydrophilic coating. The core contains cholesterol esters and triacylglycerol, because of their nonpolar nature, always avoid contact with water (Figure 1). The hydrophilic surface contains phospholipids, free cholesterol and proteins. It interacts with water in plasma, thereby promoting solubility of lipoproteins. The proteins serve to emulsify the lipid (otherwise called fat) molecules. Many enzymes, transporters, structural proteins, antigens, adhesins, and toxins are lipoproteins. Examples include the high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

The interactions between lipids and proteins in the lipoprotein particle are of purely noncovalent nature. Protein part of lipoprotein is called apoprotein or apolipoprotein.

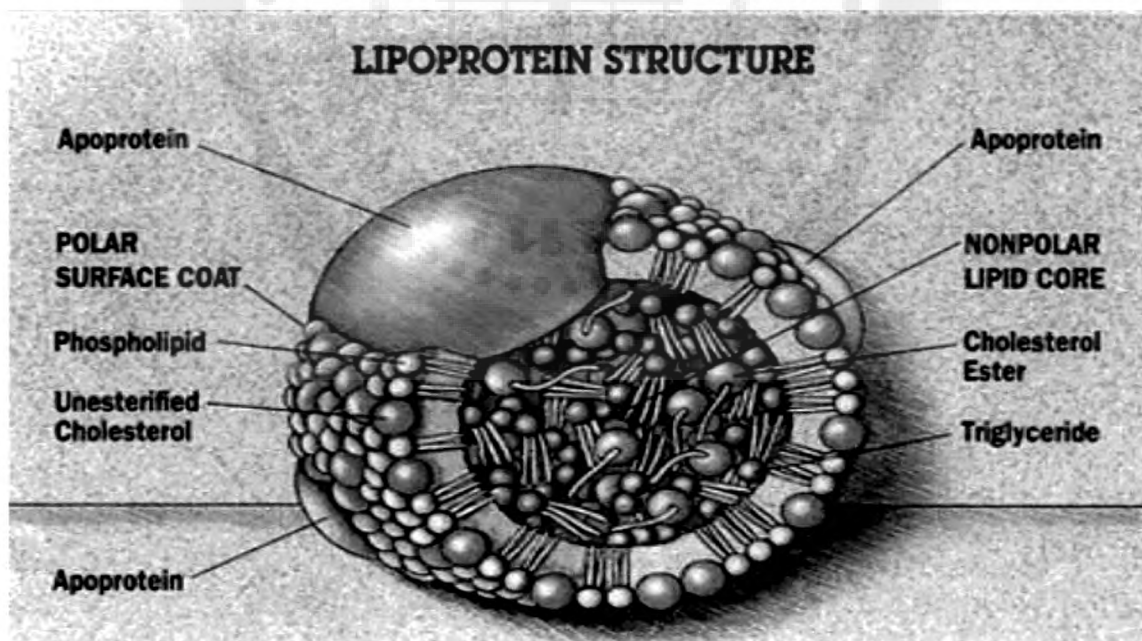


Figure 1 Structure of lipoproteins. ⁽¹⁶⁾

Classification

- Chylomicrons carry triglycerides (fat) from the intestines to the liver, to skeletal muscles, and to adipose tissues.
- Very-low-density lipoproteins (VLDL) carry (newly synthesized) triglycerides from the liver to adipose tissues.
- Intermediate-density lipoproteins (IDL) are intermediate between VLDL and LDL. They are not usually detectable in the blood.
- Low-density lipoproteins (LDL) carry cholesterol from the liver to cells of the body. LDLs are sometimes referred to as the "bad cholesterol" lipoprotein.
- High-density lipoproteins (HDL) collect cholesterol from the body's tissues, and bring it back to the liver. HDLs are sometimes referred to as the "good cholesterol" lipoprotein.

Chylomicron

Dietary cholesterol is absorbed from bile salt micelles into intestinal epithelial cells. This cholesterol, along with that synthesized by the cells, is packaged in chylomicrons, which enter the blood via the lymph. The major protein of the nascent chylomicrons is apoB-48. In the lymph and the blood, chylomicrons obtain apoCII and apoE from HDL. After digestion of the chylomicron triacylglycerols by lipoprotein lipase on capillary endothelial cells, the chylomicron remnants bind the receptors on the liver cells and are internalized via endocytosis. Lysosomal digestion occurs, and the proteins and lipids are degraded, fatty acids, cholesterol and the other products of digestion of the chylomicron remnants enter the pools of liver cells (Figure 2).

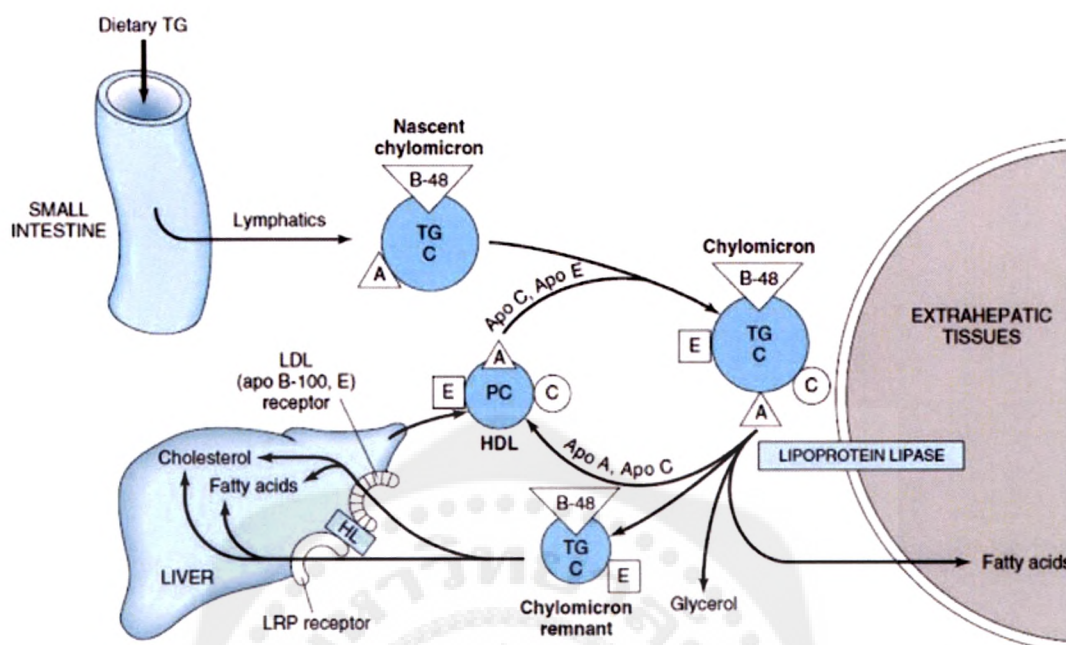


Figure 2 Fate of chylomicron. Chylomicrons, produced from dietary lipid, enter the blood by way of the lymphatic system. Chylomicron triacylglycerol (TG) from the intestinal epithelial cell and lymph are digested by LPL on capillary walls. The remnants of this digestion are much smaller and higher in density than the original chylomicrons. They are recognized by receptor on liver cells. Endocytosis followed by lysosomal action converts the remnants to their component parts. Cholesterol, obtained by the liver from the remnants, inhibits cholesterol synthesis. FA = fatty acids. ⁽¹⁷⁾

Very-low-density lipoproteins (VLDL) cholesterol

As the liver produces triacylglycerols, they are packaged along with cholesterol from the cholesterol pools, phospholipids, and apoB-100 into VLDL, which is secreted into the blood. VLDL is converted to IDL in the blood by digestion of its triacylglycerols by lipoprotein lipase (Figure 3). The triacylglycerols of IDL may be degraded to produce LDL, or IDL may return to the liver, where it binds to cell surface receptors, is taken up by endocytosis, and is degraded by lysosome enzymes. Fatty acids from IDL and LDL, amino acids, and cholesterol are returned to the pools in the liver.

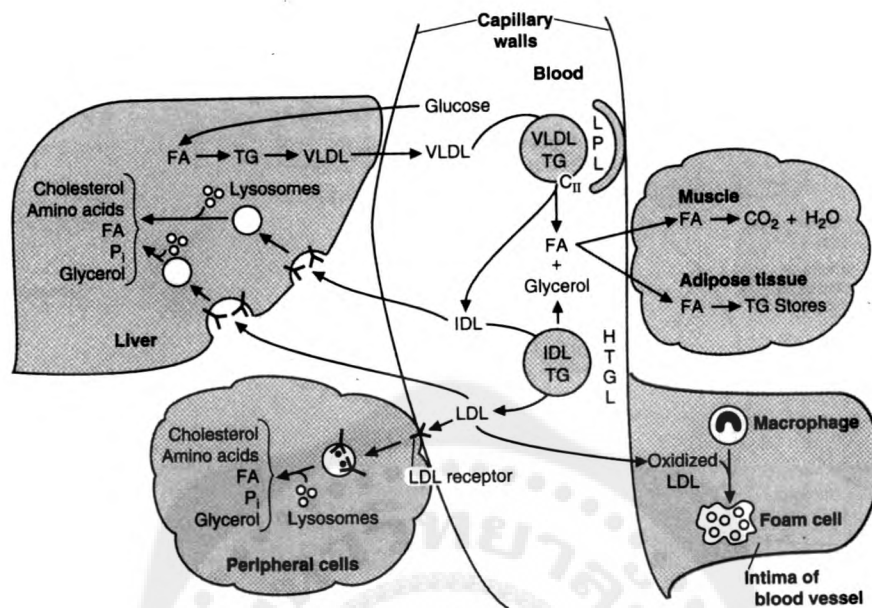


Figure 3 Fate of VLDL. VLDL triacylglycerol (TG) from the liver is degraded by LPL, forming IDL. IDL can either be endocytosed by the liver via a receptor-mediated process or further digested, mainly by hepatic triacylglycerol lipase (HTGL) to form LDL. LDL may be endocytosed by receptor-mediated processes in the liver or in peripheral cells. LDL may also be oxidized and taken up by scavenger receptors on macrophages.⁽¹⁸⁾

High-density lipoproteins (HDL)

The HDL particles are referred to as scavengers because their primary role is to remove free cholesterol from extra hepatic tissue, which is then excreted through bile. Evidently, this is a crucial mechanism that prevents the inappropriate accumulation of cholesterol in peripheral tissues. Because accumulation of cholesterol in tissues is strongly associated with the development of atherosclerosis, the level of HDL in serum is inversely related to the incidence on myocardial infarction. Thus, HDL is cardioprotective or antiratherogenic in nature, and is referred to as the "good cholesterol".

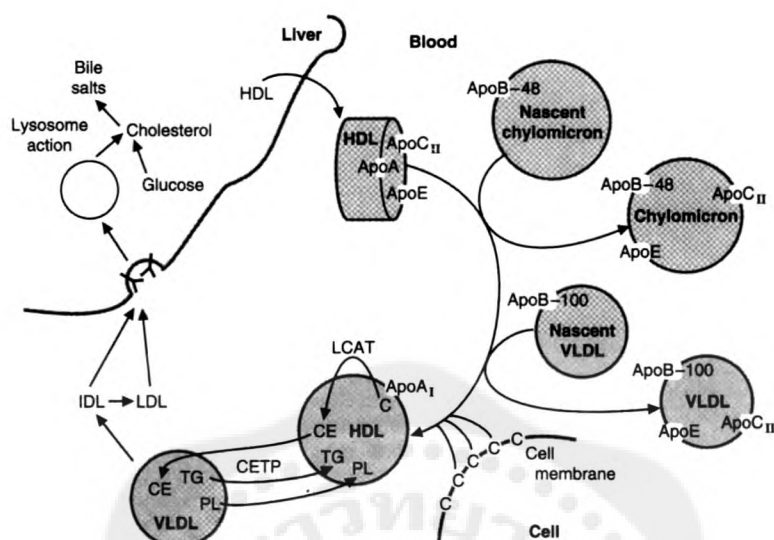


Figure 4 Functions and fate of HDL. Nascent HDL is synthesized in liver and intestinal cells. It exchanges proteins with chylomicrons and VLDL. HDL pick up cholesterol (C) from cell membranes. This cholesterol is converted to cholesterol ester (CE) by the Lecithin: cholesterol acyltransferase (LCAT) reaction. HDL transfers CE to VLDL in exchange for triacylglycerol (TG). The cholesterol ester transfer protein (CETP) mediates this exchange. ⁽¹⁸⁾

Lipoprotein (a)

Lipoprotein (a) is a lipoprotein subclass. The physiological function of Lipoprotein (a)/apo (a) is still unknown. A function within the coagulation system seems plausible, given the aspect of the high homology between apo(a) and plasminogen. The structure of Lipoprotein (a) is similar to plasminogen. Lp(a) also carries cholesterol and thus contributes atherosclerosis. In addition, Lp(a) transports the more atherogenic proinflammatory oxidized phospholipids which attract inflammatory cells to vessel walls, and leads to smooth muscle cell proliferation.

High Lp(a) in blood is a risk factor for coronary heart disease (CHD), cerebrovascular disease (CVD), atherosclerosis, thrombosis and stroke. The association between Lp(a) levels and stroke is not as strong as that between Lp(a) and cardiovascular disease. Lp(a) concentrations may be affected by disease stated, but are only slightly affected by diet, exercise and other environmental factors.

3. Apolipoprotein E (ApoE)

ApoE is a class of apolipoprotein found in the chylomicron and intermediate-density lipoprotein (IDL) that binds to a specific receptor on liver cells and peripheral cells.⁽¹⁾ ApoE is essential for the normal catabolism of triglyceride-rich lipoprotein constituents.⁽¹⁹⁾ The gene for ApoE is located on chromosome 19 in humans, as have apoC-II and the apoB, E(LDL) receptor.⁽²⁰⁻²⁴⁾ The *ApoE* gene consists of four exons and three introns with total length of 3,597 base pairs.⁽²⁵⁾ ApoE is polymorphic with three major isoforms, *ApoE2*, *ApoE3*, *ApoE4*, which translate into three alleles of the gene: Normal : ApoE- ϵ 3, Dysfunctional : ApoE- ϵ 2 and ApoE- ϵ 4. The three isoforms differ by amino acid substitutions at one or both of two sites (residues 112 and 158) on the 299 amino acid chain of the mature ApoE molecule, E4 has arginine and E2 has cysteine at both sites, while E3 has cysteine at site 112 and arginine at site 158.⁽²⁶⁻³³⁾ (Figure 5). E2 is associated with the genetic disorder hyperlipoproteinemia type III and increased risk for atherosclerosis. ApoE2 has an extremely low affinity for binding to both the LDL and ApoE receptors.⁽³⁴⁻³⁶⁾ This type III hyperlipoproteinemia (HLP III) is characterized by the reduced catabolism and accumulation of chylomicron and very low density lipoprotein (VLDL) remnants. Most HLP III patients are homozygous for the apolipoprotein E2 allele; however, several other mutations at this gene locus have been associated with this HLP. In previous studies, the association found between allele E2 and lower total cholesterol level. In this report also observed that the frequency of genotype E2/E3 and E2/E4 was significantly higher in patients with normal total serum cholesterol level compared to patients with abnormal cholesterol.⁽³⁷⁾

In humans, the structural gene locus for plasma apolipoprotein E (ApoE) is polymorphic three common alleles, designated ϵ 4, ϵ 3 and ϵ 2, code for three major ApoE isoforms in plasma. ApoE is a key protein in the modulation of the metabolism of the highly atherogenic ApoB-containing lipoproteins.⁽³⁸⁻⁴³⁾ ApoE in normal and abnormal lipoprotein metabolism, the complex interactions leading to hyperlipidemia and atherosclerosis, and the pathogenesis of type III hyperlipoproteinemia. ApoE polymorphism ranks among the major factors involved in determining interindividual differences in the initiation and progression of atherosclerosis.^(44, 45) ApoE is synthesized primarily in the liver, but it is also synthesized by a number of different tissues in the body including the spleen, kidneys, adrenals, gonads, brain, macrophages, and astrocytic glia.⁽⁴⁶⁻⁵⁰⁾ ApoE plays an important role in the particle lipoprotein and bind to LDL receptor (LDLR) on the liver cell.⁽⁵¹⁾ ApoE can bind to two distinct receptors, the LDL receptor and a lipoprotein

remnant receptor. The LDL receptor is present on most mammalian cell types. It binds both ApoE and ApoB-100-containing lipoproteins. The remnant receptor is present primarily on hepatocytes and binds only ApoE-containing lipoproteins. That chylomicron remnants will bind to the remnant receptor and to the LDL receptor, and that binding of remnant particles to the lipoprotein remnant receptor is ApoE-mediated and does not involve the binding of ApoB-48 to a cell surface receptor.^(52, 53) Allelic variations at the genetic locus responsible for the common ApoE phenotypes have been studied by many laboratories. A review of relative allele frequencies estimated for South American, India, Chinese, Japanese, and Caucasian populations.^(40, 54-63) It is also suggested that any differences in ecological factors and genetic background among these populations have little influence on the role of ApoE polymorphism in determining individual differences in plasma cholesterol that may be associated with the ApoE gene is attributable to differences in relative frequency and not to a change in the phenotypic expression of any particular ApoE phenotype. The gene coding for ApoE makes a large contribution to total phenotypic variability because the average effects of the three polymorphic alleles are moderate. The ApoE polymorphism plays an important role in determining individual differences in cholesterol levels. The effect of this structural polymorphism on the quantitative levels of the B and E apolipoprotein suggests a relationship between these gene product and levels of the lipids and lipoproteins. The ApoE phenotypes has been determined in individuals with various types of hyperlipoproteinemia, and the relationship between the ApoE phenotype and the lipid and lipoprotein concentrations has been quantified. This hyperlipoproteinemia is characterized by an accumulation of remnants of triglyceride-rich lipoprotein particles in plasma. It has been associated with several genetic abnormalities affecting lipoprotein metabolism.⁽⁶⁴⁻⁶⁹⁾ Therefore, in most individuals the type III hyperlipoproteinemia results from a complex interaction between an E2/E2 phenotype and an additional metabolic abnormality associated with hyperlipidemia. In population have demonstrated an increased prevalence of the E2 allele in individuals with hyperlipidemia.^(63, 70, 71) Other rare mutant forms of ApoE associated with type III hyperlipoproteinemia have a variable degree of decreased binding affinity to these receptors such as LDL receptor, ApoE2 receptor, VLDL receptor.^(66, 68, 69) Therefore, these *in vitro* studies indicate that ApoE modulates the binding and catabolism of chylomicron remnants, large VLDL and VLDL remnants, and that ApoE2. ApoE2 was associated less with VLDL, intermediate density lipoprotein (IDL), and LDL and more with HDL.⁽⁷²⁾ Triglyceride-rich lipoprotein particles are secreted by the intestine, and after secretion the triglycerides are hydrolyzed by lipoprotein lipase.

The ApoB-48 containing intestinal remnant particles are then catabolized by and ApoE-mediated remnant receptor endocytotic pathway in the liver. The $\epsilon 2$ allele is associated with lower levels of LDL cholesterol, but predisposes to hypertriglyceridemia; it could, therefore, play a protective role, provided no other condition interacted to raise the plasma levels of atherogenic cholesterol-enriched lipoprotein remnants, and offset its beneficial effect on LDL. The ApoE locus has a major influence on plasma LDL concentration which can be explained by differences in the metabolic behavior of the three major ApoE isoforms. Because of the close association of the ApoE gene to these other genes on chromosome 19, it may be possible to use polymorphisms in the ApoE protein or gene⁽⁷³⁾ as markers for mutations in these closely linked genes when performing genetic studies in families. The three apolipoprotein genes are closely linked and there is thus potential for their coordinate regulation. The regulatory elements for the transcription of the ApoE gene are currently being investigated to clarify the factors that regulate ApoE synthesis.⁽⁷⁴⁾

In humans, the frequencies of the ApoE alleles are quite stable among many distinct populations and it will be important to determine the factors responsible for this stability. The ApoE locus contributes to determining the variation in plasma cholesterol levels of healthy and diseased populations. It also influences the expression of hyperlipidemia and appears to modulate the susceptibility to atherosclerosis in a complex multifactorial interaction. ApoE2 has lower receptor binding affinity which results in delayed clearance of ApoE2-bearing lipoprotein particles from plasma. In addition, the relationship between the $\epsilon 2$ allele and type III hyperlipoproteinemia provides a valuable model for the study of complex genetic interactions in the pathogenesis of hyperlipidemia.⁽⁴⁴⁾ The effect of structural variability in the ApoE molecule on the levels of the apolipoproteins will help in developing an understanding of the links between genetic variation and the regulation of lipid and lipoprotein levels.

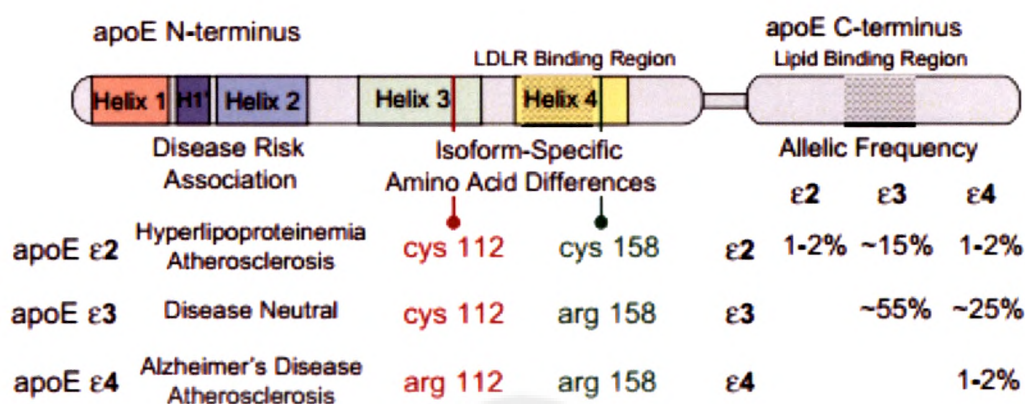


Figure 5 Linear diagram of the ApoE structure and three isoform-specific differences at residues 112 and 158, genotypic frequencies of the human isoforms⁽⁷⁵⁾ and disease risk associations for the three isoform.⁽⁷⁶⁾

4. Apolipoprotein E receptor

A fundamental role of ApoE is its function as a ligand for cell surface receptors. ApoE2 is known to be a high affinity ligand for the LDL receptor, the LDL receptor related protein 1 (LRP1), ApoE receptor 2 (ApoER2) and the VLDL receptor (Figure 6).⁽⁷⁷⁾ This gene encodes an apolipoprotein E receptor, a member of the low density lipoprotein receptor (LDLR) family. Apolipoprotein E is a small lipophilic plasma protein and a component of lipoproteins such as chylomicron remnants, very low density lipoprotein (VLDL), and high density lipoprotein (HDL). The apolipoprotein E receptor is involved in cellular recognition and internalization of these lipoproteins. Alternative splicing generates multiple transcript variants encoding distinct isoforms for this gene.⁽⁷⁸⁾ Apolipoprotein E receptor 2 is an example of a protein with such as dual function. In cultured cells it acts as an endocytic receptor for lipoprotein.

Members of the LDLR family of proteins dynamically bind and internalize apoE and apoB-containing lipoprotein, including LDL, VLDL, and chylomicron remnant. Defect in these receptors is associated with dyslipidemia and atherosclerosis in mammals. LDL defect is the most common cause of familial hypercholesterolemia, which is associated with very high plasma LDL levels accounted for by impaired LDL clearance and enhanced cholesterol biosynthesis. VLDLR modulates the binding and uptake of apoE containing lipoprotein, including chylomicron and VLDL,

and regulates TG level in plasma.⁽⁷⁹⁾ The specific receptor of three isoforms of apoE (E2, E3, E4) is apolipoprotein E receptor2 (ApoER2), that they have similar affinities for this receptor.

The Apolipoprotein E Receptor 2 (ApoER2) or Low density lipoprotein receptor-related protein 8 gene (LPR8) is a member of the low-density lipoprotein receptor gene family that plays a role in endocytosis and signal transduction. Human ApoER2 related that the gene spans approximately 60-kb and contains 19 exons.⁽⁸⁰⁾ It located on chromosome 1p32.3 that encodes 963 amino acids protein. This gene encodes a member of the low density lipoprotein receptor (LDLR) family. Low density lipoprotein receptors are cell surface proteins that play roles in both signal transduction and receptor-mediated endocytosis of specific ligands for lysosome degradation. The encoded protein plays a critical role in the migration of neurons during development by mediating reelin signaling, and also functions as a receptor for the cholesterol transport protein apolipoprotein E. Alternative splicing generates multiple transcript variants encoding distinct isoforms for this gene.

ApoER2 has the same overall domain structure as VLDLR, with several important differences. Exon 1 extends 236 bp upstream of the initial methionine codon and contains the signal sequence. Cysteine-rich repeats 1–3 and 7 of the ligand-binding domain are each encoded by individual exons. Although the ligand-binding domain of human apoER2 consists of seven cysteine-rich repeats like that of LDLR, the exon/intron organization of this domain is much more closely related to that of the human VLDLR gene, which contains an extra exon encoding an additional cysteine-rich repeat. The growth factor repeats in the apoER2 gene are each encoded by individual exons. Exons 9–13 encode the nonrepetitive sequences that are shared among LDLR, VLDLR, and epidermal growth factor precursor. The O-linked sugar domain of the apoER2 gene is encoded by a single exon of 225 bp. The transmembrane domain is interrupted by a single intron. Exon 17 contains the C-terminal half of the transmembrane domain and the first 44 amino acids of the cytoplasmic domain. The cytoplasmic domain of apoER2 contains an insertion sequence of 59 amino acids. This insertion sequence is encoded by a single exon of 177 bp (exon18). The last exon encodes the remaining 12 amino acids and the 39-untranslated region of the mRNA.⁽⁸¹⁾

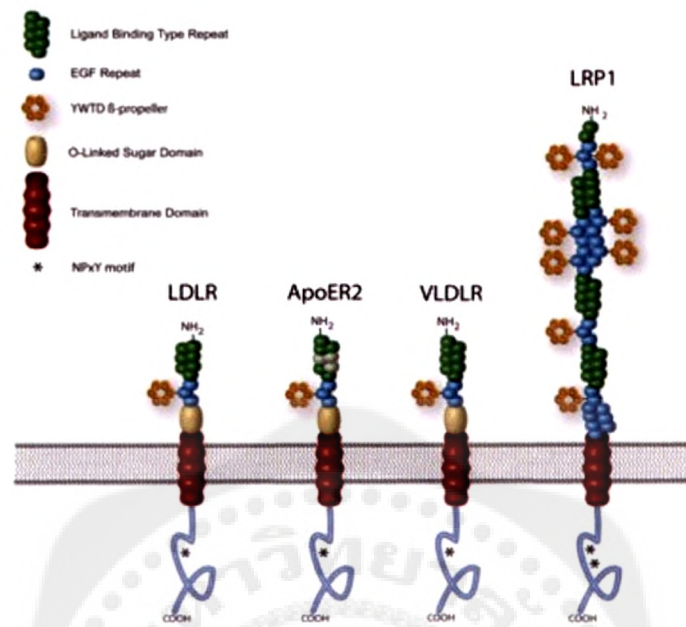


Figure 6 LDLR, ApoER2, VLDLR and LRP represent the four major ApoE receptor family members in the mammalian.⁽⁷⁷⁾

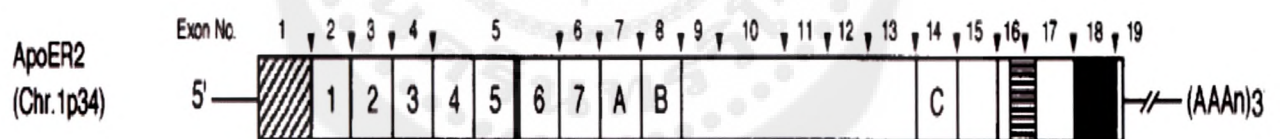


Figure 7 Specific domain and exon number of ApoER2 receptors.⁽⁸¹⁾

5. Genome-wide association study (GWAS)

The genome-wide association study (GWAS) is an examination of many common genetic variation in different individuals for identification genetic factors that influence to health and disease. These studies compare the DNA of two group of participants (patient and healthy control). DNA is extracted from cells or blood and spread on gene chips, which they can be analyzed with bioinformatics techniques. Rather than reading the entire DNA sequence, these systems usually read SNPs that are markers for group of DNA variation (haplotypes). The SNPs are more frequent in patients, the SNPs are called to be associated with the disease.^(84, 85)

Recently, a genome-wide association study (GWAS) showed the single nucleotide polymorphism (SNPs) were associated with risk of hyperlipidemia or dyslipidemia. For example, Peroxisome proliferator-activated receptor α (PPAR α), autosomal recessive hypercholesterolemia (AHR) gene, lecithin: cholesterol acyltransferase (LCAT) gene, transcription factor 7-like 2 (TCF7L2) gene, methylene tetrahydrofolate reductase (MTHFR) gene, protocadherin15 (PCDH15) gene, acyl-CoA: cholesterol acyltransferase-2 (ACAT-2) gene, adipocyte fatty acid- binding protein (FABP4), cholesteryl ester transfer protein (CETP) gene, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) gene, glucokinase regulatory protein (GCKR) gene, LDL receptor (LDLR) gene, lipoprotein lipase (LPL) gene, hepatic lipase (LIPC) gene, apolipoprotein B (ApoB) gene, apolipoprotein A1 (ApoA1) gene, apolipoprotein E (ApoE) gene.^(3, 4, 86-94)

6. Meta-analysis

The statistical analysis of a large collection of analysis results for the purpose of integrating the findings. It is to provide the same methodological rigor to a literature review that we require from experimental research. In its simplest form, this is normally by identification of a common measure of effect size (sample), of which a weighted average might be the output of a meta-analysis. The weighting might be related to sample sizes within the individual studies. More generally there are other differences between the studies that need to be allowed for, but the general aim of a meta-analysis is to more powerfully estimate the true effect size as opposed to a less precise effect size derived in a single study under a given single set of assumptions and conditions.

Advantages of meta-analysis

- Shows if the results are more varied than what is expected from the sample diversity
- Derivation and statistical testing of overall factors / effect size parameters in related studies
- Generalization to the population of studies
- Ability to control for between-study variation
- Including moderators to explain variation
- Higher statistical power to detect an effect than in 'n=1 sized study sample'
- It combines several studies and will therefore be less influenced by local findings than single studies will be.
- Makes it possible to show if a publication bias exists.

Disadvantages of meta-analysis

A meta-analysis of several small studies does not predict the results of a single large study, especially in a field like medicine where results are truly unpredictable.⁽⁹⁵⁾ Some have argued that a weakness of the method is that sources of bias are not controlled by the method. A good meta-analysis of badly designed studies will still result in bad statistics.

7. Single nucleotide Polymorphism (SNP)

A single-nucleotide polymorphism is a DNA sequence variation occurring when a single nucleotide (adenine-A, thymine-T, cytosine-C and guanine-G) in the genome differs between members of a biological species or paired chromosomes in an individual. For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide (Figure 7). Besides natural selection other factors like recombination and mutation rate can also determine SNP density. SNP density can be predicted by the presence of microsatellites as regions of thousands of nucleotides flanking microsatellites have an increased or decreased density of SNPs depending on the microsatellite sequence.⁽⁹⁶⁾

In a population, these genetic variations between the individuals (particularly in the non-coding parts of genome) are exploited in DNA fingerprinting, which is used in forensic science.

Single-nucleotide polymorphisms can be found in coding sequences of genes, non-coding regions of genes, or in the intergenic regions (regions between genes). SNPs within a coding

sequence do not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. SNP in which both alleles produce the same polypeptide sequence is called a synonymous polymorphism (sometimes called a silent mutation). If a different polypeptide sequence is produced the polymorphism is a replacement polymorphism. A replacement polymorphism change may be either missense, which results in a different amino acid, or nonsense, which results in a premature stop codon. Over half of all known disease mutations come from replacement polymorphisms.⁽⁹⁷⁾ SNPs that are not in protein-coding regions may still affect gene splicing, transcription factor binding, messenger RNA degradation, or the sequence of non-coding RNA. Gene expression affected by this type of SNP is referred to as an eSNP (expression SNP) and may be upstream or downstream from the gene.

Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, drugs, vaccines, and other agents. Therefore, the single nucleotide polymorphisms are also thought to be a key of personalized medicine. The single nucleotide polymorphisms don't usually function individually but they can work with other single nucleotide polymorphisms to develop a disease.



Figure 8 Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (A, T, C or G) in the genetic sequence is altered. In order for an altered sequence to be considered as a SNP, it must occur in at least 1% of the population. Two out of three SNPs involve the replacement of the nucleotide cytosine (C) with the nucleotide thymine (T).⁽¹¹⁶⁾

8. Single nucleotide polymorphism screening methods

Several methods have been provided to detect single nucleotide polymorphisms in dyslipidemia including DNA sequencing^(98, 99), single-strand conformation polymorphism (SSCP)⁽¹¹⁸⁾, restriction fragment length polymorphism (RFLP)⁽¹¹⁹⁾, gel electrophoresis⁽¹⁰⁰⁻¹⁰²⁾, real-time polymerase chain reaction⁽¹⁰³⁾, high resolution melting analysis (HRM)^(104, 105)

DNA sequencing

The DNA to be sequenced, called the template DNA, is first prepared as a single-stranded DNA. Next, a short oligonucleotide is annealed, or joined, to the same position on each template strand. The oligonucleotide acts as a primer for the synthesis of a new DNA strand that will be complimentary to the template DNA. This technique requires that four nucleotide-specific reactions one each for G, A, C, and T be performed on four identical samples of DNA. The four sequencing reactions require the addition of all the components necessary to synthesize and label new DNA, including: A DNA template, primer tagged with a mildly radioactive molecule or a light-emitting chemical, DNA polymerase an enzyme that drives the synthesis of DNA, four deoxynucleotides (G, A, C, T) and one dideoxynucleotide, either ddG, ddA, ddC, or ddT.

After the first deoxynucleotide is added to the growing complementary sequence, DNA polymerase moves along the template and continues to add base after base. The strand synthesis reaction continues until a dideoxynucleotide is added, blocking further elongation. This is because dideoxynucleotides are missing a special group of molecules, called a 3'-hydroxyl group, needed to form a connection with the next nucleotide. Only a small amount of a dideoxynucleotide is added to each reaction, allowing different reactions to proceed for various lengths of time, until, by chance, DNA polymerase inserts a dideoxynucleotide, terminating the reaction. Therefore, the result is a set of new chains, all of different lengths.

To read the newly generated sequence, the four reactions are run side-by-side on a polyacrylamide sequencing gel. The family of molecules generated in the presence of ddATP are loaded into one lane of the gel and the other three families, generated with ddCTP, ddGTP, and ddTTP, are loaded into three adjacent lanes. After electrophoresis, the DNA sequence can be read directly from the positions of the bands in the gel.

Single-strand conformation polymorphism (SSCP)

Single-strand conformational polymorphism (SSCP) analysis is a simple and sensitive technique for mutation detection and genotyping. The principle of SSCP analysis is based on the fact that single-stranded DNA has a defined conformation. Altered conformation due to a single base change in the sequence can cause single-stranded DNA to migrate differently under nondenaturing electrophoresis conditions. Therefore wild-type and mutant DNA samples display different band patterns. SSCP analysis involves the following four steps: (1) polymerase chain reaction (PCR) amplification of DNA sequence of interest; (2) denaturation of double-stranded PCR products; (3) cooling of the denatured DNA (single-stranded) to maximize self-annealing; (4) detection of mobility difference of the single-stranded DNAs by electrophoresis under non-denaturing conditions. Several methods have been developed to visualize the SSCP mobility shifts. These include the incorporation of radioisotope labeling, silver staining, fluorescent dye-labeled PCR primers, and more recently, capillary-based electrophoresis. Silver staining is simple, rapid, and cost-effective, and can be routinely performed in clinical laboratories. The use of SSCP analysis to discover and genotype single-nucleotide polymorphisms (SNPs) has been widely applied to the genetics of hypertension, including both monogenic (e.g., Liddle's syndrome) and polygenic disorders (e.g., essential hypertension).

Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites. The PCR product is amplified and digested into pieces by restriction enzyme. Then, the restriction fragments are checked by using gel electrophoresis, which separated according the lengths of fragments. The samples which don't have restriction enzyme sites result in the restriction enzyme can't cut it. Therefore, the gel electrophoresis shows different band of PCR products from another samples. In addition to genetic fingerprinting, RFLP was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

Gel electrophoresis

Electrophoresis is a procedure which enables the sorting of molecules based on size and charge. Using an electric field, molecules (such as DNA) can be made to move through a gel made of agar or polyacrylamide. The molecules being sorted are dispensed into a well in the gel material. The gel is placed in an electrophoresis chamber, which is then connected to a power source. When the electric current is applied, the larger molecules move more slowly through the gel while the smaller molecules move faster. The different sized molecules form distinct bands on the gel

Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

Real-time polymerase chain reaction

Real-time polymerase chain reaction is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, A DNA-binding dye binds to all double-stranded (ds)DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as Primer dimer). This can potentially interfere with, or prevent, accurate quantification of the intended target sequence. The reaction is prepared as usual, with the addition of fluorescent dsDNA dye. The reaction is run in a real-time PCR instrument, and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). With reference to a standard dilution, the dsDNA concentration in the PCR can be determined. Like other real-time PCR methods, the values obtained do not have absolute units associated with them (i.e., mRNA copies/cell). As described above, a comparison of a measured

DNA/RNA sample to a standard dilution will only give a fraction or ratio of the sample relative to the standard, allowing only relative comparisons between different tissues or experimental conditions. To ensure accuracy in the quantification, it is usually necessary to normalize expression of a target gene to a stably expressed gene (see below). This can correct possible differences in RNA quantity or quality across experimental samples. (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target. Fluorescent reporter probes detect only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification. Fluorescent probes can be used in multiplex assays for detection of several genes in the same reaction based on specific probes with different-coloured labels, provided that all targeted genes are amplified with similar efficiency. The specificity of fluorescent reporter probes also prevents interference of measurements caused by primer dimers, which are undesirable potential by-products in PCR. However, fluorescent reporter probes do not prevent the inhibitory effect of the primer dimers, which may depress accumulation of the desired products in the reaction.

High resolution melting analysis (HRM)

High resolution melting analysis (HRM) is a powerful technique in molecular biology for the detection of mutations, single nucleotide polymorphism and epigenetic differences in double-stranded DNA samples. It has advantages over other genotyping technologies. It is fast and powerful thus able to accurately genotype many samples rapidly and it is simple, a good quality HRM assay, powerful genotyping can be performed by non-geneticists in any laboratory with access to an HRM capable real-time PCR machine. HRM analysis is performed on double stranded DNA samples. Typically the user will use polymerase chain reaction (PCR) prior to HRM analysis to amplify the DNA region in which their mutation of interest lies. This region that is amplified is known as the amplicon. After the PCR process the HRM analysis begins. The process is simply a precise warming of the amplicon DNA from around 55°C up to around 95°C. At some point during this process, the melting temperature of the amplicon is reached and the double-strand DNA separate into single-strand DNA during increased temperature. This can visualize the melting behavior of the product through a fluorescent dye. The fluorescent dye binds to double-strand DNA during the amplification resulting in an increase of fluorescence. When the double-strand DNA

separates the fluorescent dye releases results in the decrease of fluorescence dye. This is visualized in a characteristic melting curve (Figure 8).

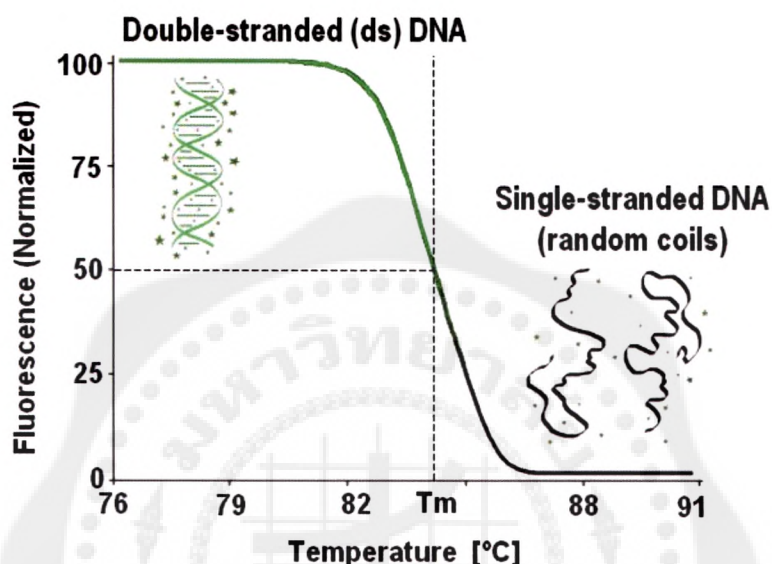


Figure 9 The melting temperature of the fluorescent dye binds to double-strand, when the DNA is heated to high temperatures, the DNA denatures and the fluorescent dye fades away as the double stranded DNA separates into single-strand DNA during resulting in a release of the fluorescent dye. This can be visualized by measuring the light emission that generates a melting curve.⁽¹⁰⁶⁾

The difference between standard and high-resolution melting techniques is shown in Figure 9, which shows derivative melting curves for different genotypes. The melting temperature (T_m) of double-strand DNA depends on the length of sequence and nucleotide distribution in the sequence. A long sequence and high concentration of GC base pair will have higher melting temperature than a shorter sequence and high concentration of AT base pairs. The differences in DNA sequence resulting in different melting temperature and shape of melting curve. The heterozygotes are identified by the low temperature melting of heteroduplexes that show the different melting temperature, shape and slope of melt curve. The samples with homozygotes are distinguished by a different in melting temperature.

The normalized melting curves of the four homozygotes and six heterozygotes are shown in Figure 10. All homozygotes melted in a single transition (change one base) (Figure 10A), and the order of melting was correctly predicted by nearest-neighbor calculations as $A/A < T/T < C/C < G/G$. The six heterozygote curves merged at high temperatures into three traces, predicted by the highest melting homoduplex present (T/T for the A/T heterozygote, C/C for the A/C and C/T heterozygotes, and G/G for the A/G, G/T, and C/G heterozygotes). All genotypes could be distinguished from each other by high-resolution melting analysis (Figure 10B).



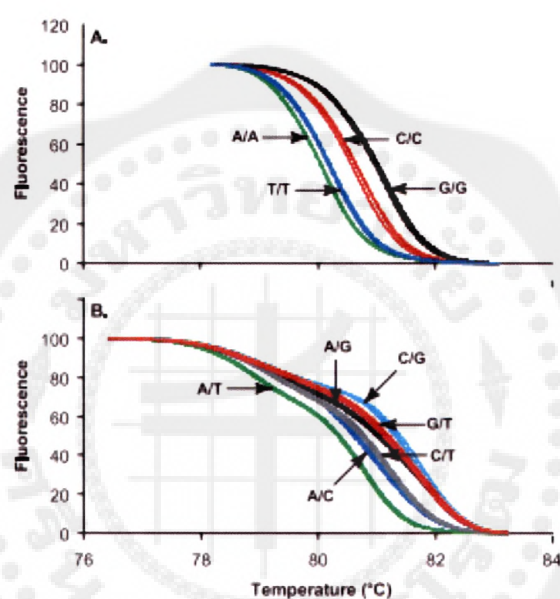


Figure 10 Normalized, high-resolution melting curves of all possible SNP genotypes. All homozygotes melted in a single transition show four homozygotes (A/A, T/T, C/C and G/G) (Figure 10A) and six heterozygotes (A/T, A/C, C/T, A/G, G/T and C/G) (Figure 10B).⁽¹⁰⁵⁾

Objectives

To investigate the single nucleotide polymorphisms (SNPs) of *ApoE* receptor 2 genes in healthy controls and dyslipidemia patients in association with risk of dyslipidemia in Thai population.



CHAPTER III

MATERIALS AND METHODS

Subjects

The dyslipidemia patients and healthy controls were randomly recruited. The subjects were divided into 2 groups; dyslipidemia patients (200) and controls (200) using level of lipids in the blood which corresponding to the criteria of National Cholesterol Education Program (NCEP). Serum lipids were analyzed at the laboratory of HRH Princess Mahachakri Sirindhorn Medical Center, Faculty of Medicine, Srinakharinwirot University, Ongkarak Campus, Nakornnayok, Thailand.

The inclusion and exclusion criteria are shown in the table 4. All patients and healthy controls have to sign an informed consent before participating in this study, which was approved by the ethical committees of the Faculty of Medicine, Srinakharinwirot University, Thailand.

Table 4 Criteria for inclusion and exclusion of subjects for this study

Inclusion	Exclusion
- Thai citizen with age range from 18-65 years - Lipid level of patient ● Triglyceride > 150 mg/dL ● Total cholesterol > 200 mg/dL ● LDL-cholesterol > 100 mg/dL ● HDL-cholesterol < 60 mg/dL - Lipid level of healthy control ● Triglyceride < 150 mg/dL ● Total cholesterol < 200 mg/dL ● LDL-cholesterol < 100 mg/dL ● HDL-cholesterol > 60 mg/dL - Sign informed consent	- Age less than 17 or more than 66 years - Refused to sign informed consent - Take the lipid lowering drugs

- The subject with age under 17 years old, the system does not stable, they are in development period whereas the subject with age over than 66 years old, the system is decrease or lose their function. Therefore, these group are excluded from this study.

Measurement of serum lipid levels

The serum of all subjects were measured for cholesterol, triglyceride, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) levels by enzymatic colorimetric methods from laboratory of HRM Princess Maha Chakri Sirindhorn Medical center, Faculty of Medicine, Srinakharinwirot University.

Genomic DNA extraction

Eight milliliters of blood sample was collected and separated into 2 tubes; 4 ml of clot blood was prepared for serum by centrifugation at 15,000 xg for 10 min and stored at -80°C; the another 4 ml of blood was collected in heparinized-tube for DNA extraction by using Flexigene DNA kit (QIAGEN, German). Briefly, the whole blood was added with 12.5 ml of lysis buffer and mix by inverting the tube. Then, centrifuge for 15 min at 5300 x g. Discarded the supernatant and left the tube inverted for 2 min that the pellets remain in the tube. Add 2.5 ml of denaturation buffer and 25 µl of protease into the tube, vortex immediately until the pellets completely dissolved and incubated at 65°C for 10 min. After that, added 2.5 ml of isopropanol (100%) and mix thoroughly by inversion. Then, centrifuged for 15 min at 5,300 xg and discarded the supernatant. DNA pellet was washed with 70% ethanol and centrifuged for 15 min at 5,300 xg. After that, DNA pellet was dried at room temperature and dissolved with FG3 buffer. The DNA concentration was measured by Qubit[®] Fluorometer (Invitrogen). Then, the DNA concentration was adjusted to 25 ng/µl for PCR amplification.

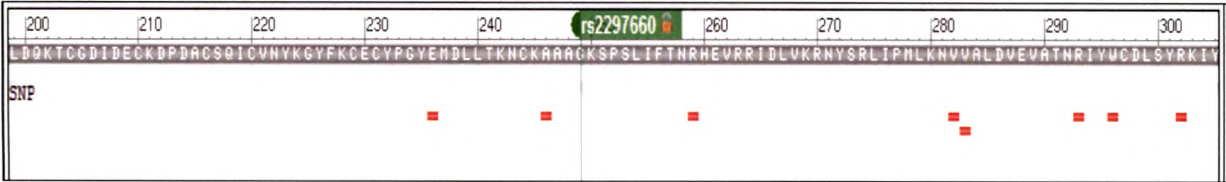
Design of SNPs primers

The primers for SNPs in the *ApoE* receptor 2 gene was designed using the program from website <http://snp.ims.u-tokyo.ac.jp/search.html> and <http://www.ncbi.nlm.nih.gov/projects/SNP/>. The primer sequences is shown in table 5. The selected SNPs position in The *ApoE* receptor 2 gene are shown in Figure 11.

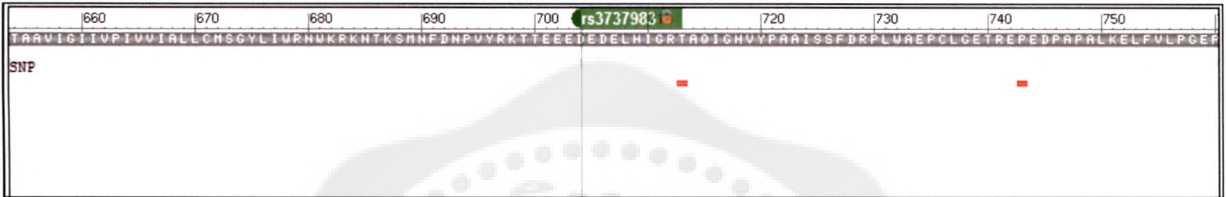
Table 5 Primers use in polymerase chain reaction of *ApoE* receptor 2 gene.

Gene	db SNPs ID	Location	Primer sequence 5'→3'	Size of PCR product (bp)
<i>ApoE</i> receptor 2297660	rs2297660	CDS*3	F1: TAGTTCCGCTTCACCAGGTC R1: AAGCCAGTGGCAGAGTCTTG	153
<i>ApoE</i> receptor 3737983	rs3737983	CDS*3	F2: GCCAATCTGAGCAGTTCTCC R2: GTGATAGCCCTCCTGTGCAT	156
<i>ApoE</i> receptor 3737984	rs3737984	intron	F3:GCATTTCTGGGAAAAGGAATTA R3: TTGAGTCTGGAGAGGGGATA	185

ApoE receptor2297660 position 249



ApoE receptor3737983 position 704



ApoE receptor3737984 position 6459 (mRNA)

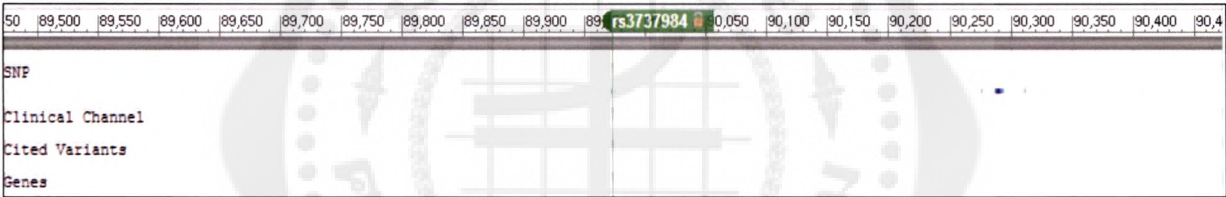


Figure 11 The position of selected SNPs (rs2297660, rs3737983, and rs3737984) in *ApoE* receptor 2 gene.

Polymerase chain reaction (PCR)

The SNPs of *ApoE* receptor2 gene were amplified by PCR with the primers listed in Table 5. The PCR reactions were performed in total volume of 10 µl containing 1µl of genomic DNA (25 ng), 1 µl of 10x buffer, 0.25 µl of 50 mM magnesium chloride (MgCl₂), 1 µl of deoxynucleotide triphosphate (dNTP), 0.1 µl of 5 U *Taq* DNA polymerase, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, and 5.65 µl of water sterile. The PCR reaction was performed for 30 cycles; Pre-denaturing at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at x°C (depending on primer to used) for 30 s, extension at 72°C for 30 s. The final extension was performed at 72°C for 5 min.

Real-time PCR, high-resolution melting analysis and sequencing

PCR reaction was carried out in total volume of 10 µl compose of 5 µl of quantiprobe, 1 µl of DNA (25 ng), 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 1 µl of Syto9 green fluorescent and 2 µl of sterile-distilled water. PCR reaction was subjected to amplification step for 40 cycles: pre-denaturing at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at x°C (depending on primer to used) for 15 s and extension at 72°C for 20 s. PCR products were genotyped by high-resolution melting (HRM) analysis. The PCR products were sequenced to confirm sequence of SNPs by DNA sequencing.

Statistical analysis

The difference in genotype distribution between patient and control was analyzed by using Pearson's Chi-square test implemented in SPSS program version 11.5 for Windows. Significant level was set at $P < 0.05$.

CHAPTER IV

RESULTS

The clinical characteristic of the 200 dyslipidemia patients and the 200 unrelated normal controls including gender, age, total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol was showed in table 6. The average age of subjects of the studied groups were not a major difference ($p < 0.22$). But the total cholesterol, triglyceride, and LDL-cholesterol of patients was significantly higher than that of unrelated normal controls ($p < 0.001$) whereas HDL-cholesterol of the patients was significantly lower than the controls ($p < 0.001$).

The SNPs in *ApoER2* gene were analyzed by amplification of the region of from genomic DNA of 200 dyslipidemia patients and 200 healthy controls by PCR and subjected to HRM analysis. The HRM results were displayed as melting curve. Generally, the homozygotes show same shape of melting curve but different in melting temperature whereas the melting curves of heterozygotes appears in different pattern. The melt curves for SNPs in *ApoER2* gene resulting from HRM analysi are shown in figure 12-14.

The SNPs for association study were selected in gene involved in lipid metabolism function that maybe contribute to dyslipidemia. The results of the association analysis are summarized in Table 7. The SNPs in *ApoER2* gene showed significant differences in the genotype distribution between patients and controls in the Thai population ($P < 0.05$). The rs3737983, rs2297660 and rs3737984 in *ApoER2* gene were significantly association with dyslipidemia ($P = 0.011$, OR and 95%CI were 0.47 (0.28-0.78)), ($P = 0.001$, OR and 95%CI were 2.38 (1.49-3.80)) and ($P = 0.001$, OR and 95%CI were 0.42 (0.28-0.65)), respectively.

Table 6 Clinical characteristic of dyslipidemia patients and unrelated normal controls.

Variables of subjects	Control group	Dyslipidemia group	<i>P</i> value
Sample number (n)	200	200	
Male	82 (42%)	78 (39%)	
Female	118 (58%)	122 (61%)	
Age (years)	47.91±0.5	47.01±0.5	>0.22
Total cholesterol (TC)	194.04±2.7 ^a	243.09±4.2 ^a	<0.001
Triglyceride (TG)	117.81±5.7 ^a	288.04±53.5 ^a	<0.001
High-density lipoprotein (HDL)	58.81±1.3 ^b	51.04±0.8 ^b	<0.001
Low-density lipoprotein (LDL)	114.49±3.2 ^b	149.31±7.3 ^b	<0.001

^a number of control =200, patients =200^b number of control =164, patients =167

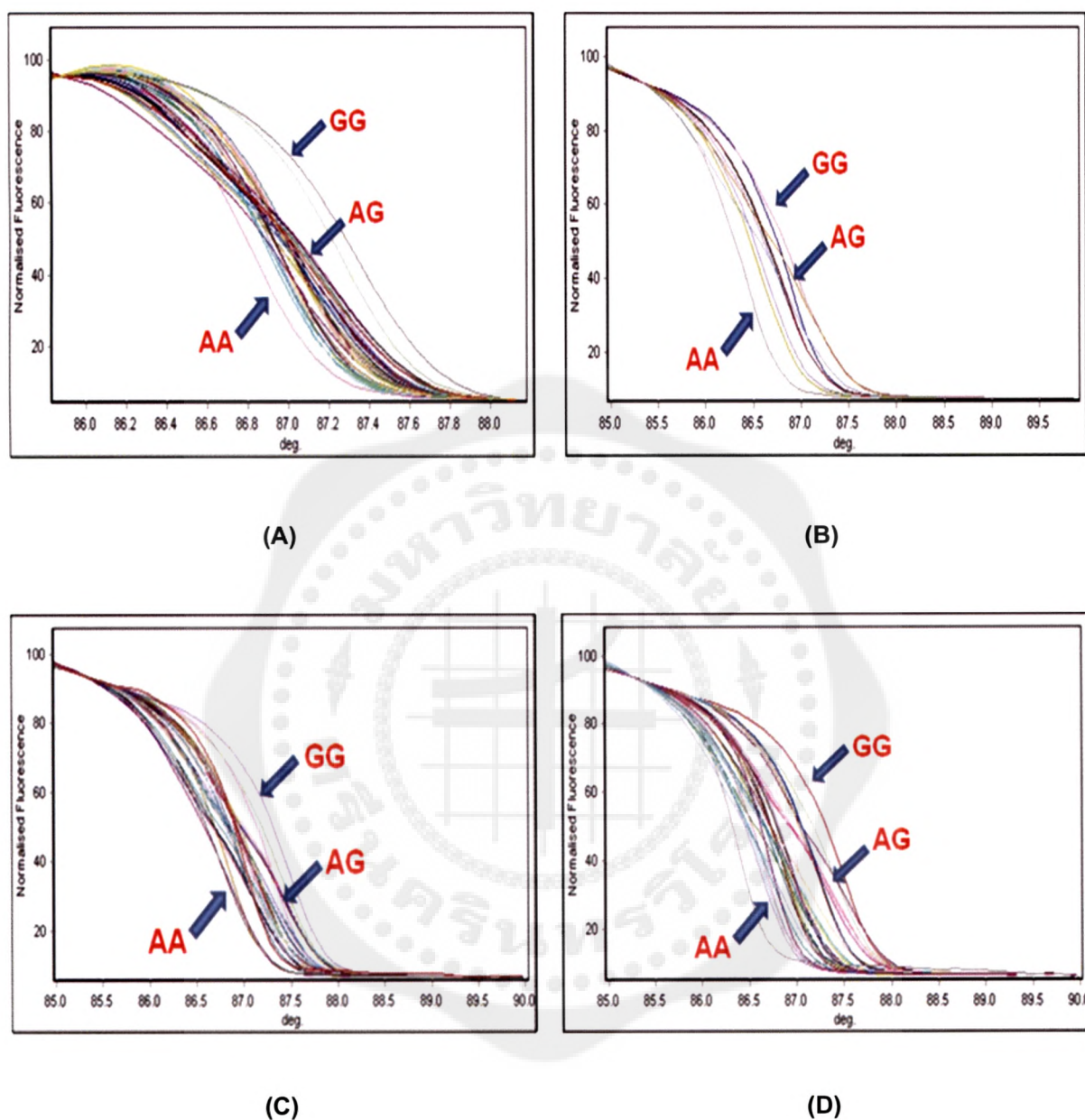
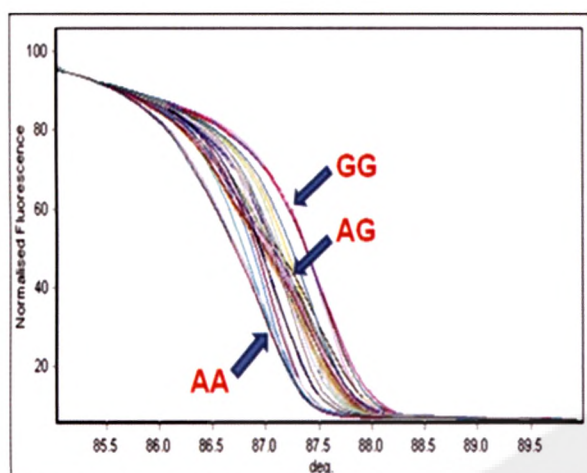
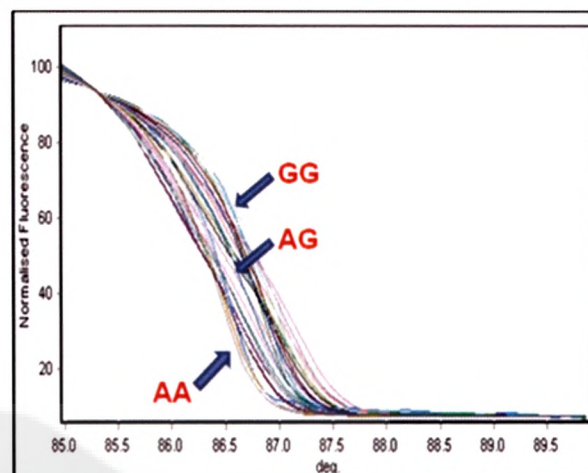


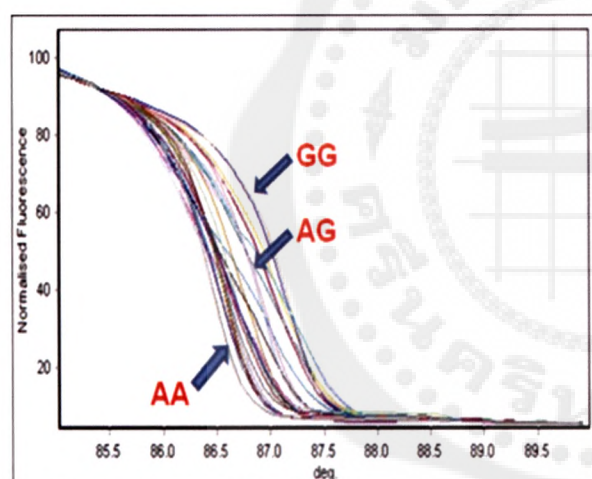
Figure 12 The melting curve of rs3737983 in *ApoE* receptor 2 gene. (A) control no. 1, 2 ,4-7, 9-15, dyslipidemia patient no. 1-6, 10, 12-16, 18, 19, 22, 24, 29, 30, 36 ; (B) control no. 3, 8, 17-23, dyslipidemia patient no. 8; (C) control no. 24-27, 29, 30, 32-37, dyslipidemia patient no. 7, 9, 11, 17, 20, 21, 23, 25-28, 30-35; and (D) control no. 38-53, dyslipidemia patient no. 36-47, 49.



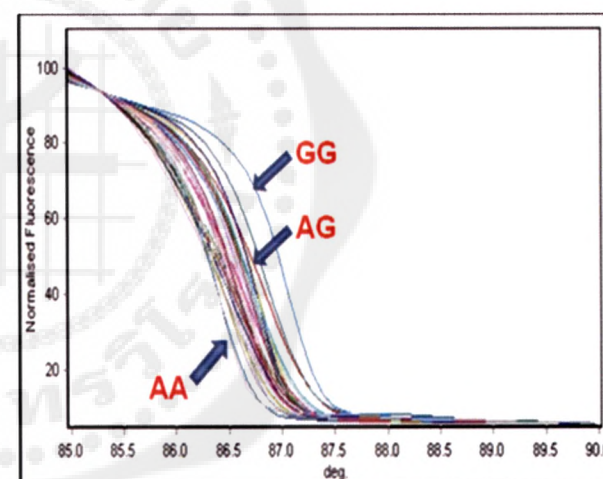
(E)



(F)



(G)



(H)

Figure 12 (continued); (E) control no. 54-69, dyslipidemia patient no. 52-56, 58-67; (F) control no. 70, 76, 79, 89, 111, 112, 168, 182-188, dyslipidemia patient no.46, 48, 50, 51, 72, 80, 98, 99, 112; (G) control no. 71-85, dyslipidemia patient no. 68-81, 83; (H) control no. 86-88, 90-95, 97-101, dyslipidemia patient no. 84-97.

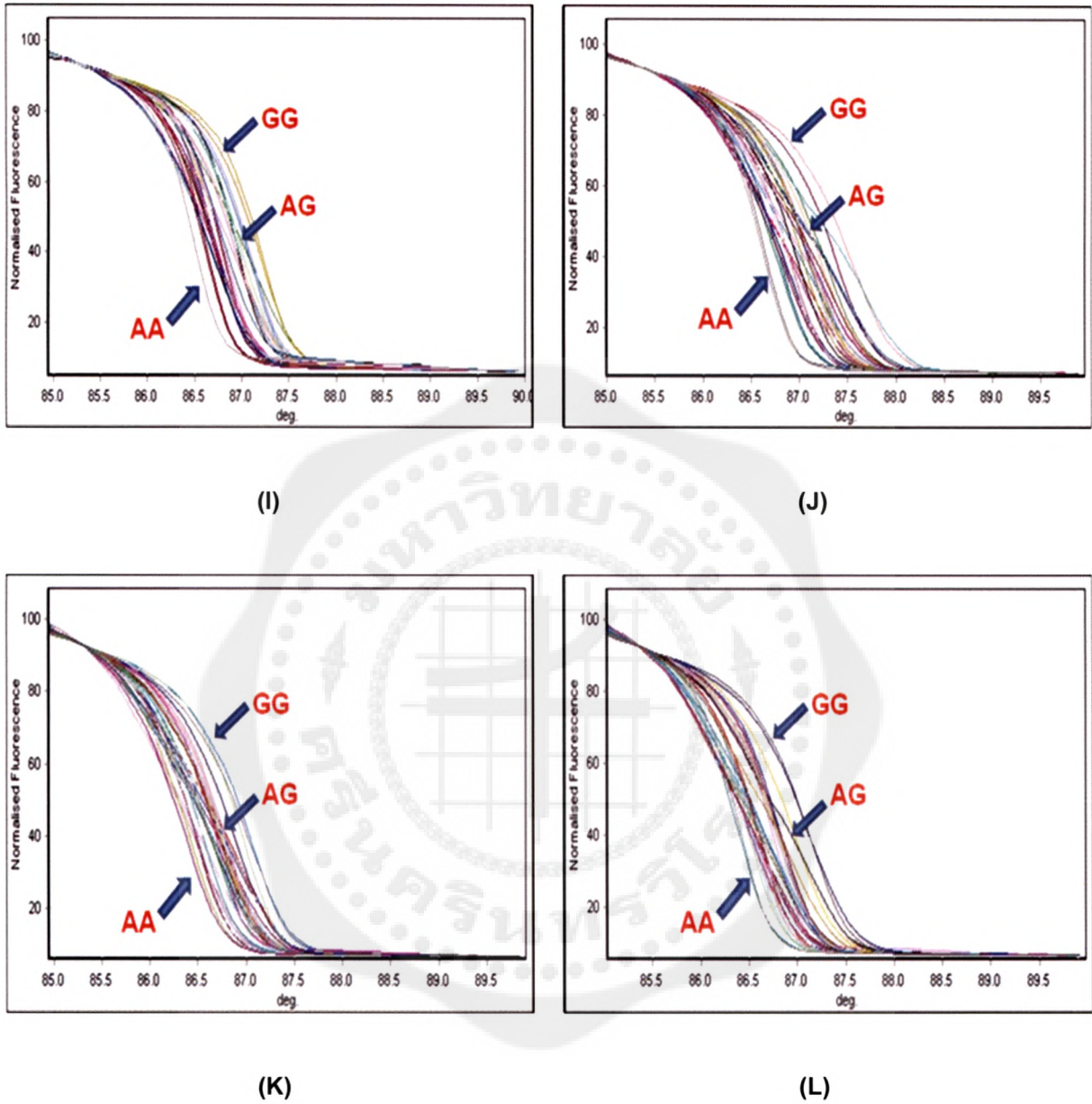


Figure 12 (continued); (I) control 102-116, dyslipidemia patient 100-111, 113; (J), control 118-133, dyslipidemia patient 116-130; (K) control 134-143, 145-149, dyslipidemia patient 132-147; (L) control 150-165, dyslipidemia patient 148-163.

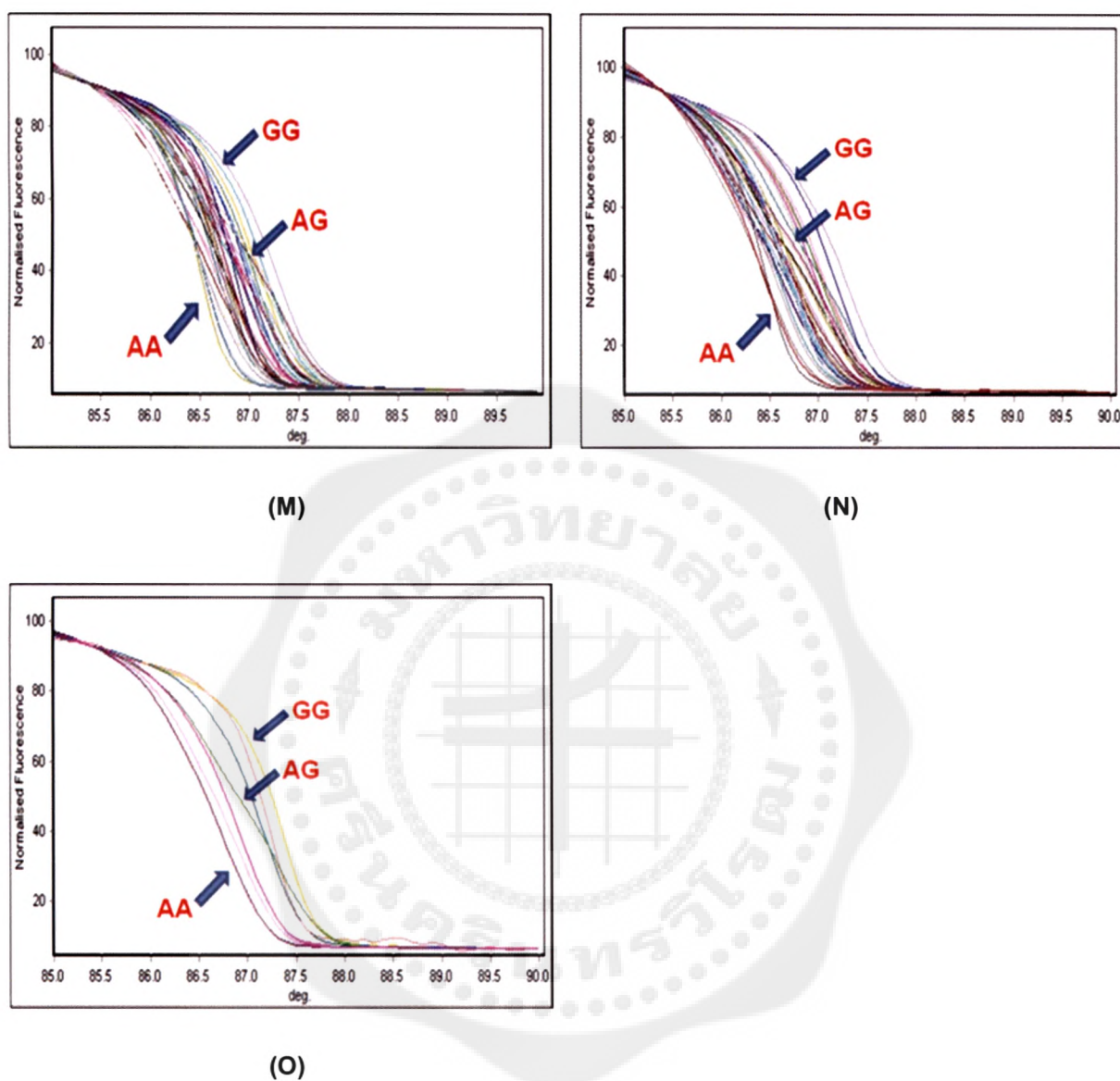


Figure 12 (continued); (M) control 166-181, dyslipidemia patient 164-179; (N) control 189- 200, dyslipidemia patient 180-200; (O) control 28, 96, 156, dyslipidemia patient 57, 181, 196.

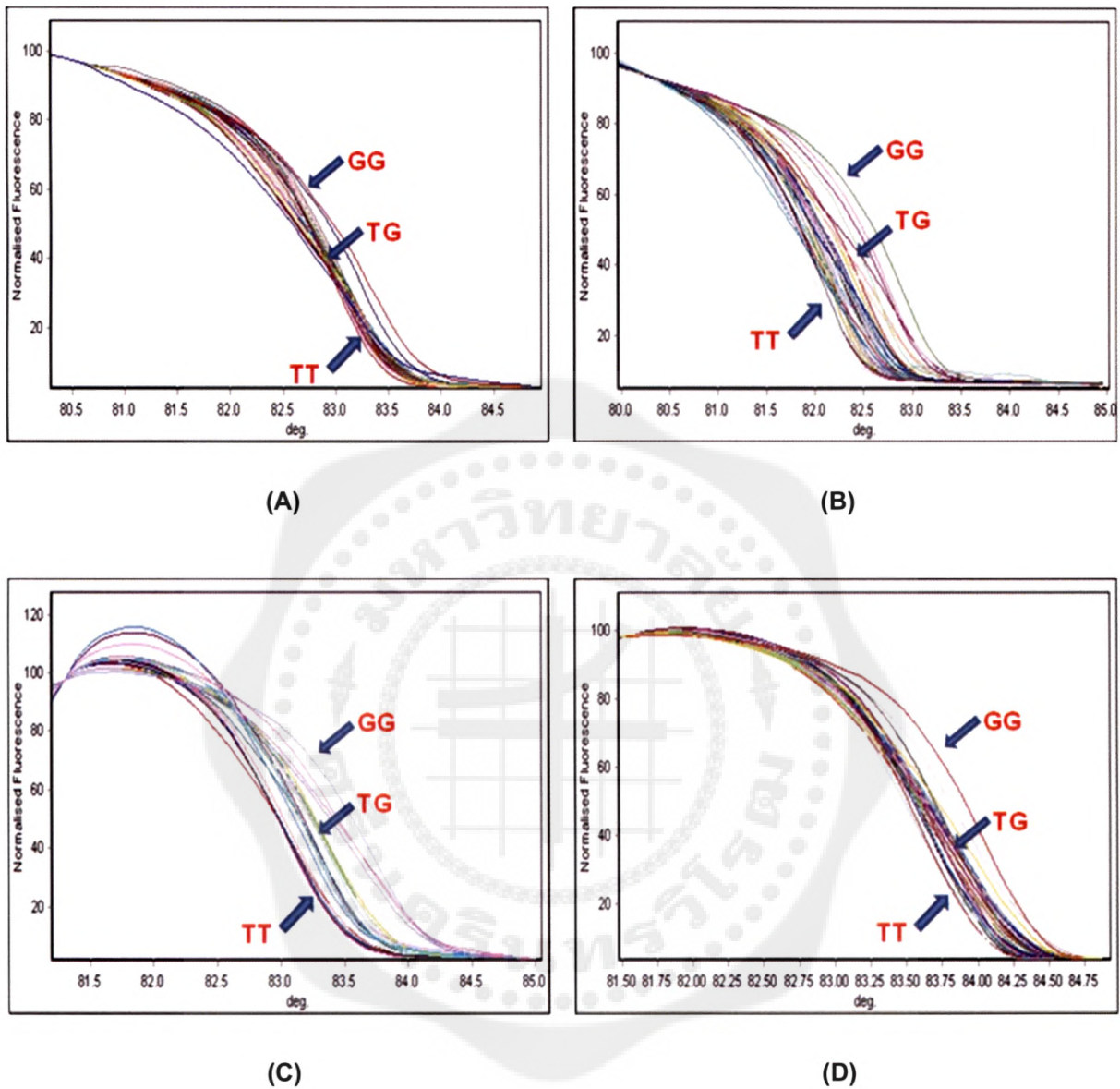


Figure 13 The melting curve of rs2297660 in *ApoE* receptor 2 gene. (A) control no. 1, 2, 4-7, 9-13, 15, 17-21, 83, 91, 123, dyslipidemia patient no. 1, 2, 5-10, 154, 155; (B) control 3, 8, 23, 27, 103, 115, dyslipidemia patient no. 128-153; (C) control 14, 16, 28, 31, 123,134,186, dyslipidemia patient no. 3, 4, 11, 17, 23, 61, 64, 171; (D) control no. 22, 24-26, 29, 30, 33-35, 39, 40, dyslipidemia patient no. 12-16, 18-22, 156-161.

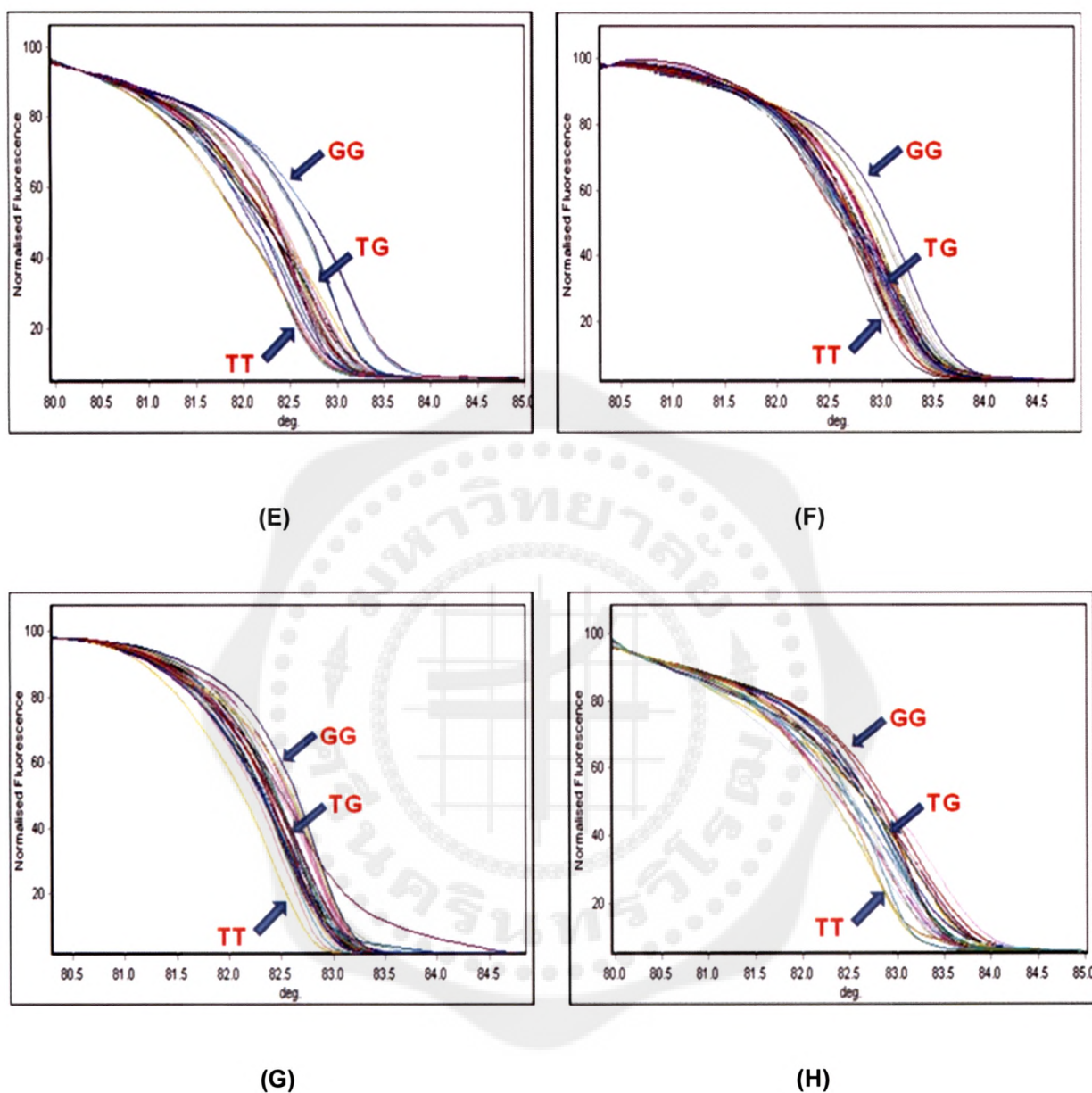


Figure 13 (continued); (E) control no. 32, 36-38, 55, 63, 70, 78, 94, 102, 129, 132, 136, 169, 170, 174, 200, dyslipidemia patient no. 177-183, (F) control no. 41-54, 56-62, 64, 83, 91, dyslipidemia patient no. 24-31, 163; (G) control no. 65-69, 71-77, 79-84, dyslipidemia patient no. 32-38, 164, 165; (H) control no. 78, 144, 145, 156, 158, 192, 198, dyslipidemia patient no. 97, 98, 104, 133, 139, 140, 142, 144, 147, 150, 151, 183.

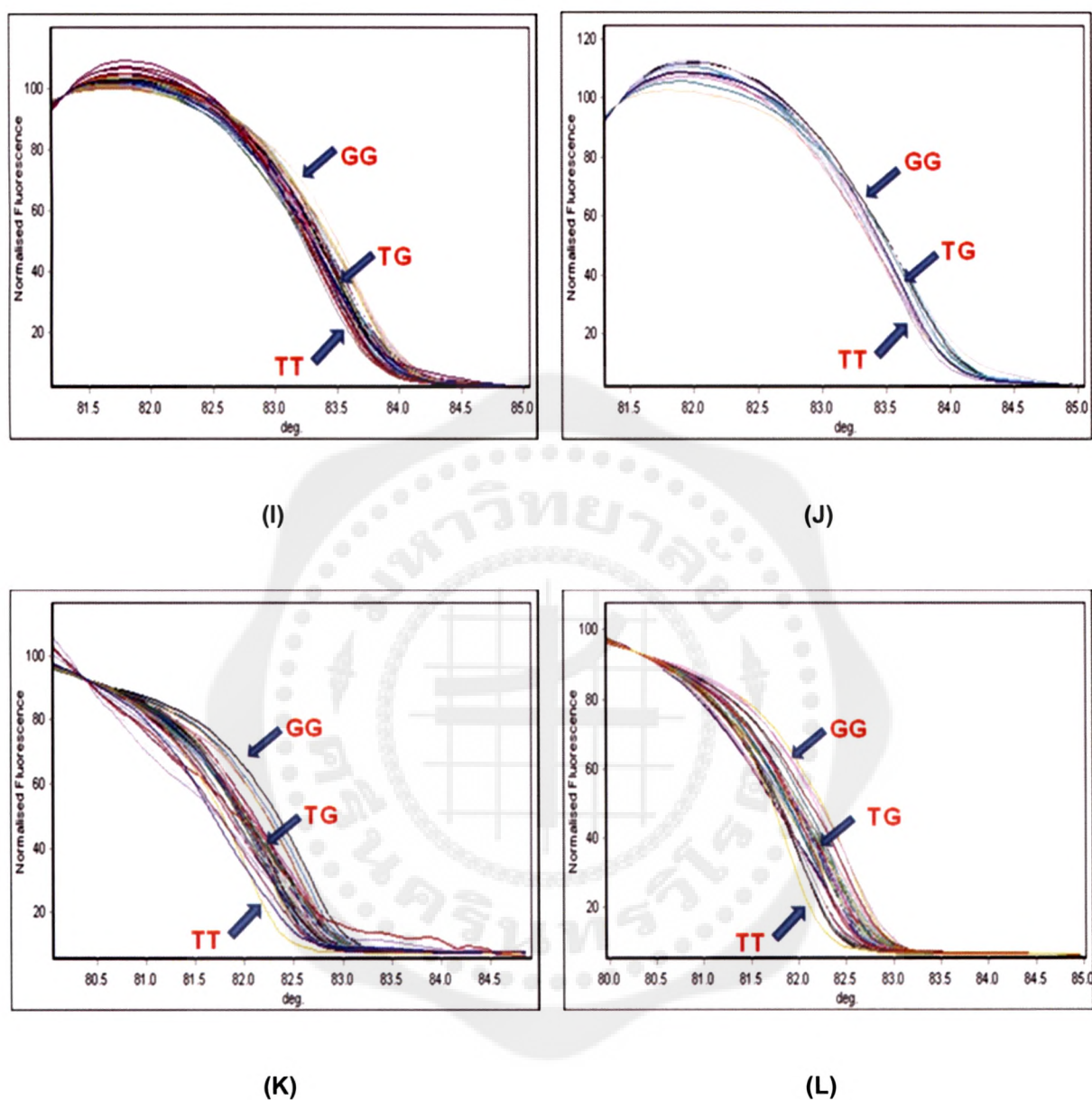


Figure 13 (continued); (I) control no. 83, 86-98, 123-125, 127, 128, 130, 131, 170, dyslipidemia patient no. 40-44, 62, 63, 166; (J) control no. 99-101, 104-114, dyslipidemia patient no.45-48, 50-59, 167-169; (K) control no. 85, 116-120, 133, dyslipidemia patient no. 39, 49, 60; (L) control no. 135-160, dyslipidemia patient no. 65-70.

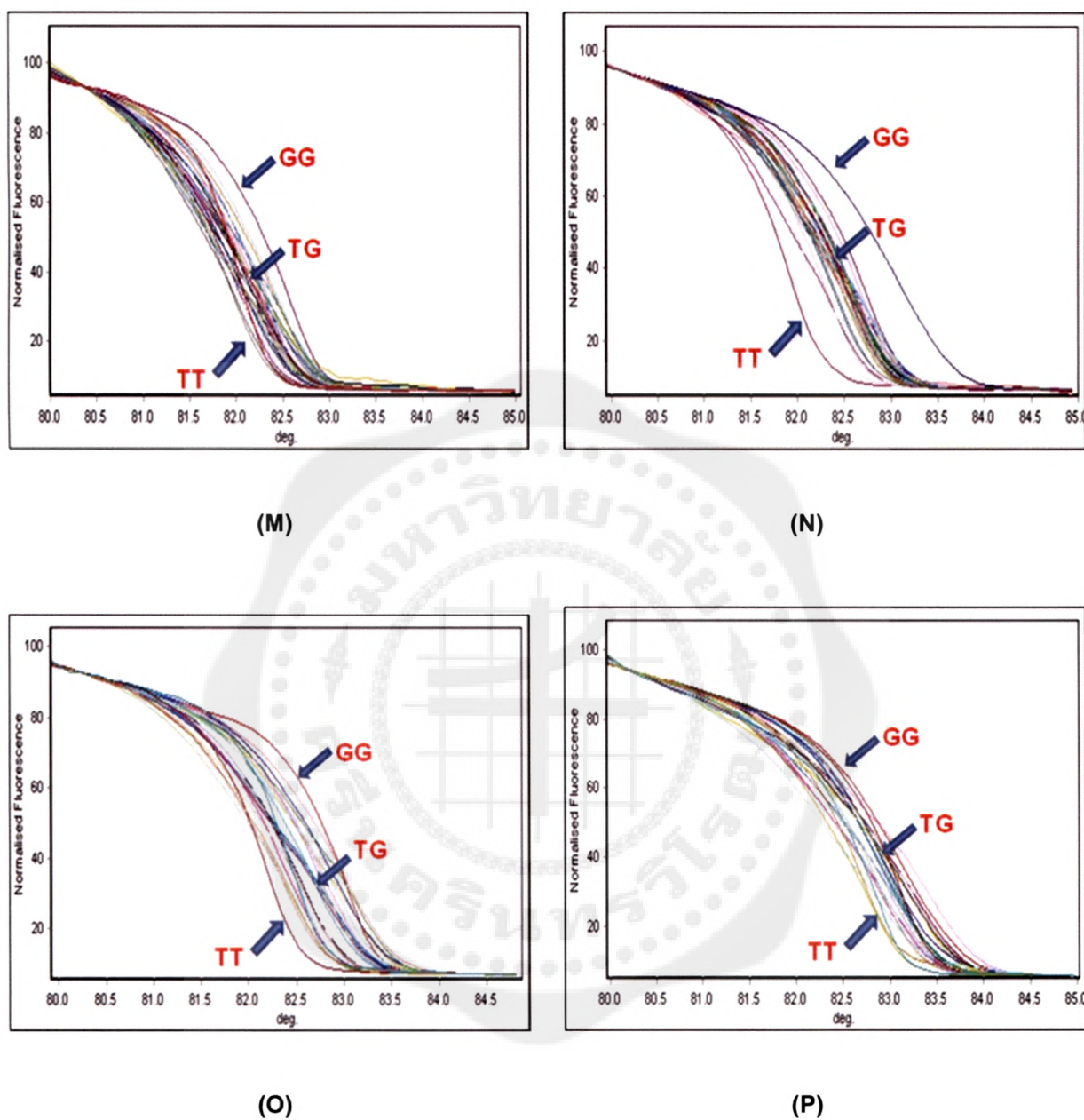


Figure 13 (continued); (M) control no. 161-172, dyslipidemia patient no. 71-90; (N) control no. 173-188, dyslipidemia patient no. 91-96, 99-106; (O) control no. 189-199, dyslipidemia patient no. 107-127; (P) dyslipidemia patient no. 172-176, 183-200.

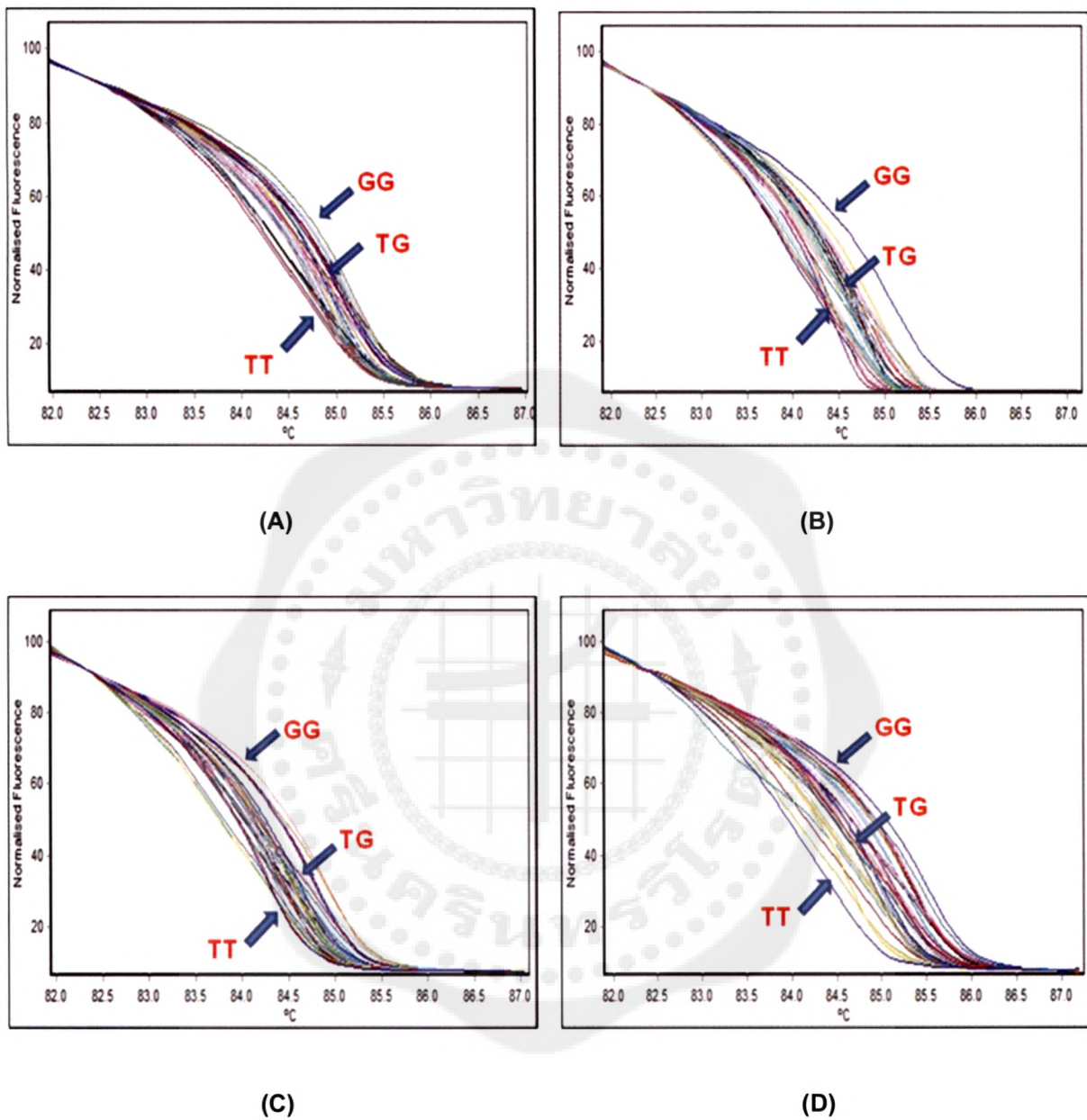


Figure 14 The melting curve of rs3737984 in *ApoE* receptor 2 gene. (A) control no. 1-17, dyslipidemia patient no. 1-18; (B) control no. 18-20, 22-35, dyslipidemia patient no. 19-30; (C) control no. 36-51, dyslipidemia patient no. 36-51; (D) control no. 52-68, dyslipidemia patient no. 52-69.

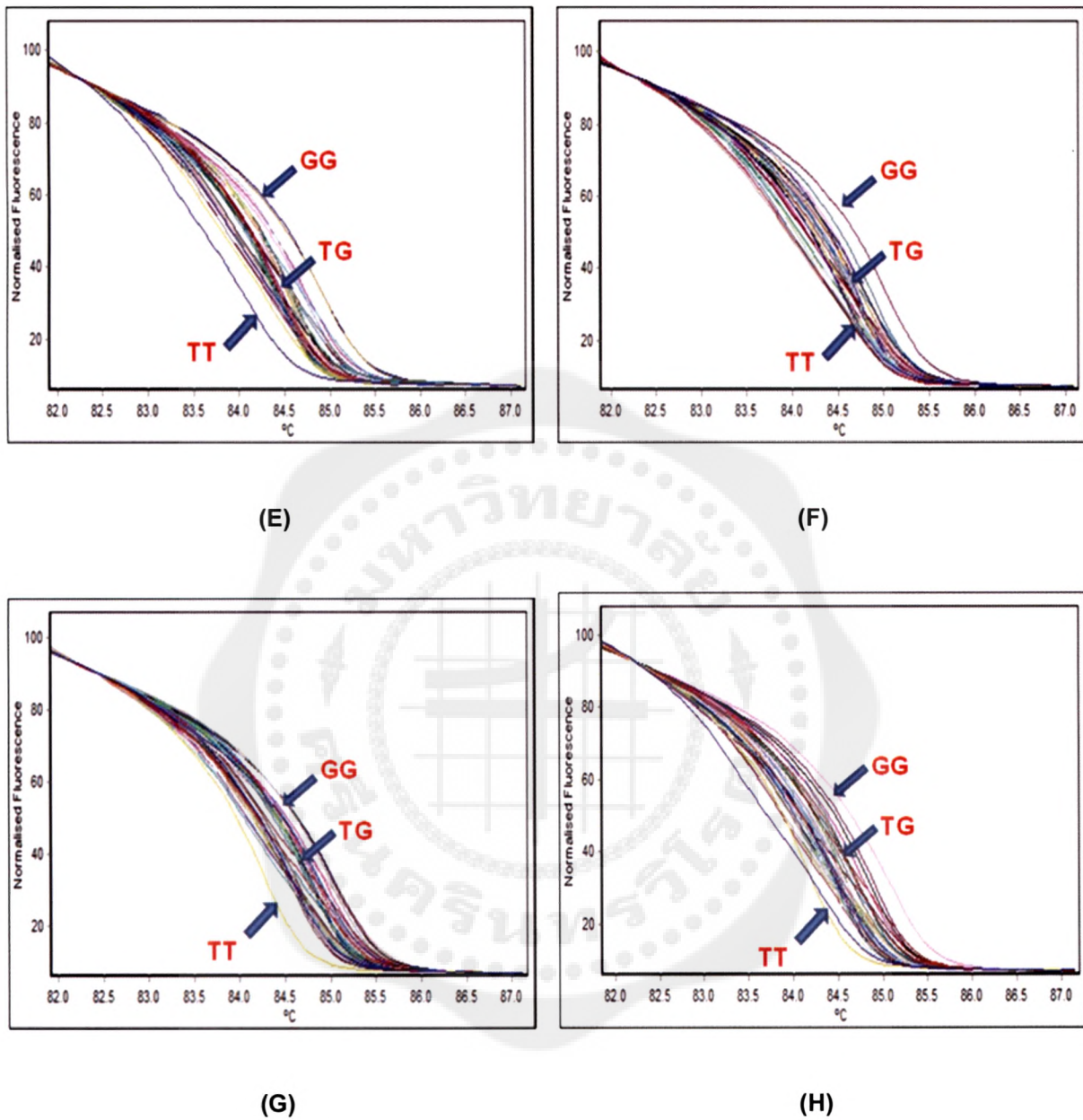


Figure 14 (continued); (E) control no. 69-86, dyslipidemia patient no. 70-86; (F), control no. 88-103, dyslipidemia patient no. 87-104; (G) control no. 104-121, dyslipidemia patient no. 105-121 (H) control 122-138, dyslipidemia patient no. 122-139.

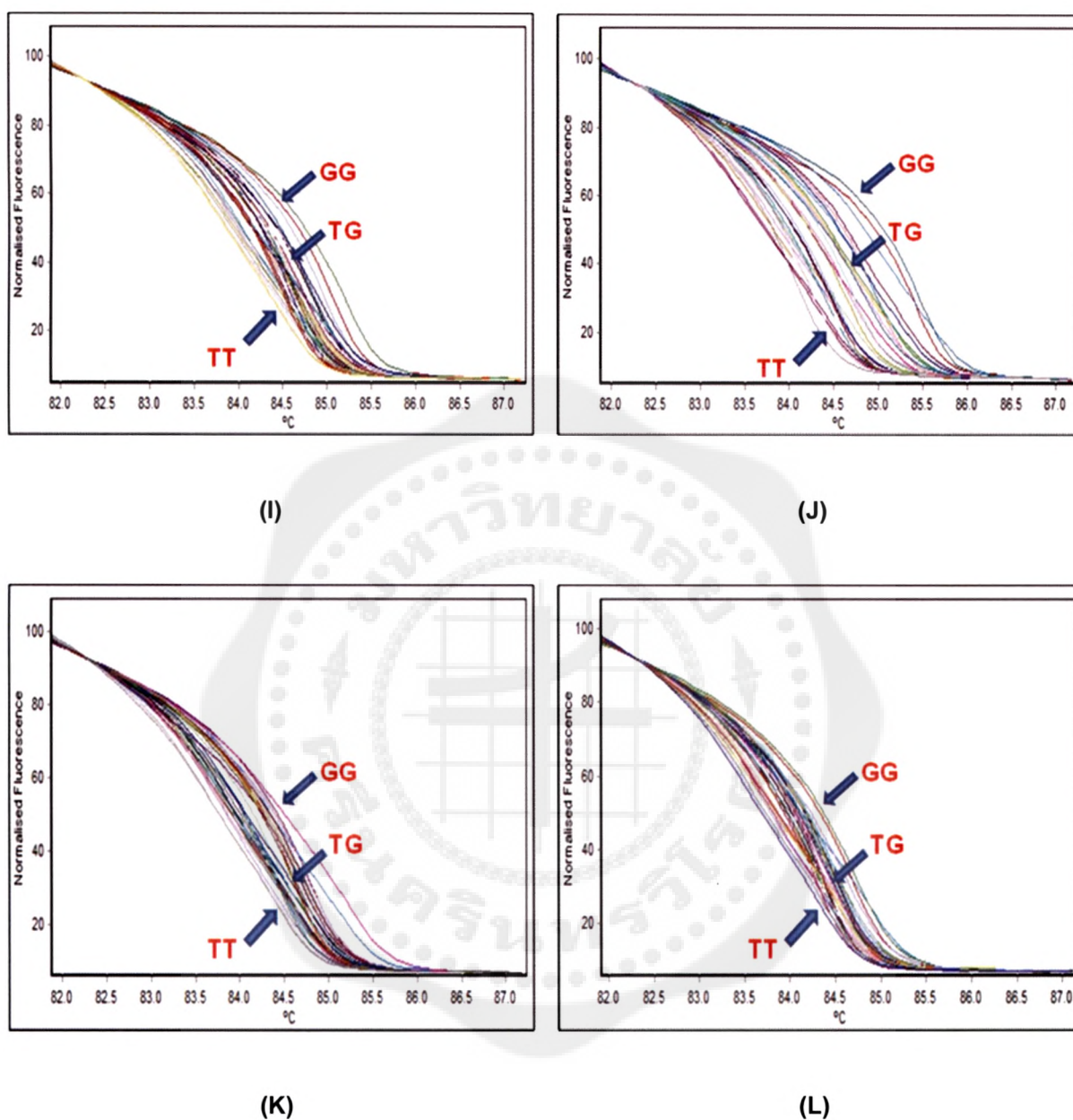


Figure 14 (continued); (I) control no. 139-155, dyslipidemia patient no. 140-155; (J) control no. 157-173, dyslipidemia patient no. 157-169; (K) control no. 174-191, dyslipidemia patient no. 175-188; (L) control no. 7, 21, 67, 87, 143, 144, 167, 192-200, dyslipidemia patient no. 17, 28, 31-35, 54, 56, 192-200.

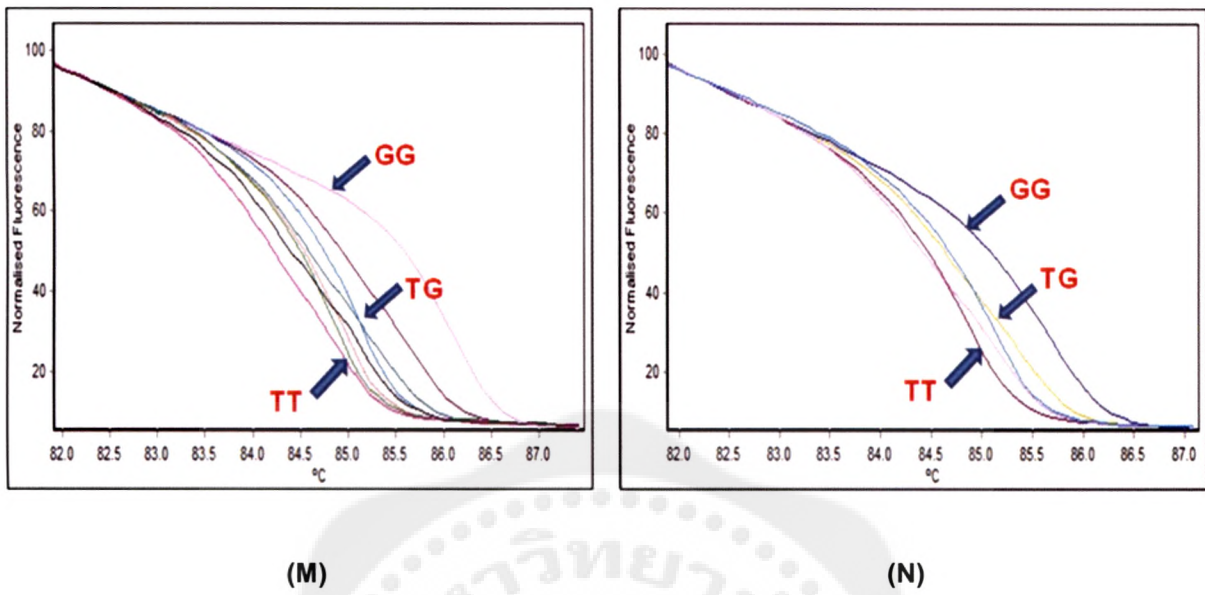


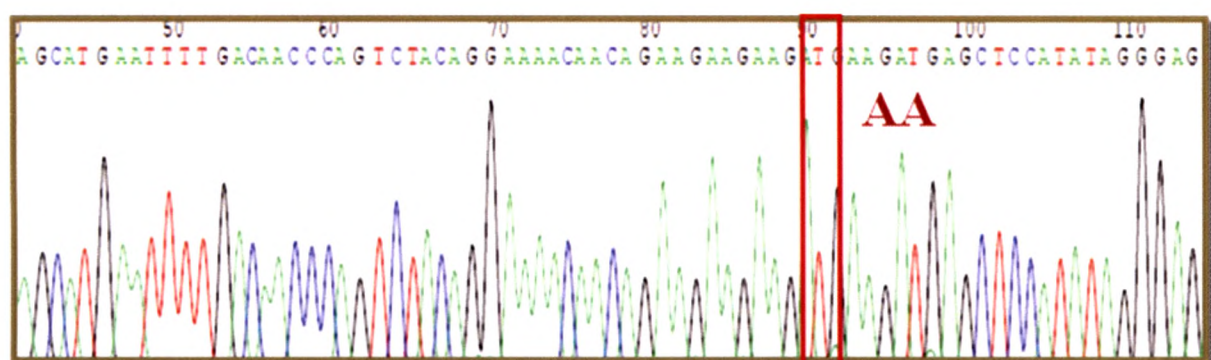
Table 7 Association analysis between polymorphisms and dyslipidemia.

Gene_db SNPs ID		Genotype		P-value ¹	OR (95%CI) ²
ApoER2_rs3737983		AA	AG	GG	
Dyslipidemia patients	71(35.5%)	76(38.0%)	53(26.5%)	0.011	0.47 (0.28-0.78)
Controls	86(43.0%)	85(42.5%)	29(14.5%)		
ApoER2_rs2297660		TT	TG	GG	
Dyslipidemia patients	109 (54.5%)	56 (28.0%)	35 (17.5%)	0.001	2.38 (1.49-3.80)
Controls	54 (27.0%)	79 (39.5%)	67 (33.5%)		
ApoER2_rs3737984		TT	TG	GG	
Dyslipidemia patients	28 (14.0%)	84 (42.0%)	88 (44.0%)	0.001	0.42 (0.28-0.65)
Controls	56 (28.0%)	94 (47.0%)	50 (25.0%)		

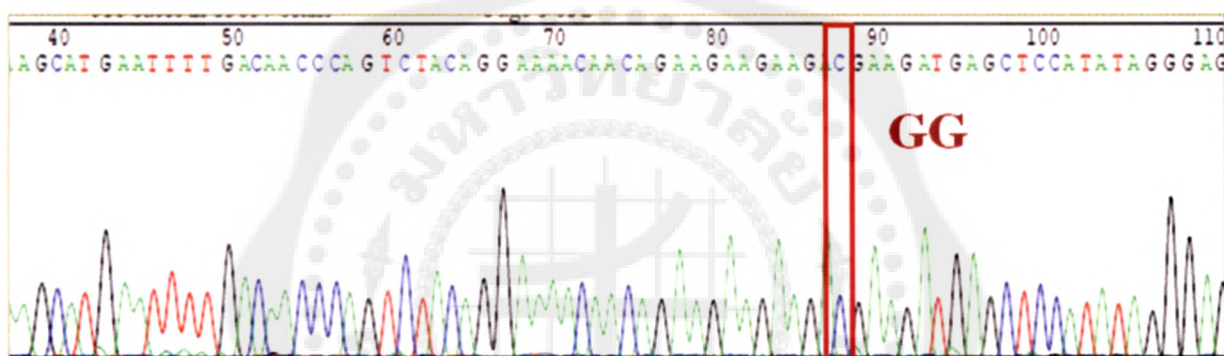
¹P-values of Chi-square test of genotype frequencies²Odds ratio and the 95%CI confidence intervals of allele frequencies

To confirm the genotype from HRM method, the three genotype of each significant SNPs (rs2297660, rs3737983 and rs3737984) in *ApoER2* gene were amplified and then performed direct sequencing. The represent samples were random selected for this method. The sequence results that showed in Figure 15-17 were agree with the HRM method.

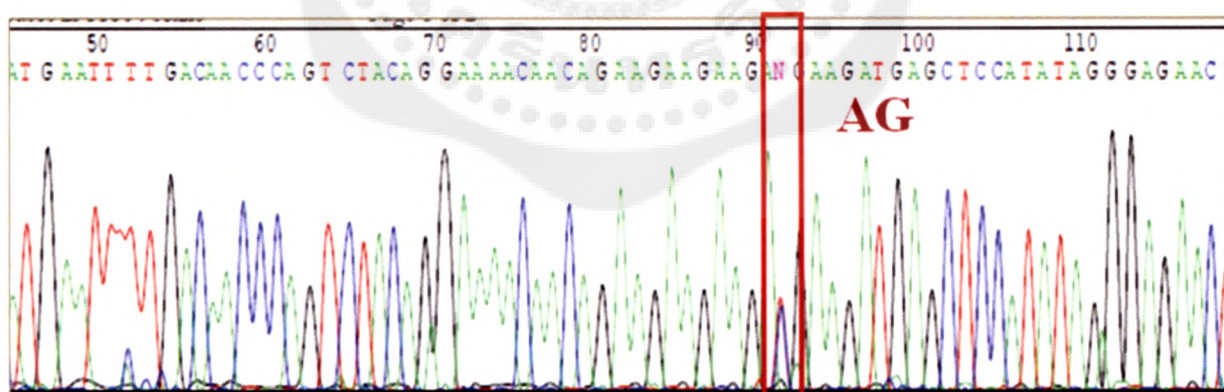




(1)

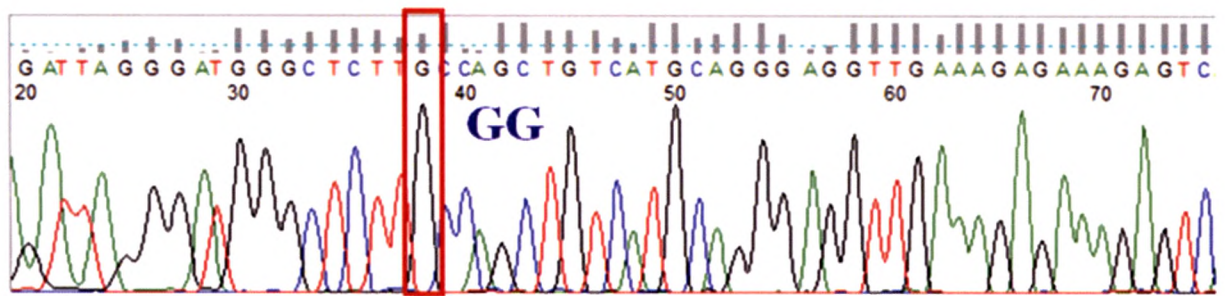


(2)

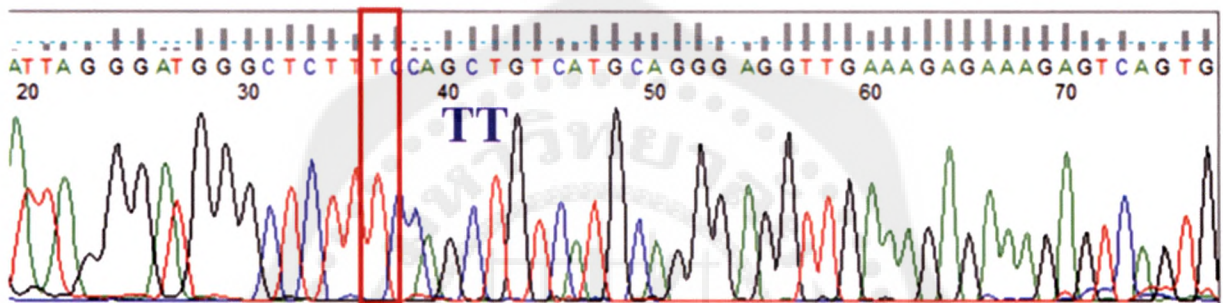


(3)

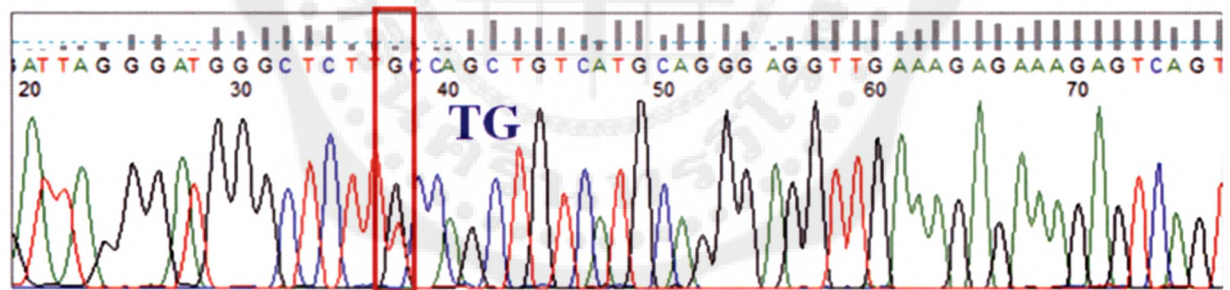
Figure 15 The chromatogram of SNP in *ApoER2* gene; rs3737983 showed homozygous AA (1), homozygous GG (2) and heterozygous AG (3).



(1)



(2)



(3)

Figure 16 The chromatogram of SNP in *ApoER2* gene; rs2297660 showed homozygous GG (1), homozygous TT (2) and heterozygous TG (3).

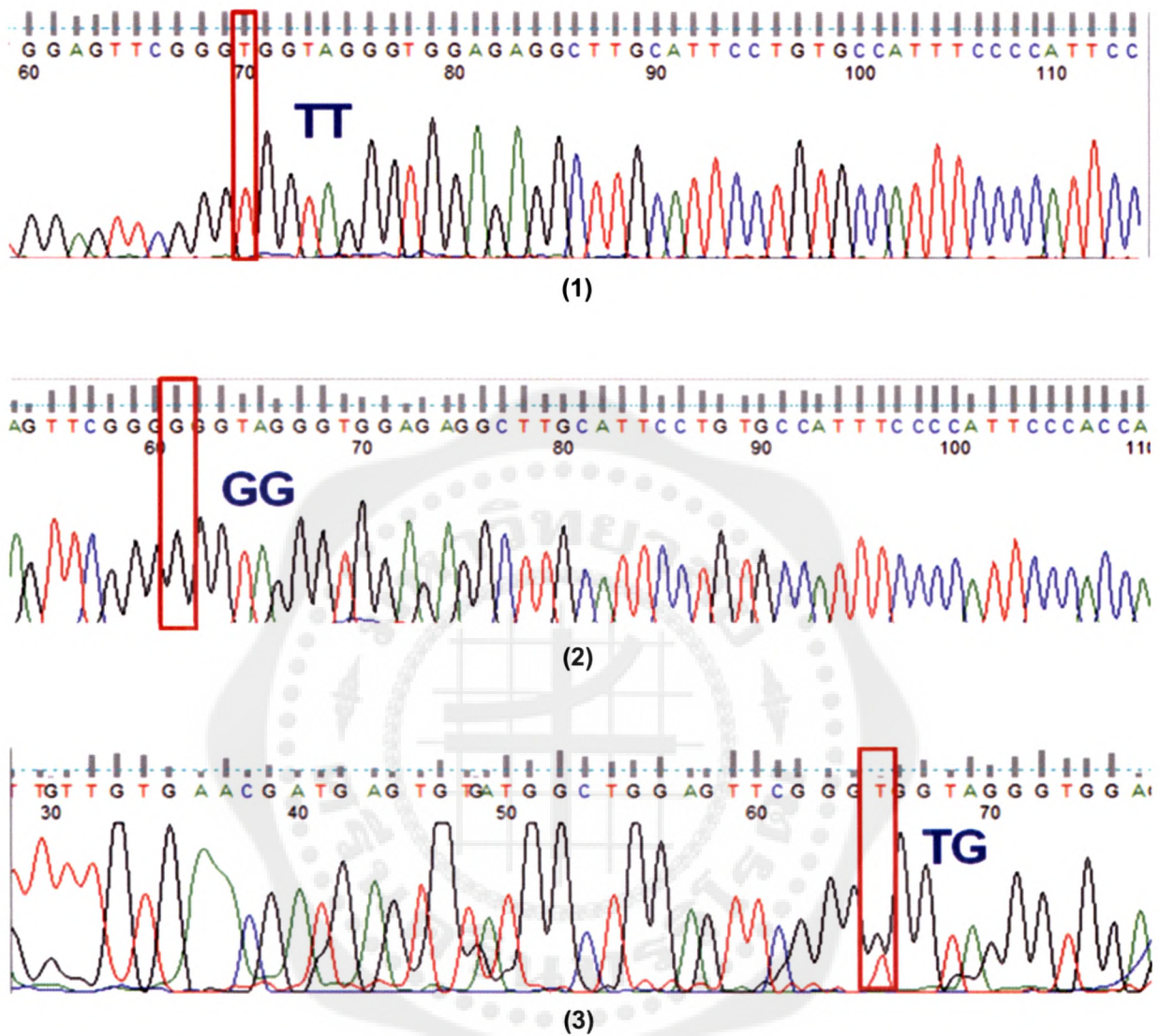


Figure 17 The chromatogram of SNP in ApoER2 gene; rs3737984 showed homozygous TT (1), homozygous GG (2) and heterozygous TG (3).

CHAPTER V

DISCUSSION

Dyslipidemia is mainly characterized by high level of total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C), but low level of high-density lipoprotein cholesterol (HDL-C). It is an important etiological factor for the development of cardiovascular disease (CVD).⁽¹⁰⁷⁾ A variety of genetic studies reported several genes associated with dyslipidemia such as *ApoA1*, *ApoC3*, *ApoA4*, *ApoA5*, *ApoB*, *ApoE*, *LPL*, *LCAT*, *LDLR*, *CETP*, etc.

This study demonstrated that dyslipidemia which characterized by lipid profile including total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels were significantly different from those of control group ($P<0.001$). In previous study showed that high level of TC, TG, LDL-C and HDL-C increase risk for coronary artery disease in most population.⁽¹¹⁷⁾ This may increase the prevalence of dyslipidemia and coronary artery disease in Thai population.

ApoE receptor 2 gene that have minor allelic frequency more than 0.2 and play an important role in the lipid metabolism was examined for its association with dyslipidemia by using HRM analysis. The HRM is a technique, which based on analysis of melting curve when double-strand DNA separate into single-strand DNA during increased temperature. This method have been reported in association study of schizophrenia, obesity and dyslipidemia.⁽¹⁰⁸⁻¹⁰⁹⁾ The results revealed that the SNPs in the gene, *ApoE* receptor 2 gene significant association with dyslipidemia ($P<0.05$). To confirm the result, all significant SNPs of *ApoE* receptor2 gene were genotyping again by direct sequencing and the result was in agreement with HRM. Therefore, HRM is a high throughput with high sensitivity and specificity for SNPs or mutation screening.

A total of three SNPs in the exon and intron region of *ApoE* receptor 2 gene – rs3737983, rs3737984 and rs2297660 were shown to be significantly associated with a risk of dyslipidemia in a Thai population ($P<0.05$). This is the first report for SNPs in *ApoE* receptor 2 gene which associate with dyslipidemia. Among the three SNPs, rs3737984 and rs2297660 showed the strong association ($P=0.001$) with dyslipidemia. But, the rs2297660 which is in coding region of the gene tend to be the strongest SNP that associated with risk of dyslipidemia in the Thai ethnic, because of the low P -value and high odd ratio value. The odds ratio and 95% confidence interval of minor G allele of rs2297660 were 2.38 (1.49-3.80) indicated that the G allele increase risk of

dyslipidemia. This SNP may be used as marker for dyslipidemia in Thai population. The rs3737984 was significantly associated with dyslipidemia ($P=0.001$), in which T allele imparted the decreased risk of dyslipidemia [odds ratio and 95% confidence interval of the T allele, 0.42 (0.28-0.65). Similarly, the G allele in the rs3737983 tend to decreased risk of dyslipidemia [odds ratio and 95% confidence interval of the G allele, 0.47 (0.28-0.78), in contrast to the other SNPs in *ApoE* receptor 2 gene including rs3820198, rs121918399, rs41382345 and rs121918393 showed no significant (data not show).

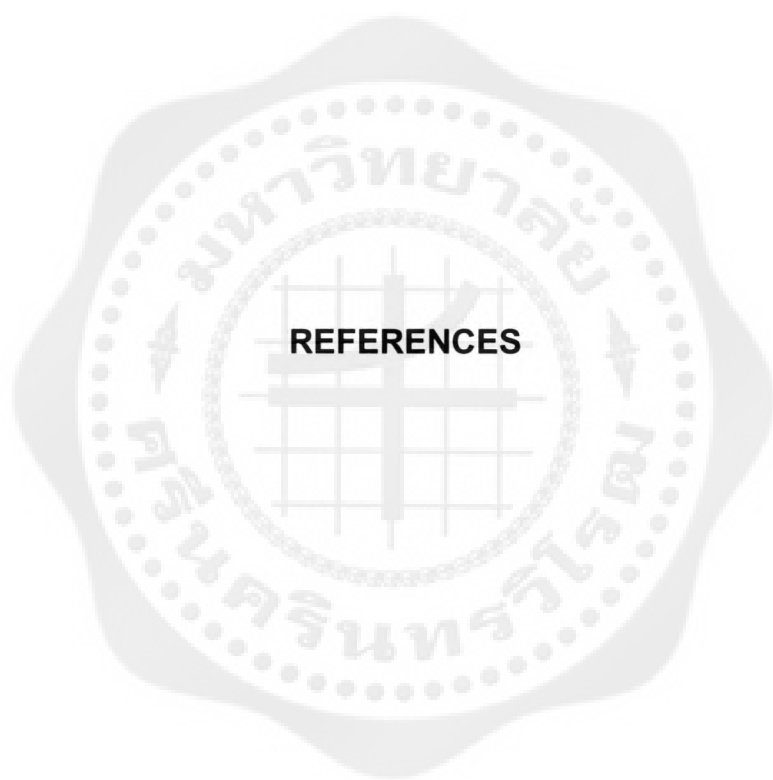
Apolipoprotein E receptor 2 (also called low density lipoprotein receptor-related protein 8 or LRP 8) is a recently identified receptor that resembles low and very low density lipoprotein receptors. Isolation and characterization of genomic clones encoding human apolipoprotein E receptor 2 revealed that the gene spans 60 kb and contains 19 exons. It is located on chromosome 1p34.⁽¹¹⁰⁾ ApoE receptor 2 is a specific receptor for apolipoprotein E (ApoE) and has been demonstrated in the liver. ApoE plays a central role in lipid metabolism, as a consequence of this important role, there is much interest in determining the receptors that the ApoE molecule interacts with lipoprotein receptors.⁽¹¹¹⁾

ApoE receptor 2 is a cell surface receptor that is part of the low density lipoprotein receptor family. These receptors function in signal transduction and endocytosis of specific ligands and can promote internalization of ApoE-containing lipoprotein, suggesting that each could be important in clearance of lipids after damage. But stimulation of these receptors by ligands, including ApoE, also mediates various neuronal signaling mechanisms.⁽¹¹²⁾ The extracellular domain can be internalized by cells and this internalization is also increased by the presence of ligand. The intracellular domains could affect kinase activation or other signaling mechanisms through adaptor protein and this signaling may be strongest for the human ApoE2 isoform.⁽¹¹³⁾

A major function of ApoE is its mediation of the cellular uptake of specific lipoproteins through an interaction with ApoB, E (LDL) receptors on extrahepatic and hepatic cell surfaces and with distinct hepatic ApoE receptors.⁽¹¹⁴⁾ In human ApoE has a key role in lipid transport both in the plasma and in the central nervous system. Its three common structural isoforms differentially affect the risk of developing atherosclerosis and neurodegenerative disorder, including Alzheimer's disease.⁽¹¹⁵⁾ The failure of the ApoE receptor 2 will affect the functions of ApoE in transportation lipid and cause diseases. Because it is a major risk factor for this disease, therefore, in the future it may be used as biomarker for atherosclerosis or Alzheimer's disease.

In conclusion, this study demonstrated that three SNP in exon and intron regions of *ApoE* receptor 2 gene, rs3737983, rs3737984 and rs2297660, significantly associated with dyslipidemia whereas the rs2297660 showed the strongest association with risk of dyslipidemia in the Thai population. Further study is required for clarification the role of genetic variation in expression pattern of the *ApoE* receptor 2 gene which may be involved in dyslipidemia.





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APPENDIX

Materials

Absolute ethanol	Merck
Agrose	Takara
Buffer FG1 (lysis buffer)	Qiagen
Buffer FG2 (denaturation buffer)	Qiagen
Buffer FG3 (hydration buffer)	Qiagen
Deoxynucleotide trophosphates (dNTPs)	Takara
100 bp DNA ladder	BioRad
Ethidium bromide	Sigma
Ethylenediamine tetraacetic acid (EDTA)	QRec
Syto [®] 9 green fluorescent	Invitrogen
6 x Loading dye	BioRad
10 x PCR buffer	Takara
Taq DNA polymerase	Takara
Protease	Qiagen
MgCl ₂	Takara
Quantimix easy probes	Biotools
Boric acid	QRec
Tris-HCl	Vivants

Reagents

0.5x TBE buffer, pH 8.0

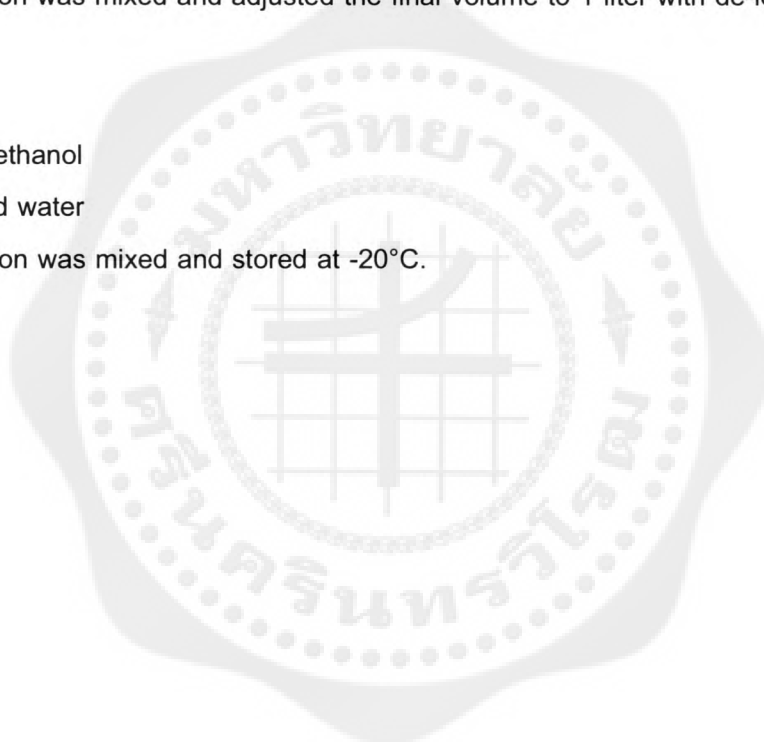
Tris-HCl	5.4	g
Boric acid	2.73	g
EDTA	0.47	g
dH ₂ O	1,000	ml

The solution was mixed and adjusted the final volume to 1 liter with de-ionized water.

70% Ethanol

Absolute ethanol	70	ml
De-ionized water	30	ml

The solution was mixed and stored at -20°C.





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