SINGLE NUCLEOTIDE POLYMORPHISMS OF MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN (*MTTP*) AND APOLIPOPROTEIN B (*APOB*) GENES IN OBESE THAI CHILDREN

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ABSTRACT

Childhood obesity has become the public health problem worldwide. It is an important risk factor of many chronic diseases. Dyslipidemia, which is a risk factor of cardiovascular disease, is a serious complication of childhood obesity. Dyslipidemia results from disorder of lipoprotein metabolism. Microsomal triglyceride transfer protein (*MTTP*) and apolipoprotein B (*APOB*) genes have important roles in lipoprotein metabolism. The aim of this study was to study the association between single nucleotide polymorphism (SNP) of *MTTP* and *APOB* genes and plasma low-density lipoprotein cholesterol (LDL-C) level in obese Thai children. Subjects were children aged between 4 to18 years. Their BMI for sex and age were above the 95th percentile. Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and amplification-refractory mutation system (ARMS) were used for gene analysis. This study found that the presence of 891C/G SNP in *MTTP* gene was significantly associated with elevated plasma LDL-C level but it found no significant association of *APOB* SNP with elevated plasma LDL-C level in obese children. The *MTTP* and *APOB* SNPs had no effect on abnormal LDL-C level.

KEY WORDS: OBESITY/ CHILDREN/ DYSLIPIDEMIA/ MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN (MTTP)/ APOLIPOPROTEIN B (APOB)

83 pp.

Single nucleotide polymorphism ของยีน microsomal triglyceride transfer protein (*MTTP*) และ apolipoproteinB (*APOB*) ในเด็กอ้วน

(SINGLE NUCLEOTIDE POLYMORPHISMS OF MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN (*MTTP*) AND APOLIPOPROTEIN B (*APOB*) GENES IN OBESE THAI CHILDREN)

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บทคัดย่อ

โรคอ้วนในเด็กมีอุบัติการณ์เพิ่มสูงขึ้น และได้กลายเป็นปัญหาสาธารณสุขระดับโลก โรคอ้วนเป็นปัจจัยสำคัญของโรคเรื้อรังต่างๆมากมาย ปัญหาแทรกซ้อนที่สำคัญอย่างหนึ่งของโรคอ้วนในเด็กคือความผิดปกติของระดับไขมันในเลือดซึ่งเกิดจากความผิดปกติของ lipoprotein metabolism และเป็นปัจจัยเสี่ยงที่ก่อให้เกิดโรคหัวใจและหลอดเลือด ยีนที่ควบคุมการสร้าง microsomal triglyceride transfer protein (*MTTP*) และ apolipoprotein B (*APOB*) มีบทบาทสำคัญในกระบวนการ lipoprotein metabolism วัตถุประสงค์หลักของการศึกษานี้คือศึกษาความสัมพันธ์ของ single nucleotide polymorphism (SNP) ของยีน *MTTP* และยีน *APOB* ต่อระดับ low-density lipoprotein cholesterol (LDL-C) ในเด็กอ้วน ทำการศึกษาในเด็กอายุระหว่าง 4 ถึง 18 ปี ซึ่งมี BMI มากกว่า เปอร์เซ็นไทล์ที่ 95 เมื่อเทียบกับเกณฑ์อ้างอิงตามอายุและเพศของเด็กคนนั้น การวิเคราะห์ทางพันธุกรรมใช้วิธี polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) และ amplification-refractory mutation system (ARMS) ผลการศึกษาพบว่า 891C/G SNP ของยีน *MTTP* มีความสัมพันธ์กับระดับของ LDL-C ในเด็กอ้วนอย่างมีนัยสำคัญทางสถิติ แต่ SNP ของ ยีน *APOB* ไม่มีผลต่อระดับของ LDL-C และพบว่าความผิดปกติของระดับ LDL-C ไม่มีความสัมพันธ์กับ SNP ของยีน *MTTP* และยีน *APOB*

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LIST OF ABBREVIATIONS

A	= adenine
AAP	= American Academy of Pediatrics
ABCA1	= ATP-binding cassette transporter
ABL	= abetalipoproteinemia
ANOVA	= analysis of variance
apoA-I	= apolipoprotein AI
apoB	= apolipopotein B
APOB	= apolipopotein B gene
apoB-48	= apolipopotein B-48
apoB-100	= apolipopotein B-100
apoC-I	= apolipoprotein CI
apoC-II	= apolipoprotein CII
apoC-III	= apolipoprotein CIII
apoE	= apolipoprotein E
ARMS	= amplification-refractory mutation system
bp	= base pairs
BMI	= body mass index
C	= cytosine
^{o}C	= degree Celcius
CDC	= Centers for Disease Control and Prevention
CETP	= cholesteryl ester transfer protein
Chol	= cholesterol
CM	= chylomicron
DM	= diabetes mellitus
DNA	= deoxyribonucleic acid
dNTPs	= deoxyribonucleotide triphosphate
EDTA	= ethylenediamine tetraacetic acid

LIST OF ABBREVIATIONS (CONT.)

G	= guanine
HDL-C	= high-density lipoprotein cholesterol
HT	= hypertension
IDL	= intermediate-density lipoproteins
IL-6	= interleukin-6
LDL-C	= low-density lipoprotein cholesterol
LDLR	= low-density lipoprotein receptor
LPL	= lipoprotein lipase
$MgCl_2$	= magnesium chloride
mg/dl	= milligram per deciliter
MTTP	= microsomal triglyceride transfer protein
MTTP	= microsomal triglyceride transfer protein gene
N	= number
NEFA	= nonesterified fatty acid
NH_4	= ammonium
NA	= not available
PCR	= polymerase chain reaction
PL	= phospholipid
RFLP	= restriction fragment length polymorphism
SNPs	= single nucleotide polymorphisms
T	= thymine
TRLs	= triglyceride-rich lipoproteins
VLDL	= very-low-density lipoprotein
WHO	= World Health Organization
WHR	= waist-hip ratio
wk	= week

CHAPTER I
INTRODUCTION

Background and significance of the problem

Childhood obesity is a public health problem worldwide. Its prevalence is increasing in Thailand and many countries at an alarming rate. The fundamental cause of obesity is an energy imbalance due to excessive food intake and decreased physical activity, both of which are significantly influenced by environmental factors and lifestyle changes.[1-4] Genetic factors also play important roles in energy regulation and nutrient metabolism. Obesity is one of the major risk factors of many chronic diseases such as type 2 diabetes, hypertension, dyslipidemia, cardiovascular disease, musculoskeletal problems, cancer, and psychological problem.[1, 3] Obese children also have increased risk to become obese adults.[5] Therefore, obese children suffer from both short-term and long-term complications.

Dyslipidemia is caused by disorders of lipoprotein metabolism,[6] which result from genetic factors, diseases or drugs.[7] The other cause includes environmental factors, namely excess saturated fat diet and sedentary lift style. Dyslipidemia affects cardiovascular system because fat accumulation in the arterial wall leads to narrow artery which interrupts blood flow resulting in cardiovascular disease, coronary artery disease, cerebrovascular disease, and stroke. Increased low-density lipoprotein cholesterol (LDL-C) is one of the main risk factors of cardiovascular disease (CVD). Low-density lipoprotein (LDL) transports cholesterol from the liver to peripheral tissues. The interaction between LDL and the LDL receptor is essential for the regulation of plasma cholesterol.[8]

Obesity affects lipoprotein metabolism, leading to dyslipidemia, i.e. increased triglyceride, LDL-C and decreased HDL-C.[9, 10] In addition, dyslipidemia in obesity may result from SNPs of some genes that influence lipoprotein metabolism such as genes which code for microsomal triglyceride transfer protein (MTTP), apolipoprotein B (apoB), and low density lipoprotein receptor (LDLR).

Microsomal triglyceride transfer protein and apolipoprotein B are lipid transfer protein and lipid carrier, respectively. MTTP is important for the assembly and secretion of apoB-containing lipoproteins.[11] The polymorphism or mutation in *MTTP* gene causes abnormal function of apoB leading to diseases such as abetalipoproteinemia.[12] *APOB* gene polymorphism or mutation is a cause of abnormal lipid profile.[13, 14] The recent study found that apoB concentrations had effect on small, dense LDL particles in plasma.[15]

Previous studies suggested that polymorphisms in *MTTP* and *APOB* genes may lead to hyperlipidemia in obese children.[16, 17] In Thailand, there has been no study regarding the effect of single nucleotide polymorphisms (SNPs) in *MTTP* and *APOB* genes on obesity and dyslipidemia. Therefore, this study was undertaken to assess the influence of *MTTP* and *APOB* SNPs on the occurrence of dyslipidemia in Thai obese children.

Research objectives

Primary objective

To assess association between single nucleotide polymorphisms (SNPs) of *MTTP* and *APOB* genes and plasma LDL-C level in obese children and adolescents, by comparing mean LDL-C between genotypes of polymorphisms.

Secondary objective

To assess association between single nucleotide polymorphisms (SNPs) of *MTTP* and *APOB* genes and abnormal LDL-C

Expected benefits and application

We postulated that (1) SNP frequencies of *MTTP* and *APOB* genes in obese pediatric patients are not different from those of the general population and (2) obese children with elevated LDL-C level have higher prevalence of SNPs than those without dyslipidemia.

The results from this study should provide data for future research concerning genetic influence and management of dyslipidemia in childhood obesity.

CHAPTER II
LITERATURE REVIEW

2.1 Obesity

2.1.1 Definition and classification

Obesity is defined as excessive fat accumulation in the body. Body mass index (BMI) is a simple criteria to identify and classify overweight and obesity in adults.[18] BMI is calculated as a person's weight (in kilograms) divided by the square of his or her height (in meters) as following.

BMI = Weight (kg)/ Height^2 (m^2)

The cut-off point of BMI in Asian population is proposed to be lower than those of the WHO (Table 2.1). The waist-hip ratio (WHR), another method to classify obesity in adults, is the ratio of the circumference of the waist to the hip. It is calculated by measuring the waist circumference, above the upper hip bone, and dividing by the hip circumference, the widest part of hip (waist circumference /hip circumference). High waist-hip ratio (WHR>1 in men and WHR>0.85 in women) can indicate abdominal fat accumulation[19] which is related to cardiovascular disease and chronic diseases.[20]

Table 2.1 Comparison of the WHO's and the Asian BMI cut-off points[18]

Classification	BMI cut-off point (kg/m^2)	
	WHO	Asian
Underweight	<18.5	<18.5
Normal	18.5-24.9	18.5-22.9
Overweight	25-29.9	23-24.9
Class I obesity	30-34.9	25-29.9
Class II obesity	35-39.9	≥30
Class III obesity	≥40	

In children, classification of obesity is based on BMI reference for age and sex. The 85th percentile is a cut-off point of overweight and the 95th percentile is a cut-off point of obesity[21] (Table 2.2). The WHO 2007 reference BMI charts are shown in Figure 1 and Figure 2.[22]

Table 2.2 Classification of childhood obesity

Classification	BMI(kg/m^2)	
	WHO	CDC and AAP[23]
Underweight	<-2SD	<5th percentile
Normal		5th-85th
At risk overweight		85th -95th
Overweight	>+1SD	>95th
Obesity	>+2SD	

AAP, the American Academy of Pediatrics; CDC, the Centers for Disease Control and Prevention; SD, standard deviation; WHO, the World Health Organization

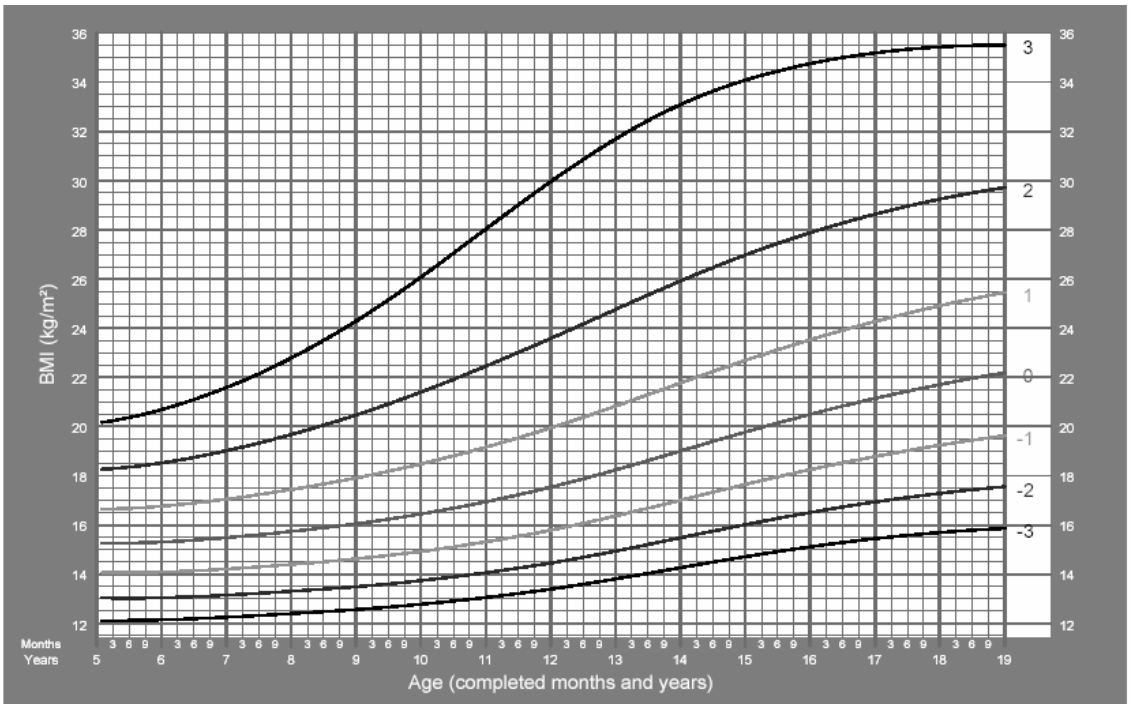

Figure 2.1 The WHO 2007 reference chart of BMI for age in boys.[22]

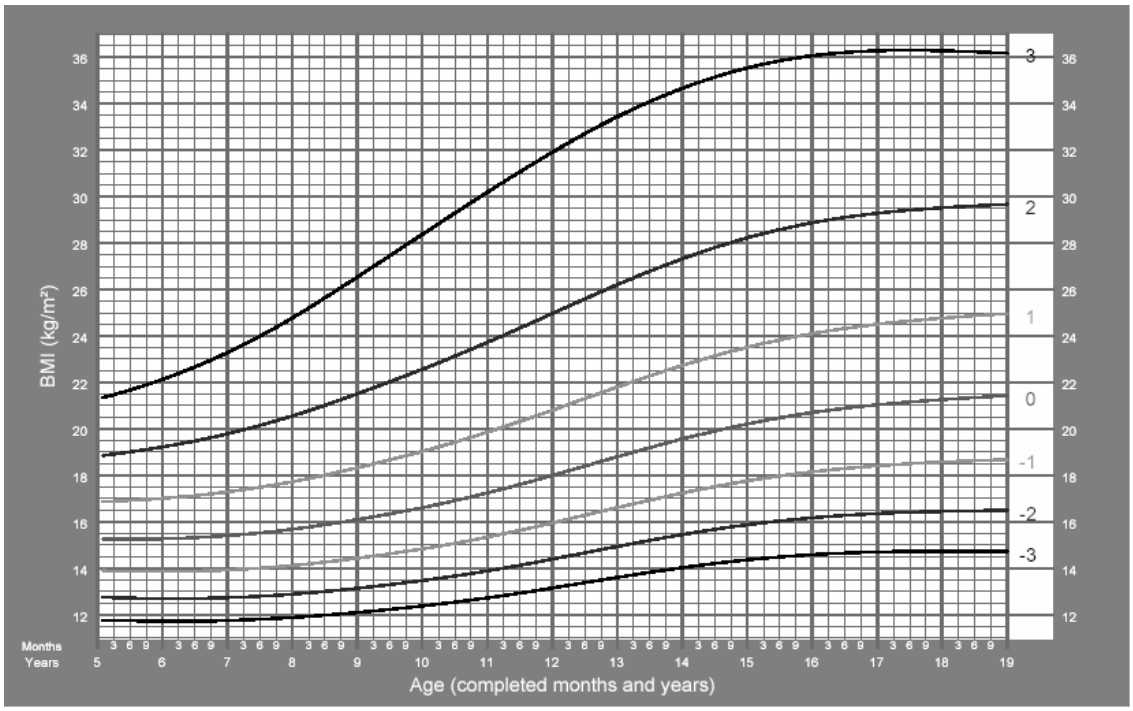

Figure 2.2 The WHO 2007 reference chart of BMI for age in girls.[22]

The severity of childhood obesity is determined by the percentage of ideal body weight for height (Table 2.3).

Table 2.3 Severity of childhood obesity

Severity of obesity	Percent ideal body weight for height
Mild	120-139%
Moderate	140-159%
Severe	160-199%
Morbid	≥200%

2.1.2 Causes of obesity

Obesity results from genetic, environmental, and behavioral or lifestyle factors, which are involved with energy imbalance.[24, 25] Excess dietary intake, particularly of unhealthy foods and drinks such as fast food, junk food, solf drink, and high calorie diet, leads to childhood obesity.[1, 26] Physical activity and exercise are main components of energy output factor. Lacking physical activity and exercise also causes childhood obesity.[1, 27] Lifestyle change is another primary cause of obesity.

Twenty years ago, portion sizes and energy intake of diet were smaller than at present.[28] Nowadays, children have less physical activity than in the past. For example, they use cars and elevators rather than walking or climbing stairs and spend time sitting in front of television rather than playing outside.[29] The other cause is genetics. For example, morbid obesity results from mutations in human genes coding for leptin (*LEP*), leptin receptor (*LEPR*), proopiomelanocortin (*POMC*), and melanocortin-4 receptor (*MC4R*).[30-32] In the Quebec Family Study, analysis of the markers in the α2-, ß2-, and ß3-adrenergic receptor genes (*ADR*s) found that *ADR* genes contribute to body fat and plasma lipid variability in abdominal obesity.[33] In Denmark, the study in twins regarding hereditary influence found that twin analysis indicates a heritability of fat mass.[34] Adoption study found that adopted children did not have BMI and fat mass that correlated to their adoptive parents.[35] From past studies, findings have suggested that both environmental and genetic factors play important roles in the origin and consequences of childhood obesity.[36]

2.1.3 Obesity and other diseases

Hypertension: Collection of fat in the blood vessel wall is the major cause of hypertension because the heart must pump blood through narrow arteries with increased resistance. The recent study suggests that an obese or overweight person is more hypertensive than normal person.[37] The study of obese Irish children found that subjects who had BMI above the 95th percentile for age had blood pressure measurements in the hypertensive range. In boys, correlation was found between degree of obesity and systolic blood pressure.[38]

Type 2 diabetes mellitus: Obesity is the major cause of type 2 diabetes that results from the failure of respond to insulin or insulin resistance. The increasing prevalence of overweight and obesity is parallel to the increasing prevalence of type 2 DM. A study in Costa Rica found that children with obesity (BMI > 95th percentile for age) and high body fat tissue had higher serum insulin level as well as insulin resistance. One study in Israel found that obese children and adolescents had high prevalence of insulin resistance.

Heart disease: Atherosclerosis develops from accumulation of plaque in arteries. Arteries will be narrow and reduced blood flow to the heart can cause chest pain (angina) or a heart attack. The obese person has fat in vessels that catch plaque in

blood circulation. The study regarding abdominal obesity found that abdominal obesity is associated with accelerated progression of atherosclerosis, supporting the view that it is an important cardiovascular risk factor.[39]

Osteoarthritis: Obesity is a common cause of osteoarthritis because excess weight overloads joints, particularly of knees and hips. The joints are damaged by stress placed on them.[40]

Sleep apnea and respiratory problems: Sleep apnea is stopping breathing while a person is sleeping. During sleep, excess weight of chest presses the lungs and respiratory system of obese patients.

Cancer: Overweight women have increased risk for some cancers such as cancer of breast, colon, gallbladder, and uterus than normal weight women.[41] Overweight men have higher risk of colon and prostate cancers than normal weight men.

Metabolic syndrome: Metabolic syndrome is a defined risk factor for cardiovascular disease and type 2 diabetes. Metabolic syndrome consists of six major components: abdominal obesity, dyslipidemia, hypertension, insulin resistance with or without glucose intolerance, proinflammatory state, and prothrombotic state.[42] Obese children have higher risk to develop metabolic syndrome because obesity is the cause of some components of metabolic syndrome. The study in childhood obesity found that the prevalence of metabolic syndrome increased with the severity of obesity.[43]

2.2 Dyslipidemia

2.2.1 Definition and classification

Dyslipidemia is defined as unusual level of lipid profile, i.e. high level of total cholesterol, LDL cholesterol (LDL-C), triglyceride, and reduced HDL-cholesterol (HDL-C).[44] In adults, lipid levels are classified according to the Adult Treatment Panel III (ATP III)[42], as shown in Table 2.4. In children, the classification of lipid levels is based on the adaptation from the National Cholesterol Education Program (NCEP)[23] as shown in Table 2.5.

Table 2.4 ATP III classification of triglyceride, total cholesterol, LDL-C and HDL-C[42]

Lipid profile (mg/dl)	Low	Normal	Borderline high	High
Triglyceride	NA	<150	150–199	200–499
Total cholesterol	NA	<200	200–239	≥240
LDL cholesterol	NA	<100	130–159	≥160
HDL cholesterol	<40	40-60	NA	≥60

Table 2.5 Adaptation from National Cholesterol Education Program (NCEP) classification of triglyceride, total cholesterol, LDL-C and HDL-C in children aged 5-19 years[23]

Lipid profile (mg/dl)	Age (year)	Percentile							
		5th		50th		75th		95th	
		Boy	Girl	Boy	Girl	Boy	Girl	Boy	Girl
Triglyceride									
	5-9	31	33	53	57	67	73	104	108
	10-14	33	38	61	72	80	93	129	135
	15-19	38	40	71	70	94	90	152	136
Total cholesterol									
	5-9	125	130	164	168	180	184	209	211
	10-14	123	128	160	163	178	179	208	207
	15-19	116	124	150	160	170	177	203	209
LDL cholesterol									
	5-9	65	70	93	101	106	118	133	144
	10-14	66	70	97	97	112	113	136	140
	15-19	64	61	96	96	112	114	134	141
HDL cholesterol									
	5-9	39	37	56	54	65	63	76	75
	10-14	38	38	57	54	63	60	76	72
	15-19	31	36	47	53	54	63	65	76

2.2.2 Causes of dyslipidemia

Causes of dyslipidemia are disorders of lipoprotein metabolism, including lipoprotein overproduction and deficiency that result from genetic disorders, diseases, or drugs that affect lipoprotein metabolism. These factors lead to increasing total cholesterol, low-density lipoprotein cholesterol, and triglyceride levels or reducing high-density lipoprotein cholesterol level.

Dyslipidemia is closely associated with cardiovascular diseases, stroke, and atherosclerosis. The prospective study on dyslipidemia and coronary heart disease

in women found that women with obesity, metabolic syndrome, or diabetes had lipid profiles that affected CHD risk.[45]

2.3 Dyslipidemia and obesity

Obesity has association with increasing of plasma triglycerides, LDL-C, total cholesterol and decreasing of HDL-C. Analysis of data from the Second National Health and Nutrition Examination Survey (NHANES II) suggested that increasing obesity in white men and women was associated with increased triglyceride levels, LDL-C levels and decreased HDL-C levels.[9, 10]

The recent study regarding BMI and dyslipidemia found that subjects having BMI over 25 had higher cholesterol level than those with BMI below 25.[46] One study found that each 1 unit of increasing BMI resulted in 3 mg/dl decreasing in HDL-C level.[47] Another study showed that the effect of 1-unit increase in BMI was equal to 5.5-mg/dl increase in LDL-C levels.[48] The recent studies conclude that obesity has strong association with dyslipidemia.[46, 49, 50] In addition, many genes have association with obesity and dyslipidemia (Table 2.6).

Table 2.6 Single nucleotide polymorphism study concerning dyslipidemia

Author(year)	Gene	SNP	Study population	Result
Porchay I, et al. (2006)[51]	ABCA1	C69T G378C	Adult French	T allele associated with higher HDL-C in BMI <25 kg/m^2 C allele associated with lower HDL-C in BMI >25 kg/m^2
		G1051A		A allele associated with higher HDL-C in BMI <25 kg/m^2
Huang A, et al. (2006) [52]	LPL	s447x	Adult Chinese twins	Significantly associated with serum lipids (TG/HDL-C and HDL-C).
Henningsson S, et al. (2006) [53]	IL-6	-174G/C	Adult Swedish	CC genotype of the IL-6 polymorphism was associated with lower levels of cholesterol and LDL-C and low triglycerides in women. The association between the C allele and lipid pattern found in women was not found in men.

A, adenine; ABCA1, ATP-binding cassette transporter; C,cytosine; G,guanine; IL-6, interleukin-6; T, thymine

Increased triglycerides: Very-low-density lipoprotein (VLDL) is over produced by liver defect in obesity and the insulin-resistance state. Insulin resistance in liver, muscle, and adipose tissue results from the incapability to extinguish hepatic glucose production, impaired muscle glucose uptake and oxidation, and the incapability to extinguish release of nonesterified fatty acids (NEFA) from adipose tissue, respectively. Hepatic VLDL production are regularized by increase NEFA and glucose flux to the liver.[54]

The rate of apolipoprotein B-100 (apoB-100) degradation is another factor of regulation of VLDL secretion. The apoB-100 is translocated into the lumen and incorporated into lipid-poor VLDL precursors or is degraded by ubiquitin/proteasome system.

Lipids and microsomal triglyceride protein are important factors in the translocation of apoB-100. Microsomal triglyceride protein is heterodimeric lipid transfer protein that is required for the assembly of apoB-containing lipoproteins. The apoB -100 is degraded because translocation does not happen.[50]

Triglyceride-rich lipoproteins (TRLs) decrease in obesity. Lipoprotein lipase (LPL) activity is stimulated by insulin. In skeletal muscle of insulin-resistant people, LPL activity is lower than normal. The decreased LPL activity is resulting from decreased clearance of VLDL.[55] VLDL particles are cleared by LDL receptor (LDLR). In obesity, LDLR activity dose not work, so VLDL particle clearance delays.

Increased small, dense low-density lipoprotein: The formation of small, dense LDL depends on the metabolism of VLDL particles. Therefore, small, dense LDL concentration is associated with fasting triglyceride levels. In obesity, an increased exchange between cholesterol esters in LDL and triglycerides in VLDL results from increased concentration and delayed clearance of VLDL particles that cholesterol ester transfer protein (CETP) is the mediator.

In triglyceride, this exchange process produces LDL particles that are immediately lipolyzed by hepatic lipid. The CETP activity and hepatic lipolysis activity are increased. The exchange process leads to high VLDL particles. The small, dense LDL particles are more prone to modification. The modifications result from a decreased LDLR-mediated clearance of small, dense LDL particles.[56]

Decreased high-density lipoprotein cholesterol: In obese person with insulin resistance, the decreased HDL-C can be explained by many mechanisms. Inverse relationship between VLDL triglycerides and HDL cholesterol was shown in lipoprotein studies. The decreased transfer of apolipoproteins and phospholipids from TRL to the HDL compartment is the causes of decreased TRL lipolysis leading to reduced HDL concentration. Additionally, the delayed clearance of TRLs helps the CETP-mediated exchange between cholesterol esters in HDL and triglycerides in VLDL. In obesity, the increased of hepatic lipids activity produces smaller HDL particles and helps HDL clearance.[57]

2.4 Lipoprotein metabolism

2.4.1 Structure and classification

A lipoprotein is a macromolecule that contains proteins and lipids. The general structure of a lipoprotein contains a central core consisting of nonpolar lipid (triacylglycerols and cholesteryl esters) and a surface monolayer that consists of polar lipids (phospholipid) and apolipoproteins.[58]

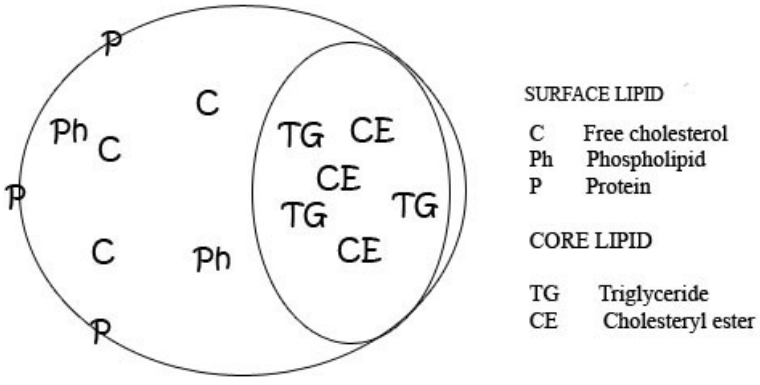

Figure 2.3 Structure of lipoprotein[59]

The classification of lipoprotein is based on its density (Table2.7).

Chylomicrons carry dietary triglyceride from the small intestine to the liver, skeletal muscle, and adipose tissue.

Very-low-density lipoproteins (VLDL) carry endogenously synthesized cholesterol and triglycerides from the liver to adipose tissue.

Intermediate-density lipoproteins (IDL) are intermediate between VLDL and LDL.

Low-density lipoprotein (LDL) transports cholesterol from the liver to peripheral tissues. Sometimes it is called the bad cholesterol.

High-density lipoprotein (HDL) is involved in reverse transport of cholesterol from peripheral tissues to the liver. Sometimes it is called the good cholesterol.

Table 2.7 Classification of lipoproteins [60]

Lipoprotein	Density (g/dl)	Diameter (nm)	Lipid (%)		
			TG	Chol	PL
Chylomicron	0.95	75-1200	80-95	2-7	3-9
VLDL	0.95-1.006	30-80	55-80	5-15	10-20
IDL	1.006-1.019	25-35	20-50	20-40	15-25
LDL	1.019-1.063	18-25	40-50	40-50	20-25
HDL	1.063-1.210	5-12	15-25	15-25	20-30

TG, triglyceride; Chol, cholesterol; PL, phospholipid

Functions of lipoprotein are the first transport of dietary fat after digestion and absorption at intestinal mucosa to the other tissues in form of chylomicron. The second transfer of triglycerides from liver to other tissues is in the

form of VLDLs. After VLDLs release triglyceride to tissues, VLDLs go back to the liver in the form of IDL and LDL. The last is removal of cholesterol from the tissues and return to the liver.

2.4.2 Apolipoproteins

Apolipoprotiens are the protein components of lipoproteins which play an important role in lipid transport and metabolism. Classification and presentation of each lipoprotein are shown in Table 2.8.

Table 2.8 Properties of some human apolipoproteins[61]

Apolipoprotein	Molecular weight	Site of synthesis	Lipoprotein	Function
ApoA-I	28,100	Intestine, liver	CM ,HDL	Activates lecithin:cholesterol acyltransferase (LCAT)
ApoA-II	17,400	Intestine, liver	HDL	
ApoB-48	241,000	Intestine	CM	Triglyceride transport
ApoB-100	512,000	Liver	LDL, VLDL	Binds to LDL receptor
ApoC-I	7,600	Liver	VLDL, CM	Activates LCAT
ApoC-II	8,900	Liver	VLDL, CM	Activates lipoprotein lipase
ApoC-III	8,700	Liver	VLDL, CM	Inhibits lipoprotein lipase
ApoE	34,000	Liver, intestine, macrophage	HDL	Binds to LDL receptor

ApoA-I, apolipoprotein A-I; ApoB-48, apolipoprotein B-48; ApoB-100, apolipoprotein B-100; ApoC-I, apolipoprotein C-I; ApoC-II, apolipoprotein C-II; ApoC-III, apolipoprotein C-III; ApoE, apolipoprotein E; CM, chylomicron

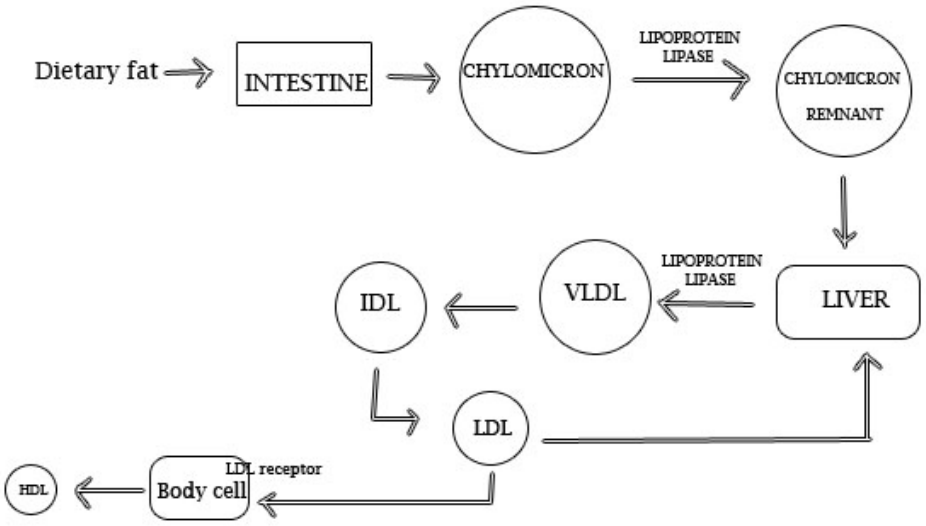

Figure 2.4 Lipoprotein metabolisms[60]

2.4.3 Metabolism of lipoprotein[60, 61]

There are 3 main pathways responsible for the lipoprotein metabolism. These pathways include the exogenous pathway, the endogenous pathway, and the pathway of reverse cholesterol transport.

Exogenous lipid pathway: After digestion and absorption of dietary fat, triglyceride and cholesterol are packaged to chylomicron form in the epithelial cells of the intestines. Chylomicrons are secreted into lymphatic system and sent to bloodstream. In blood circulation, chylomicrons are hydrolyzed with lipoprotein lipase (LPL) enzyme at the capillaries of adipose tissue and muscle cells to release triglyceride to the adipose tissue to store for the body's energy.

Endogenous lipid pathway: The endogenous pathway involves the liver synthesizing lipoproteins. Triglyceride and cholesterol esters are generated by the liver and packaged into VLDL form and then released into the blood circulation. In tissues, VLDL is hydrolyzed by LPL to release fatty acids and glycerol. VLDL becomes IDL and then IDL particles are hydrolyzed by hepatic-triglyceride lipase to LDL form that is the main carrier of circulating cholesterol within the body.

Reverse cholesterol transport: Reverse cholesterol transport involves HDL. In this pathway, cholesterol is removed from the tissues and returned to the liver.

2.5 Microsomal triglyceride transfer protein (*MTTP*) gene

2.5.1 Structure

Microsomal triglyceride transfer protein (*MTTP*) gene, at chromosome 4 location 4q24, encodes MTTP protein which is heterodimer lipid transfer protein. MTTP has a large unique 97 kDa subunit (M subunit) that is essential for the lipid transfer and small unique 55 kDa subunit (P subunit). P subunit is ER resident enzyme protein disulfide isomerase (PDI).[62] MTTP is found in high concentration in the endoplasmic reticulum (ER) lumen of liver and intestinal cells.[63] MTTP protein plays important role in the assembly of lipoprotein, allowing apoB to fold correctly and assembly lipoprotein with a neutral lipid core before secretion

The M subunit is a single polypeptide that has 894 amino acids[64] and contains 3 domains: N-terminal ß-barrel, middle α-helical domain, and C-terminal lipid-binding cavity.[12, 65] N-terminal, ß-barrel at residues 22–297, mediates the interaction with the N terminus of apoB (MTTP binding domain).[65] Middle, α-helical at residues 298–603, mediates the interaction with both PDI and apoB.[66] C-terminal domains, residues 604–894, mediate the lipid-binding and transfer catalytic activity of MTTP. The ß-barrel domain and α-helical domain are globular.

2.5.2 Function and polymorphism

MTTP transfers lipids by a shuttle mechanism.[67] MTTP molecule momentary interacts with a membrane, lipid molecules are extracted and dissociated from the membrane. MTTP molecule binds transiently with another membrane and lipids are delivered to the second membrane.[11]

Variation of *MTTP* gene may have impact on plasma lipoprotein level[68, 69] as shown in Table 2.9. Polymorphisms of *MTTP* gene involve with accumulation of adipose tissue[68] and polymorphisms of the *MTTP* promoter gene regulate the development of dyslipidemia and atherosclerotic disease in overweight subjects.[16] Absence of *MTTP* causes abetalipoproteinemia, a disease characterized by a defect in the assembly and secretion of apolipoprotein B (apoB) containing plasma lipoproteins.

Table 2.9 Single nucleotide polymorphisms of microsomal triglyceride transfer protein gene and plasma lipid level

Author(year)	SNP	Study population	Result
Chen SP, et al (2003) [70]	-493 G/T	Adult Chinese	In type 2 diabetes −493 G/T polymorphism has less effect on LDL.
Berthier MT, et al (2004) [68]	-400A>T 891C>G	Adult French Canadian	-400A>T and 891C>G were associated with plasma lipoprotein and lipid level.
Ledmyr H, et al (2002) [69]	Q94H I128T H297Q	Adult Swedish	Q94H, I128T, and H297Q coding single nucleotide polymorphisms were associated with variations in plasma total and LDL cholesterol levels.
Ohashi K, et al (2000) [71]	Asp780Tyr	Adult Japanese	Defects of the *MTTP* gene cause ABL.

ABL, abetalipoproteinemia

2.6 Apolipoprotein B (*APOB*) gene

2.6.1 Structure (Figure 2.5)

Apolipoprotein B, at chromosome 2 location 2p24-p23, is a non-exchangeable apolipoprotein associated with plasma lipoproteins and consists of 29 exons. In the human genome, apoB has 45 kb. It consists of 2 isoforms. The first isoform is apoB-48 which is produced by small intestine which apoB mRNA is post-transcriptionally edited, resulting in the conversion of a glutamine codon into a stop codon. The edited mRNA is translated into a single polypeptide of 2,152 amino acids. The second isoform is apoB-100 which is produced in the liver. ApoB-100 is transcribed into a single mRNA of 15 kb and is translated into a single polypeptide of 4,536 amino acids[72]. ApoB-100 contains 3 amphiphatic α-helical domains interchanging with 2 amphiphatic ß-sheet domains in NH2-α1-ß1-α2-ß2-α3-COOH. The ßα1 domain consists of a ß-barrel at residues 1–263 and an α-helical at residues 294–592. This domain contains MTTP binding site. The α1 domain, at residues 58–795 is globular shape that comprises of 12 cysteine residues. The disulfide bond formation between some cysteine residues is essential for the assembly of apoB-containing lipoproteins.[73] The ß1, α2, ß2 and α3 domains consist of several short amphiphatic ß-strands and α-helices. The ß1 domains, at residues 827–2001 and ß2 domains, at residues 2571–4032 are essential for lipoprotein assembly and lipid binding. The ß2 domain has LDL receptor binding and heparin binding sites. ApoB-48 contains only ß1 domain that is used for chylomicron assembly. ApoB-100 contains ß1 and ß2 domains that are used for VLDL assembly. The α2, at residues 2045–2587 and α3, at residues 4017-4515 domains consist of several amphiphatic helices that associate with property of exchangeable apolipoproteins. Their role in lipoprotein assembly is not clear.[11]

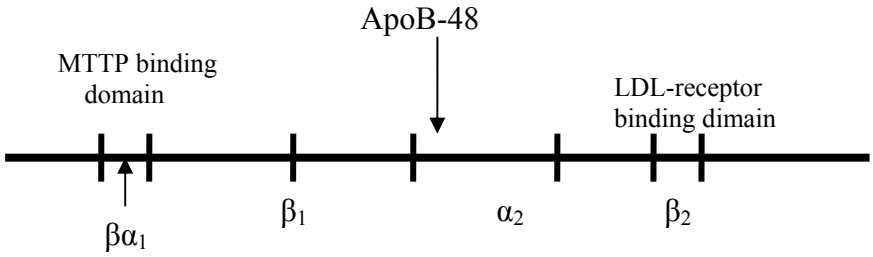

Figure 2.5 Structure of apoB [14]

2.6.2 Function and polymorphism

The interaction between LDL and the LDL receptor has association with plasma cholesterol levels in humans.[74] The LDL-receptor-binding domain has been localized to the carboxyl-terminal site of apoB-100 that is a component of LDL and is a ligand for binding to the LDL receptor.[75] ApoB-100 binds to the LDL receptor at residues 3359–3369. Conformation change is associated with interaction between amino acid residues Arg-3500 and Trp-4369 which is important to induce the structure of apoB-100 necessary for binding to the LDL receptor. It leads to increasing LDL-C level in plasma. Mutation of the arginine or the tryptophan inhibits receptor binding, which is the cause of familial defective apolipoprotein B-100 (FBD).[76]

The interaction at apoB-MTTP binding is the cause of absence of apoB-containing lipoproteins in the plasma, leading to reduced plasma lipid.[14] The recent study suggests that serum level of apolipoprotein B was predictor for dyslipidemia and metabolic syndrome.[15] Single nucleotide polymorphism of *APOB* gene was the cause of abnormal plasma lipid level and coronary artery disease (CAD), as shown in Table 2.10.

Table 2.10 Association between Single nucleotide polymorphisms of apolipopoteinB gene and plasma lipid level and coronary artery disease

Author (year)	SNP	Study population	Result
Kallel. A, et al. (2007) [13]	Ins /Del	Tunisian	Del/Del allele had higher LDL-C levels than Ins/Ins allele and Ins /Del alleles.
Sposito AC, et al. (2004) [77]	C516T	French	TT allele had higher plasma levels of apoB and LDL-C than TC allele and both had higher plasma levels of apoB and LDL than CC allele.
Benn M, et al. (2005) [78]	C7673T	Danish	Associated with increases in total cholesterol, LDL cholesterol, and apoB in males and females

CHAPTER III
MATERIALS AND METHODS

3.1 Subjects

3.1.1 Inclusion criteria

Studied subjects were obese children aged 4 to18 years who attended pediatric nutrition clinic, Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital during April-October 2007. Their body mass indexes were above the 95th percentile for age and sex of the WHO 2007 reference.

3.1.2 Exclusion criteria

Subjects were excluded if they had underlying causes of obesity (e.g. Prader Willi syndrome, Down's syndrome, hypothyroidism, and growth hormone deficiency) or regularly took medication which affected blood pressure, glucose level, and lipid profiles.

The study protocol was approved by the Ethical Committee of the Faculty of Medicine Ramathibodi Hospital, Mahidol University. Written informed consent was obtained from parents or guardians of all subjects

3.1.3 Sample size analysis

Sample size calculation was performed based on testing correlation coefficient (or slope) of genotypes and LDL-C level. Type I & II errors were set at 0.05 and 0.2 respectively. The slopes that we wanted to detect for correlation were set at 5 and 10. Power and Sample Size Calculation (version 2.1.31) was used and estimated sample size for each SNP is shown in Table 3.1.

Table 3.1 Sample size of each SNP

SNPs	Sample size (n)	
	Slope (λ) = 5	Slope (λ) = 10
MTTP		
453T/C	370	94
IVS6 - 116A/G	518	131
891C/G	360	91
APOB		
T71I (293C/T)	1050	264
T2488T (7545C/T)	1402	352
R3611Q (10913G/A)	22104	5527
IVS18 + 1708C/A	592	150

3.2 Methods

3.2.1 Anthropometric measurement

3.2.1.1 Body weight

Body weight of each subject was measured by the researcher, using Seca scale. Before measurement, the scale was calibrated with standard weight and checked for zero-balance. The subject wore light-clothing without shoes and stood straight with minimal movement during the measurement. The body weight was distributed between both feet. The measurement was recorded to the nearest 100 g.

3.2.1.2 Height

Height was measured by using Seca scale. The subject stood with the back and head straight and the eyes were focused forward. His or her feet, knees, buttocks and shoulder blades were in contact with the vertical surface of the scale. The arms hanged loosely with palms facing the thighs. The subject took a deep breath; shoulders relaxed and took off their shoes. The headboard of the scale touched the crown of the head. The measurement was recorded to the nearest 0.1 cm.

3.2.1.3 Waist circumference

The subject stood straight with abdomen relaxed, arms at the sides, feet together. The tape was placed around midway between the lower rib margin and the iliac crest. The horizontal plane of the tape was parallel to the floor and the tape was snug, but did not compress the skin. The measurement was performed at a normal respiration. The measurement was recorded to the nearest 0.1 cm.

3.2.1.4 Hip circumference

The subject stood erect with arms at the sides, feet together, and the gluteal muscles relaxed. The researcher sat at the side of the subject. The tape was placed around the buttocks in a horizontal plane. The tape contacted with the skin but did not compress it. The measurement was recorded to the nearest 0.1 cm.

3.2.2 Collection of subject information

History taking and physical examination were performed in each subject by one pediatrician. Symptoms and signs of obesity complications such as acanthosis nigrican, bone and joint deformities were assessed. Then, subjects and their parents were asked to fill in the questionnaires their family information, dietary information and physical activity. The information was checked and completed by the researcher using interviewing technique. Questionnaires are shown in appendix.

3.2.3 Blood sample collection

Each subject was fasted for 8 hours before blood sample collection. The subject sat and put their relaxed arm on a table. Twenty-one ml of venous blood was gently withdrawn from the forearm by a nurse. Fifteen ml of fasting blood was sent to central laboratory of Ramathibodi Hospital for analysis of C-reactive protein, insulin level, lipid profiles, and oral glucose-tolerance test (glucose1.75 g/kg, maximum 75 g). Three ml of blood was put into an EDTA tube for genetic study and another 3 ml was put into a non-EDTA tube for collected serum.

Three ml of blood from the regular blood donors of Ramathibodi Hospital blood bank was collected in EDTA tubes.

3.2.4 DNA extraction from human blood sample by phenol-chloroform extraction method

On the first day, blood lysis was performed by adding 3 ml of buffer to 3 ml of whole blood in an EDTA tube. The EDTA tube was rotated for 15 min and

centrifuged at 4,500 rpm for 10 min at 20°C. The supernatant (blood waste) was removed from the EDTA tube and 7 ml of Nonidet was added into EDTA tube. Then, EDTA tube was rotated for 30 min and centrifuged at 4,500 rpm for 10 min at 20°C. The supernatant was removed from the EDTA tube. One ml of sterile water, 25 µl of proteinase K, 25 µl of 10% SDS, and 50 µl of 10X STE were subsequently added and then mixed by vortex machine and incubated overnight at 56°C in a water bath.

On the second day, 1 ml of saturated phenol was added into the EDTA tube and the tube was rotated for 30 min, and then centrifuged at 4,500 rpm for 10 min at 4°C. The supernatant was transferred from the EDTA tube into a microtube and 0.5 ml of chloroform was added. Then, the microtube was rotated for 10 min and centrifuged at 6,000 rpm for 10 min at 4°C. The supernatant was transferred into a new microtube and 50 µl of sodium acetate plus 1 ml of absolute ethanol was added. Then, the microtube was gently shaked until the DNA precipitated. The microtube was subsequently centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was removed from the microtube and 70% ethanol was added. The microtube was centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was removed from the microtube and DNA was dried by heat box. The TE buffer was added into DNA and then incubated overnight at 56°C in a water bath. On the last day, the DNA concentration was measured in a spectrophotometer.

3.2.5 Polymerase chain reaction (PCR)

3.2.5.1 Principle

The polymerase chain reaction (PCR) is a technique for amplifying a particular piece of target DNA in the test tube. The cycle of PCR has 3 steps. The first step (denaturation) is heating at high temperature to separate double strand to single strand. The second step (annealing) is cooling to temperature about 50-60°C for primer (short DNA strand) to find complementary base on single strand to bind. The final step (extension) is heating temperature to 72°C which each single strand is duplicated by tag DNA polymerase. The amplification of DNA is started at denaturation and then annealing and extension and back to the denaturation step again. The process of amplification of DNA is repeated for multiple cycles of PCR.[79, 80] The polymerase chain reaction is determined by agarose gel electrophoresis technique.

3.2.5.2 Polymerase chain reaction of *MTTP* and *APOB* polymorphism

The following polymorphisms were amplified by PCR: the 453T/C, in exon 4 of *MTTP* gene; the Ivs6-116A/G, the 891C/G, in exon 7 of *MTTP* gene; the 293C/T (T71I), in exon 4 of *APOB* gene; the 7545C/T (T2488T) and the 10913G/A (R3611Q), in exon 26 of *APOB* gene, The primers, forward primer, and reverse primer were designed by primer 3 program. The primer sequences are shown in Table 3.2. The primers were used to amplify the whole PCR product. The amplified PCR product sizes were 334 bp (453T/C), 467 bp (Ivs6-116A/G and 891C/G), 546 bp (T71I), 453 bp (T2488T), and 274 bp (R3611Q).

Table 3.2 Oligonucleotide primers and annealing temperature of *MTTP* and *APOB* SNPs

Oligonucleotide primers	Annealing temperature	Forward primer 5'……3'	Reverse primer 5'……3'
***MTTP* primers**			
453T/C	60 °C	GAATCTGACC TTGCCTGACA	CATAGGCAAAT GCGACTTCA
IVS6 - 116A/G	51 °C	TGTTGCTCCAG AAAGACTTCA	GCTGTCATCAC AACTCTGTGG
891C/G	51 °C	TGTTGCTCCAG AAAGACTTCA	GCTGTCATCAC AACTCTGTGG
***APOB* primers**			
T71I	65 °C	CACTCCTAGTC TGCAGCGCTTC	CAAGTTCATAC CTCAGCGGACA C
T2488T	60 °C	GATGAAACCA ATGACAAAAT CC	AACAGTGAACC CTTGCTCTACC
R3611Q	60 °C	AGAACATACA AGCAAAGCCA	GAGGAACCTTA GGTGTCCTTC

A, adenine; C, cytosine; G, guanine; T, thymine

The polymerase chain reaction (PCR) was modified from the method of Bentzen et al[81] and Marianne et al.[82] The PCR master mix, as shown in Table 3.3, was carried out in 20 µl final volume.

Table 3.3 Composition of polymerase chain reaction master mix

PCR master mix	1X volume
Sterile water	10.3 µl
NH_4 buffer	2 µl
$MgCl_2$	0.6 µl
dNTPs	2 µl
Forward primer	2 µl
Reverse primer	2 µl
Tag DNA polymerase	0.1 µl
DNA	0.1 µl

DNA, deoxyribonucleic acid; dNTPs, deoxyribonucleotide triphosphate; $MgCl_2$, magnesium chloride; NH4, ammonium

The master mix was mixed for few seconds by vortex machine. A total of 30 cycles were run under this condition, then denaturation at 95 ^{o}C for 1 minute. The annealing followed (Table 3.2) for 1 minute and the extension at 72 ^{o}C for 1 minute.

PCR product of the 453T/C, the Ivs6-116A/G, and the 891C/G of *MTTP* gene, the T71I, the T2488T, and the R3611Q of *APOB* gene were determined by agarose gel electrophoresis technique as following. The DNA was run in plate that had 1.5% agarose mixed with 1X TBE for about 30 minutes. Then, gel was put into ethidium bromide for about 10 minutes and subsequently put into clean water for about 10 minutes. Then, gel photo was taken by UV transilluminator.[83]

3.2.6 Restriction fragment length polymorphism (RFLP)

3.2.6.1 Principle

Restriction fragment length polymorphism (RFLP) is a technique that uses restriction endonuclease which can recognize and digest DNA at spacific site. The variation of sequence base of DNA can distinguish different fragment length of interesting DNA from another DNA. The size of DNA restriction fragment is separated by agarose gel electrophoresis.[83]

3.2.6.2 Restriction fragment length polymorphism of *MTTP* and *APOB* genes

The 453T/C, the Ivs6-116A/G, and the 891C/G of *MTTP* gene, the T71I, the T2488T, and the R3611Q of *APOB* gene were digested by restriction enzyme (Table 3.4).

Table 3.4 Restriction enzymes of *MTTP* and *APOB* SNPs

SNPs	Restriction enzyme
MTTP	
453T/C	StuI
IVS6 - 116A/G	Bmg BI
891C/G	BsrI
APOB	
T71I	ApaLI
T2488T	XbaI
R3611Q	MspI

The final volume of master mix was 10 µl. The RFLP master mix is shown in Table 3.5. The master mix was mixed for few seconds by vortex machine and put into 56^{o}C water bath overnight.

Table 3.5 Composition of restriction fragment length polymorphism master mix

RFLP master mix	1X volume
Sterile water	3.6 µl
Buffer	1 µl
Restriction enzyme	0.4 µl
PCR product	5µl

Agarose gel electrophoresis technique was used for separating DNA restriction fragment by size. The DNA was run in a plate that had 2% agarose mixed with 1X TBE for about 60 minutes. Then, gel was put into ethidium bromide for about 10 minutes and subsequently put into clean water for about 10 minutes and then photo was taken by UV transilluminator.[83] Genotype distribution of SNPs was counted by 3 researchers.

3.2.7 Amplification-refractory mutation system (ARMS)

3.2.7.1 Principle

Amplification-refractory mutation system (ARMS), known as allele-specific polymerase chain reaction (ASPCR) or PCR amplification of specific alleles or allele-specific amplification (ASA), is a method that easily and rapidly detects single nucleotide polymorphism (SNP) or small changes of base. This technique uses specific sequence PCR primers that amplifies only target allele and will not amplify non-target allele. The presence or absence of PCR product from ARMS reaction is used to diagnose the presence or absence of target allele.[84]

3.2.7.2 Amplification-refractory mutation system of IVS18 + 1708C/A

IVS18 + 1708C/A, in intron 18 of *APOB* gene was amplified by PCR. The primers, i.e. forward primer, normal reverse primer and mutant reverse primer, were designed by primer 3 program. Primer sequences are shown in Table 3.6. The primers were used to amplify the whole PCR product. The amplified PCR product size was 283 bp.

Table 3.6 Oligonucleotide primers of IVS18 + 1708C/A

Forward primer 5'……3'	Normal reverse primer 5'……3'	Mutant reverse primer 5'……3'
GGAGTAGAAAGGA AAGATTCCGGGTTA CCT	TCAAATCAATATGT TCTTAGGTATTTTTC T	TCAAATCAATATGT TCTTAGGTATTTTTC G

The final volume of master mix was 25 µl. The ARMS master mix for normal tube and mutant tube are shown in Table 3.7.

Table 3.7 Composition of amplification-refractory mutation system master mix

Amplification-refractory mutation system master mix		1X volume
Normal tube	Mutant tube	
Sterile water	Sterile water	11.3 µl
NH_4 buffer	NH4 buffer	2.5 µl
$MgCl_2$	$MgCl_2$	1.5 µl
dNTPs	dNTPs	2.5 µl
Forward primer	Forward primer	1 µl
Normal reverse primer	Mutant reverse primer	1 µl
Forward primer control*	Forward primer control*	2 µl
Reverse primer control**	Reverse primer control**	2 µl
Tag DNA polymerase	Tag DNA polymerase	0.2 µl
DNA	DNA	1 µl

** I71T Forward primer; ** I71T Reverse primer*

The mixture was mixed for few seconds by vortex machine. A total of 30 cycles were run under this condition, and then denatured at 95 ^{o}C for 1 minute. Subsequently, the annealing was performed at 58 ^{o}C for 1 minute and the extension followed at 72 ^{o}C for 1 minute.

PCR products of the IVS18 + 1708C/A were determined by agarose gel electrophoresis technique. The DNA was run in plate that had 1.5% agarose mixed

with 1X TBE for about 30 minutes and then the gel was put into ethidium bromide for about 10 minutes and picked into clean water for about 10 minutes. Then gel photo was taken by UV transilluminator. Genotype distribution of SNPs was counted by 3 researchers.

3.2.8 Statistical analysis

Continuous data (e.g. age, height, weight, waist-hip ratio (WHR), lipid profile) were described using means and standard deviations whereas categorical data were describe using frequency and percentage. Hardy-Weinberg equilibrium was assessed using Exact test.

The means of LDL-C levels according to genotype was compared using one-way ANOVA or unpaired t-test where only two genotypes were identified. Logistic regression was used to estimate association between SNPs and abnormal LDL-C in obesity. Analyses were performed using SPSS version 13 and STATA version 10. P value of less than 0.05 was considered as statistically significant.

CHAPTER IV
RESULTS

4.1 Subject characteristics

A total of 103 subjects were initially screened and 4 were excluded because their BMI for age and sex were below the 95th percentile of WHO 2007 reference. Figure 4.1 shows diagram of subject recruitment in this study.

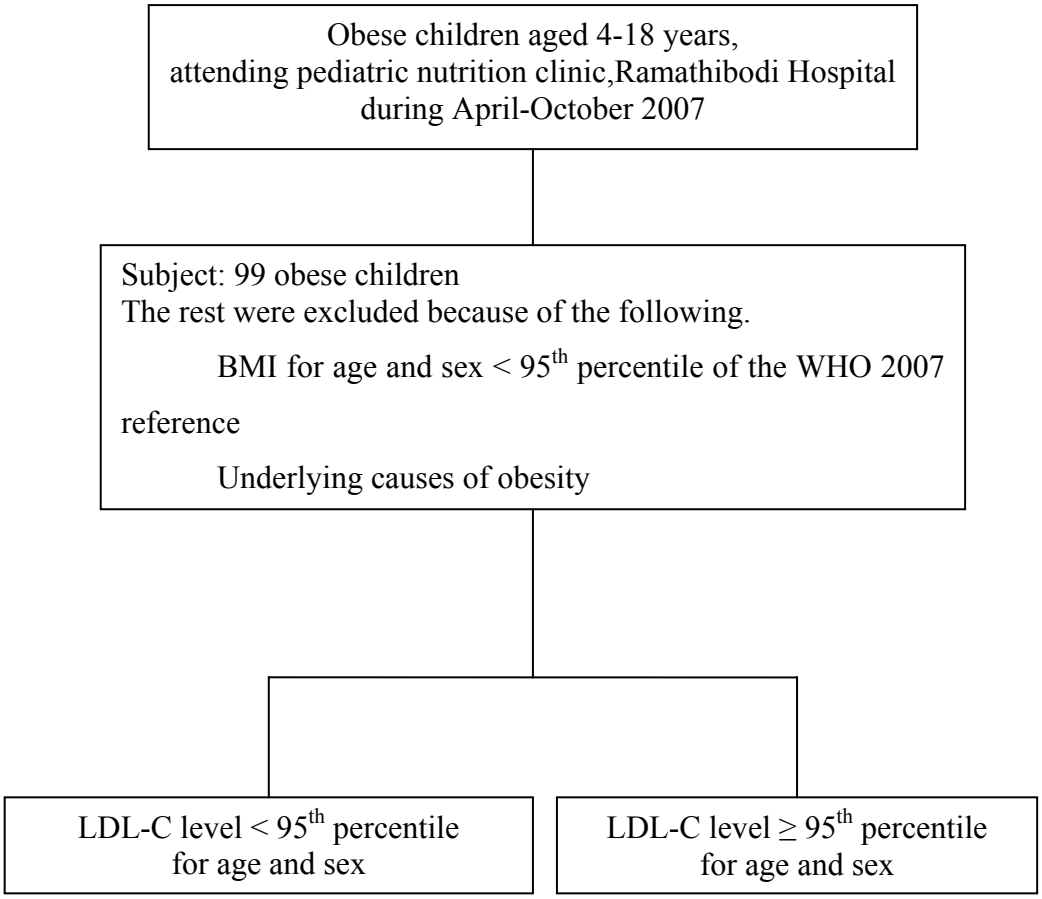

Figure 4.1 Diagram of subject recruitment

Characteristics of subjects were described as in Table 4.1. Their age range was 5-17 years and most of them were adolescents (72.7%). The majority of them were males (58.6%) and all had abdominal obesity.

The family history revealed that 93.9%, 68.7%, 64.6%, and 37.4% of the subjects had obesity, diabetes, hypertension, and dyslipidemia, respectively, in their family.

Table 4.1 Subject characteristics

Characteristic	Mean ± SD
Age(yr)	11.1 ± 2.7
Sex, number (%)	
Male	58(58.6%)
Female	41(41.4%)
Weight (kg)	70.2 ± 24.1
Height (cm)	151.5 ± 15.6
Waist (cm)	90.5 ± 12.6
Waist-hip ratio (WHR)	0.89 ± 0.07

Table 4.2 shows cooking oil that was consumed by subjects. The majority of them consumed soybean oil (78.79%). Five subjects consumed both soybean oil and palm oil; 6 subjects consumed soybean oil and rice-bran oil; 1 subject consumed soybean oil and olive oil; 1 subject consumed soybean oil, rice-bran oil, and olive oil; 1 subject consumed soybean oil and corn oil; 1 subject consumed palm oil and olive oil.

Table 4.2 Consumption of cooking oil in obese children

Cooking oil	N	%
Soybean	78	78.79
Rice-bran	10	10.10
Coconut	0	0.00
Palm	13	13.13
Lard	0	0.00
Sunflower	4	4.04
Olive	3	3.03
Corn	1	1.01
Unknown	6	6.06

Lipid profile was described as in Table 4.3. We found that mean total cholesterol, triglyceride, HDL-C, and LDL-C were 183.5 (SD = 29.9) mg/dl, 105.8 (SD = 64.2) mg/dl, 44.6 (SD = 8.2) mg/dl, and 118.4 (SD = 26.2) mg/dl, respectively. Among these 99 subjects, proportions of hypertriglyceridemia and hypercholesterolemia were quite high, 24.24% and 21.21% respectively.

Table 4.3 Lipid profile of obese children

Lipid profile	Mean $\pm$ SD	Subjects with abnormal lipid profile, number (%)
TC(mg/dl)	183.5 ± 29.9	21 (21.2)
HDL-C(mg/dl)	44.6 ± 8.2	17 (17.2)
LDL-C(mg/dl)	118.4 ± 26.2	20 (20.2)
TG(mg/dl)	105.8 ± 64.2	24 (24.2)

The severity of obesity was classified using percentage of ideal weight for height. We found that the majority of subjects were moderately and severely obese, 45.5% and 35.5%, respectively. The LDL-C levels according to severity of obesity were shown in Table 4.4. Surprisingly, mildly obese subjects had the highest mean LDL-C level (124.5± 25.2 mg/dl) whereas those with moderate obesity had the lowest

mean LDL-C level (113.5 ± 30.2 mg/dl). However, these means were not significantly different among various classes of obesity severity.

Table 4.4 LDL-C levels according to severity of obesity

Severity of obesity	N (%)	LDL-C (mg/dl)	P-value
Mild	10 (10.1%)	124.5 ± 25.2	0.391
Moderate	45 (45.5%)	113.5 ± 30.2	
Severe	35 (35.5%)	121.97 ± 19.3	
Morbid	9 (9.1%)	122.8 ± 29.2	

Values are mean ± SD

4.2 Single nucleotide polymorphism of 453T/C in *MTTP* gene

PCR product containing 453T/C site, in exon 4 of *MTTP* gene, is shown in Figure 4.2

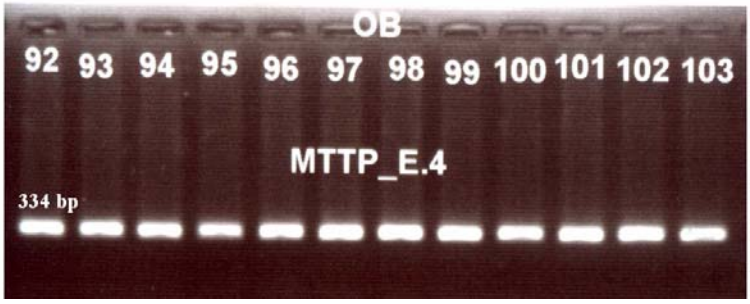

Figure 4.2 Polymerase chain reaction product of 453T/C, in exon 4 of *MTTP* gene

PCR products of exon 4 of *MTTP* gene was digested by StuI. The StuI is restriction enzyme that cut 5'…..AGG▼CCT…..3'. The largest fragment or wild type (334 bp) was defined as WT/WT. The homozygote (MT/MT) had 2 bands, 188 bp and 146 bp. The heterozygote (WT/MT) had 3 bands, 334 bp, 188 bp, and 146 bp. Figure 4.3 showed pattern of PCR-RFLP of the 453T/C SNP. The 453T/C SNP creates a StuI restriction site while the wild type allele remains uncut.

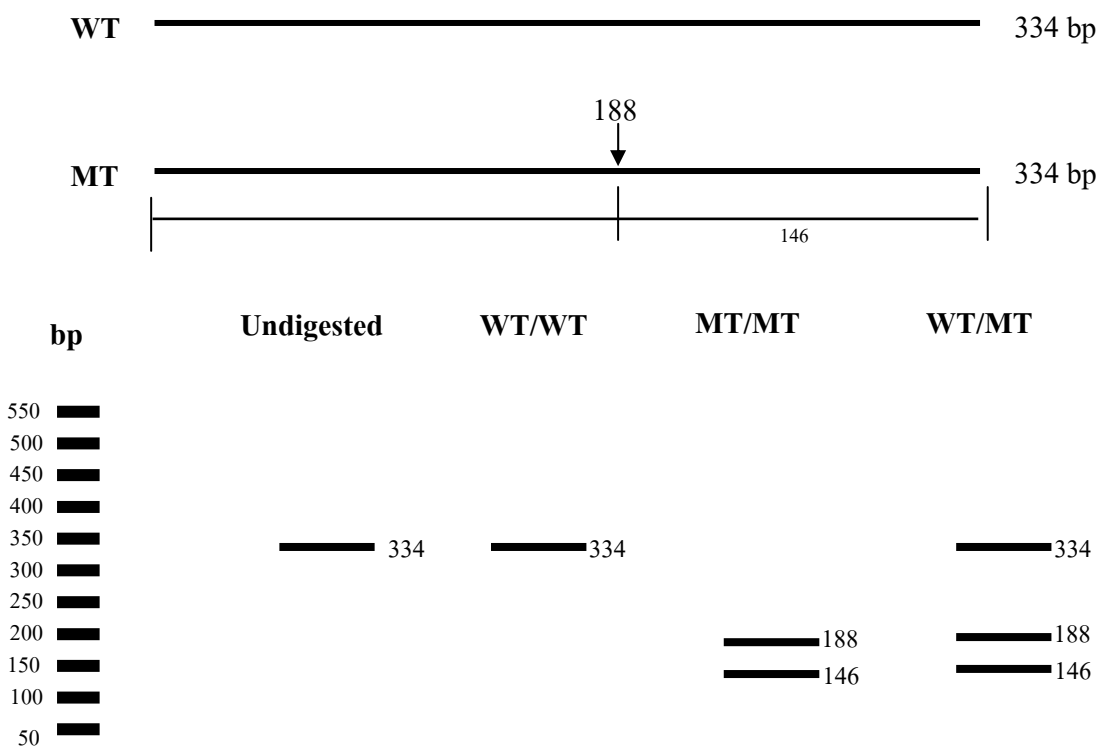

Figure 4.3 Diagram showing PCR-StuI digest of 453T/C SNP

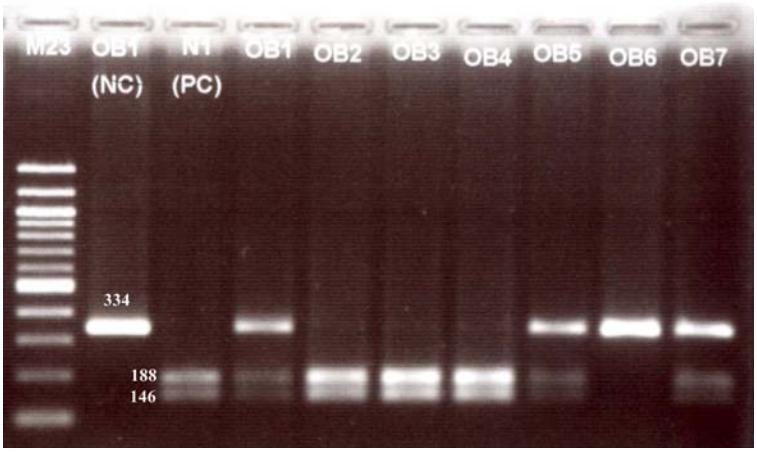

Figure 4.4 Restriction fragment length polymorphism of 453T/C in *MTTP* gene. Lane M23 is 100 bp marker. Lane OB1 (normal control, NC) represents undigested product. Lane N1 is positive control (PC). Lanes OB2, OB3, and OB4 are homozygous mutant. Lanes OB1, OB5, and OB7 are heterozygous mutant. Lane OB6 had only wild type allele.

4.3. Single nucleotide polymorphism of IVS6 - 116A/G in *MTTP* gene

PCR product containing IVS6 - 116A/G site, in exon 7 of *MTTP* gene, is shown in Figure 4.5.

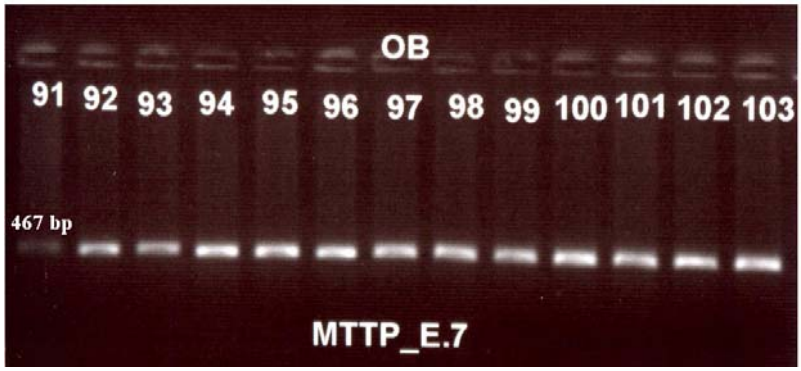

Figure 4.5 Polymerase chain reaction products of IVS6 - 116A/G and 891C/G, in exon 7 of *MTTP* gene

PCR products of IVS6 - 116A/G were digested by BmgBI. The BmgBI is restriction enzyme that cut 5'…..CAC▼GTC …..3'. The largest fragment or wild type (467 bp) was defined as WT/WT. The homozygote (MT/MT) had 2 bands, 400 bp and 67 bp. The heterozygote (WT/MT) had 3 bands, 467 bp, 400 bp, and 67 bp. Figure 4.6 showed pattern of PCR-RFLP of IVS6 - 116A/G SNP. The IVS6 - 116A/G SNP creates a BmgBI restriction site while the wild type remains uncut.

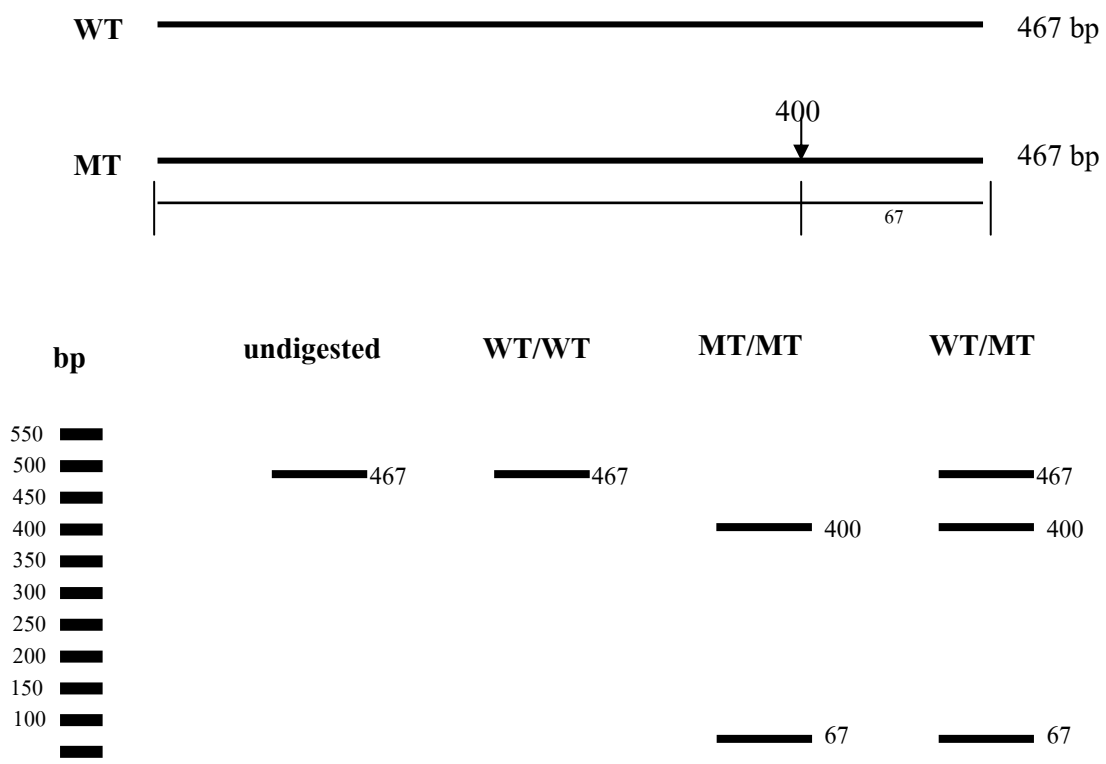

Figure 4.6 Diagram showing PCR-BmgBI digest of IVS6 - 116A/G SNP

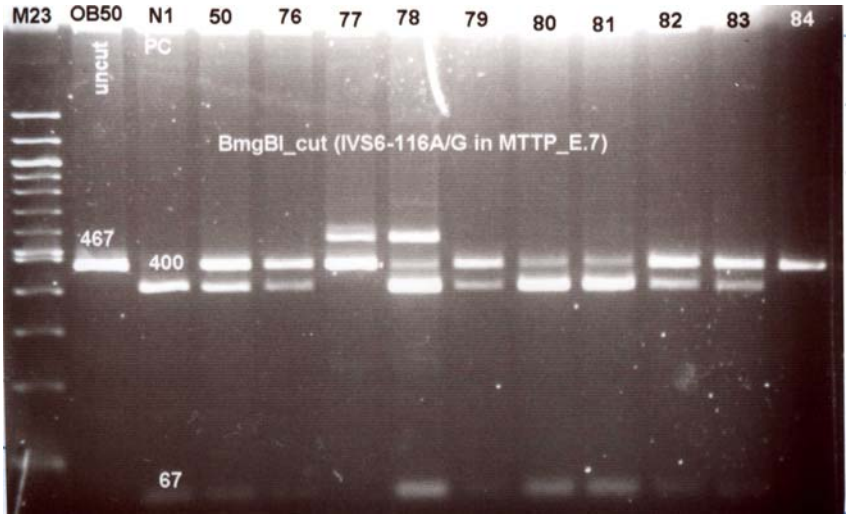

Figure 4.7 Restriction fragment length polymorphism of IVS6 - 116A/G in *MTTP* gene. Lane M23 is 100 bp marker. Lane OB50 (uncut) represents undigested PCR product. Lane N1 is positive control (PC). Lanes OB78 is homozygous mutant. Lanes OB50, OB76, and OB79-OB83 are heterozygous mutant. Lane OB77 is homozygous wild type. The upper most band in OB77 and 78 probably represent nonspecific products.

4.4 Single nucleotide polymorphism of 891C/G in *MTTP* gene

PCR containing 891C/G site, in exon 7 of *MTTP* gene, were shown in Figure 4.4. PCR product of 891C/G was digested by BsrI. The BsrI is restriction enzyme that cut 5'…ACTGGN▼...3'. The largest fragment was undigested (467 bp). Wild type (WT/WT) had 2 bands, 393 bp and 74 bp. The homozygote (MT/MT) had 3 bands, 313 bp, 80 bp and 67 bp. The heterozygote (WT/MT) had 4 bands, 393 bp, 313 bp, 80 bp and 67 bp. Figure 4.8 showed pattern of PCR-RFLP of 891C/G SNP. The 891C/G SNP creates an additional BsrI cut site, while the wild type allele has only one cut site.

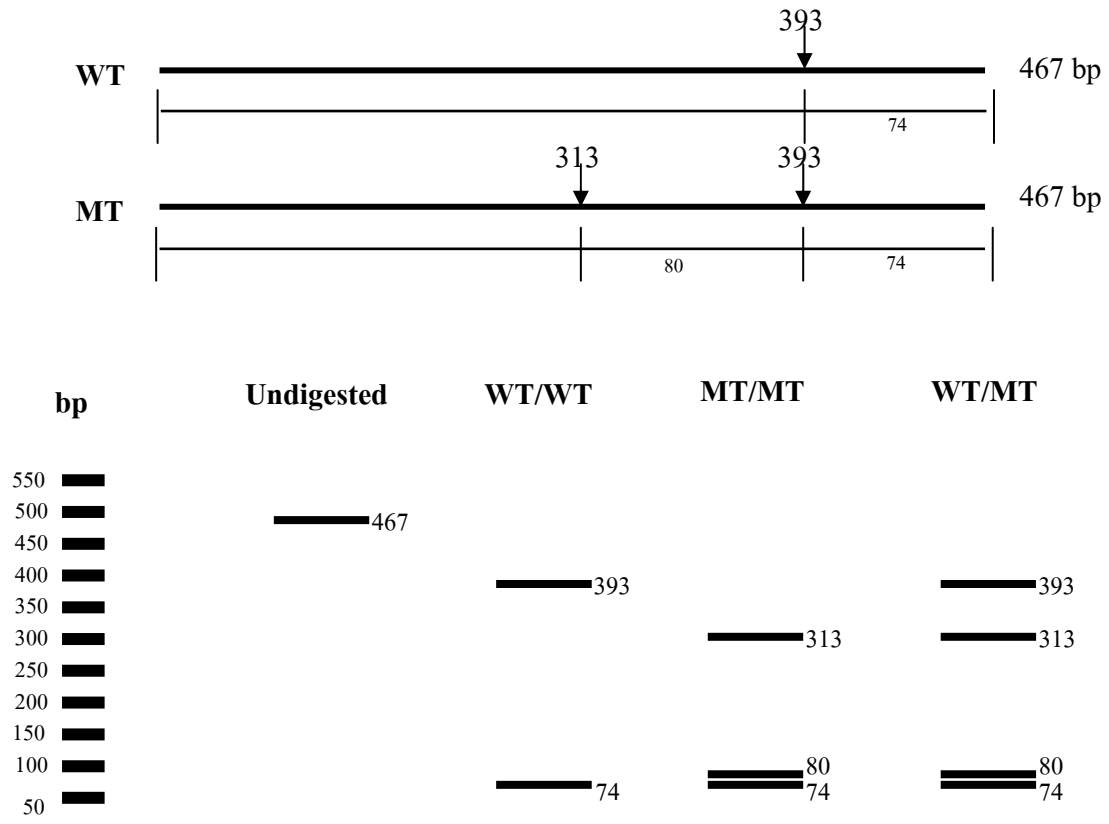

Figure 4.8 Diagram showing PCR-BsrI digest of 891C/G SNP

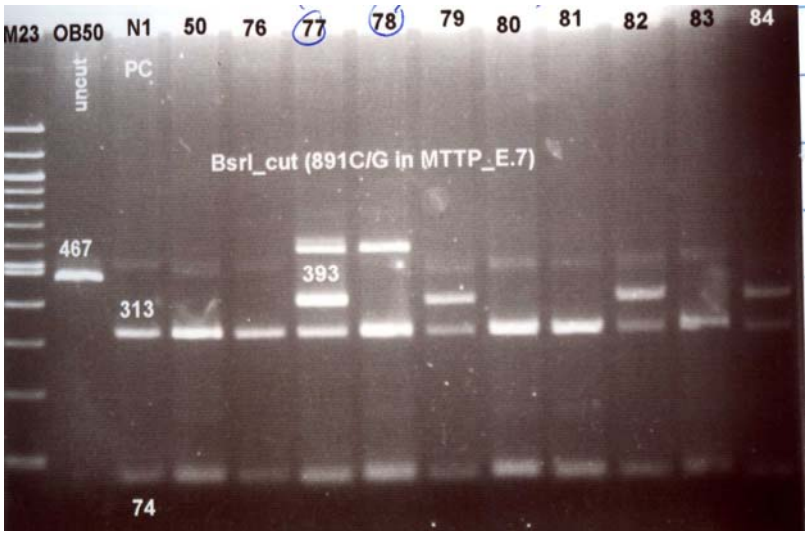

Figure 4.9 Restriction fragment length polymorphism of 891C/G in *MTTP* gene. Lane M23 is 100 bp marker. Lane OB50 (uncut) represented undigested PCR product. Lane N1 is positive control (PC). Lanes OB50, OB76, OB78, OB80, OB81 and OB83 are homozygous mutant. Lanes OB77, OB79, OB82 and OB84 are heterozygous mutant. There is no homozygous wild type shown in this picture.

4.5 Single nucleotide polymorphism of 293C/T (T71I) in *APOB* gene

PCR containing T71I site, in exon 4 of *APOB* gene, are shown in Figure 4.10.

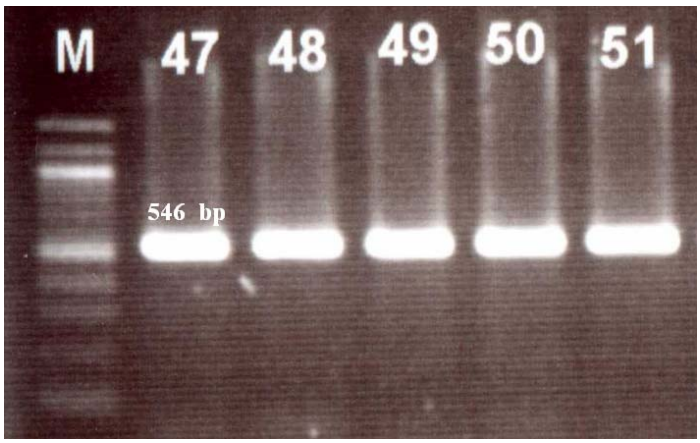

Figure 4.10 Polymerase chain reaction product of 293C/T (T71I), in exon 4 of *APOB* gene; Lane M is 100 bp marker; Lane.47-51 are subjects' PCR products.

PCR product of T71I was digested by ApaLI. The ApaLI is restriction enzyme that cut 5'…G▼TGCAC...3'. The largest fragment was undigested (546 bp). Wild type (WT/WT) had 3 bands, 225 bp 168 bp and 153 bp. The homozygote

(MT/MT) had 2 bands, 378 bp and 168 bp. The heterozygote (WT/MT) had 4 bands, 378 bp, 225 bp, 168 bp and 153 bp. Figure 4.11 showed pattern of PCR-RFLP of the 293C/T (T71I) SNP. The wild type allele contains two ApaLI restriction site. The 293C/T allele abolishes an ApaLI restriction site.

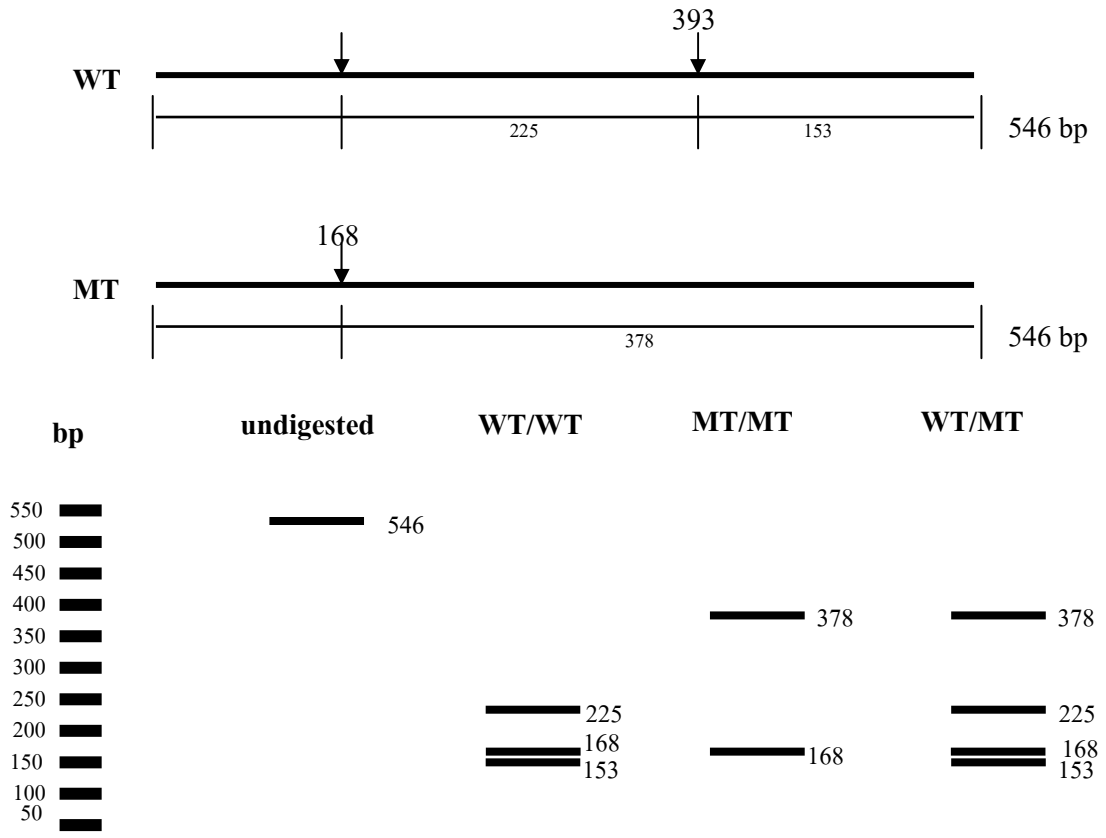

Figure 4.11 Diagram showing PCR-ApaLI digest of 293C/T (I71T) SNP

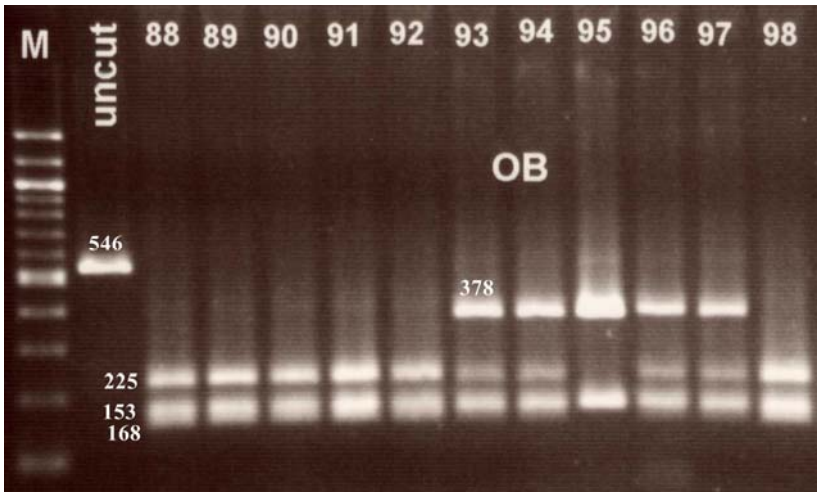

Figure 4.12 Restriction fragment length polymorphism of T71I in *APOB* gene. Lane M is 100 bp marker. Lane uncut represent undigested PCR product. Lanes OB95 is homozygous mutant. Lanes OB93, OB94, OB96 and OB97 are heterozygous mutant. Lane OB80-OB92 and OB98 are wild type.

4.6 Single nucleotide polymorphism of 7545C/T (T2488T) in *APOB* gene

PCR containing T2488T site, in exon 26 of *APOB* gene, are shown in Figure 4.13.

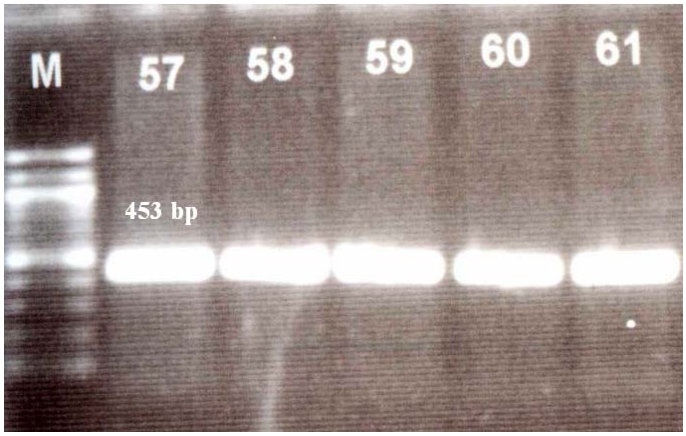

Figure 4.13 Polymerase chain reaction product of 7545C/T (T2488T), in exon 26 of *APOB* gene; Lane M is 100 bp marker; Lane 57-61 are subjects' PCR products.

PCR product of T2488T was digested by XbaI. The XbaI is restriction enzyme that cut 5'…T▼CTAGA...3'. The largest fragment or wild type (453 bp) was defined as WT/WT. The homozygote (MT/MT) had 2 bands, 238 bp and 215 bp. The heterozygote (WT/MT) had 3 bands, 453 bp, 238 bp and 215 bp. Figure 4.14 showed

pattern of PCR-RFLP of the 7545C/T (T2488T) SNP. The mutant creates an XbaI restriction site.

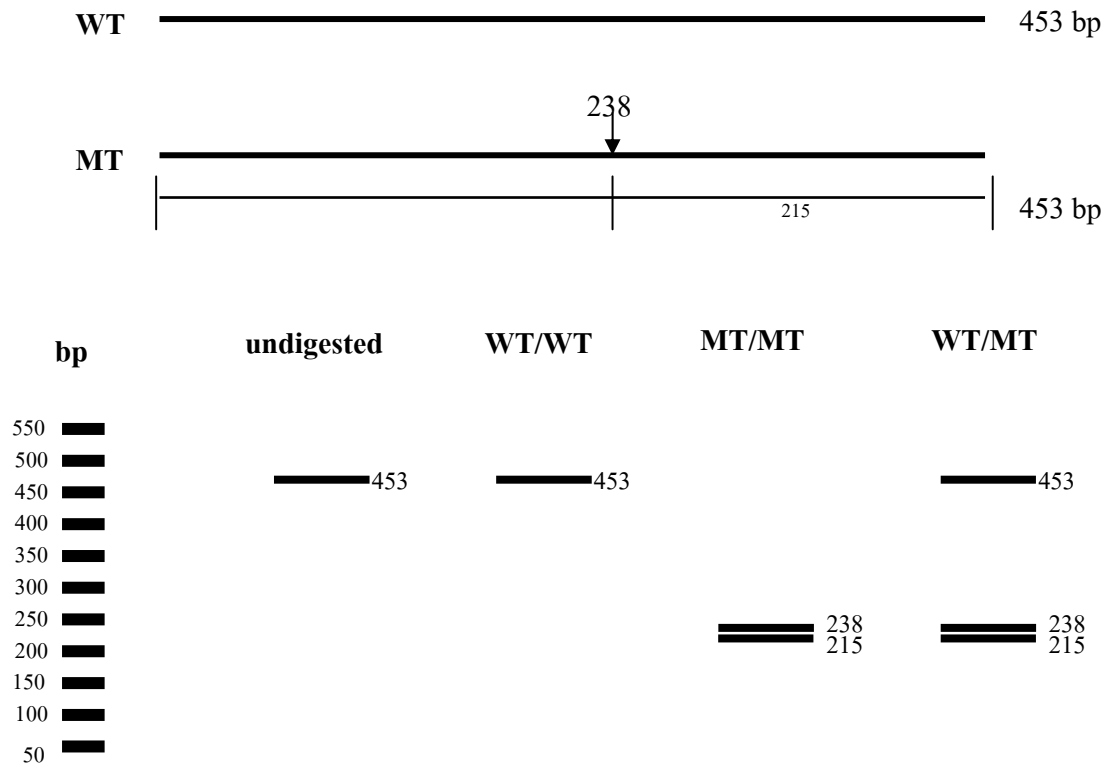

Figure 4.14 Diagram showing PCR-XbaI digest of 7545C/T (T2488T) SNP

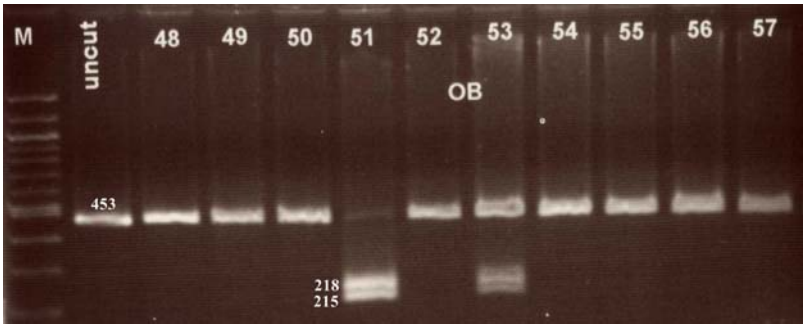

Figure 4.15 Restriction fragment length polymorphism of T2488T in *APOB* gene. Lane M is 100 bp marker. Lane uncut represent undigested PCR product. Lanes OB51 is homozygous mutant. Lanes OB53 is heterozygous mutant. Lane OB48-OB50, OB52 and OB54-OB57 are wild type.

4.7 Single nucleotide polymorphism of 10913G/A (R3611Q) in *APOB* gene

PCR containing R3611Q site, in exon 26 of *APOB* gene, were shown in Figure 4.16.

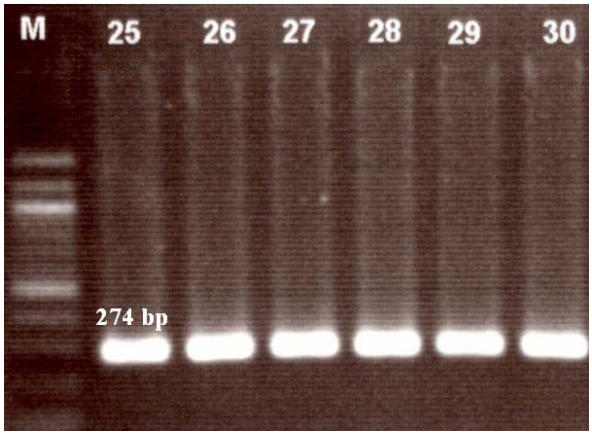

Figure 4.16 Polymerase chain reaction product of 10913G/A (R3611Q), in exon 26 of *APOB* gene; Lane M is 100 bp marker; Lane 25-30 are subjects' PCR products.

PCR product of R3611Q was digested by MspI. The MspI is restriction enzyme that cut 5'…C▼CGG...3'. The largest fragment or homozygote (274 bp) was defined as MT/MT. The Wild type (WT/WT) had 2 bands, 170 bp and 104 bp. The heterozygote (WT/MT) had 3 bands, 274 bp, 170 bp, and 104 bp. Figure 4.17 showed pattern of PCR-RFLP of the 10913G/A SNP. The mutant lose MspI restriction site.

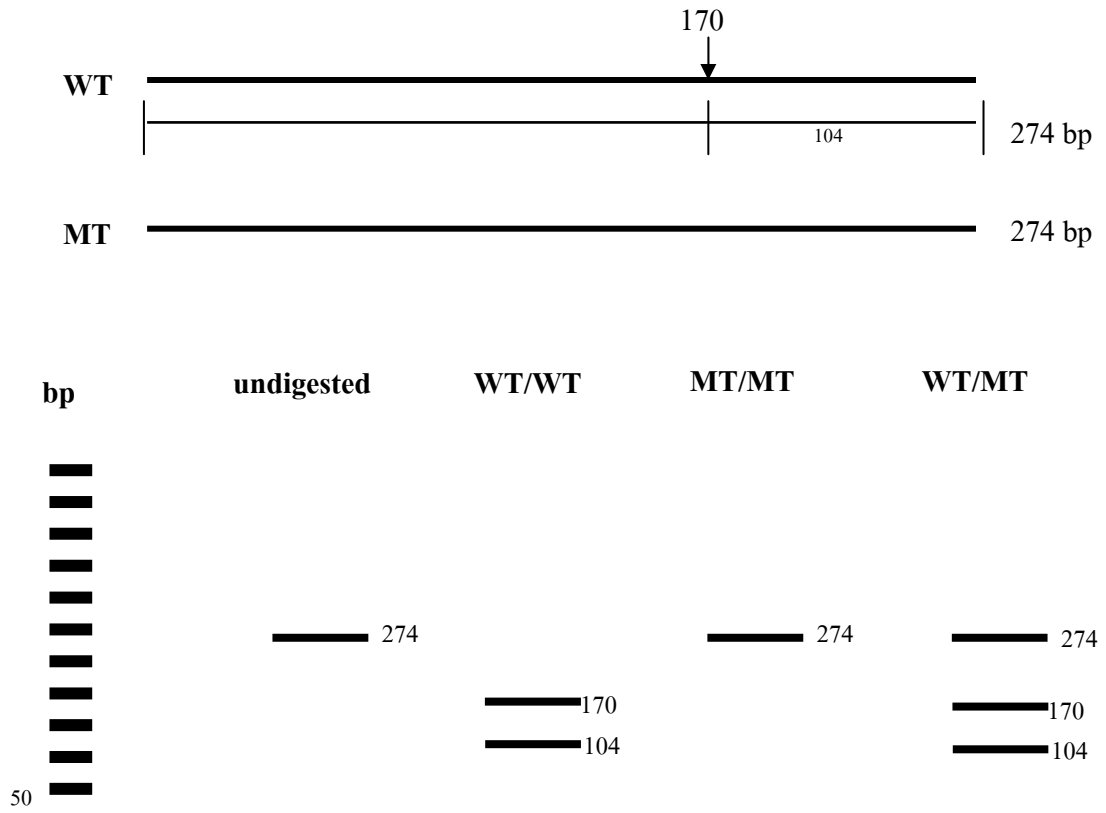

Figure 4.17 Diagram showing PCR-MspI digest of 10913G/A SNP

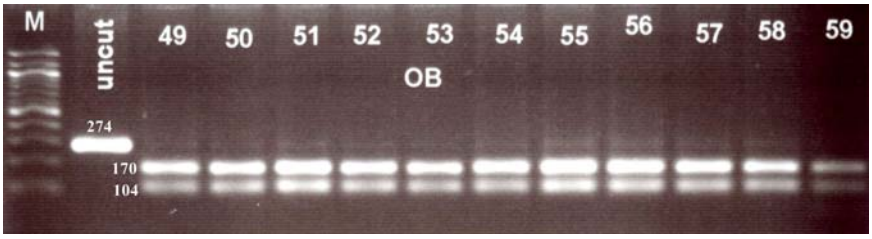

Figure 4.18 Restriction fragment length polymorphism of R3611Q in *APOB* gene. Lane M is 100 bp marker. Lane uncut represent undigested PCR product. Lanes OB49-OB59 are wild type.

4.8 IVS18+1708 C/A single nucleotide polymorphism of *APOB* by ARMS

The extracted DNA was prepared 2 tubes per 1 sample. One tube was normal tube and another tube was mutant tube. Both tubes were amplified by polymerase chain reaction (PCR) at same condition.

The wild type (WT/WT) had band only in normal tube and the homozygouse mutant (MT/MT) had band only in mutant tube. The heterozygote (WT/MT) revealed PCR product in both tubes. Figure 4.19 showed pattern of PCR product of IVS18 + 1708C/A.

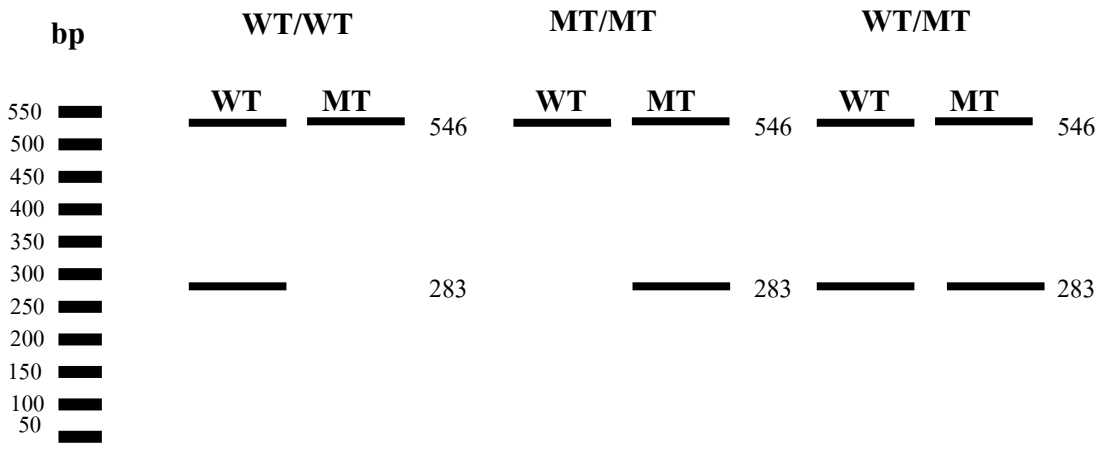

Figure 4.19 Diagram showing pattern of verified PCR amplification produced by amplification-refractory mutation system (ARMS). The 546 bp product represent internal control.

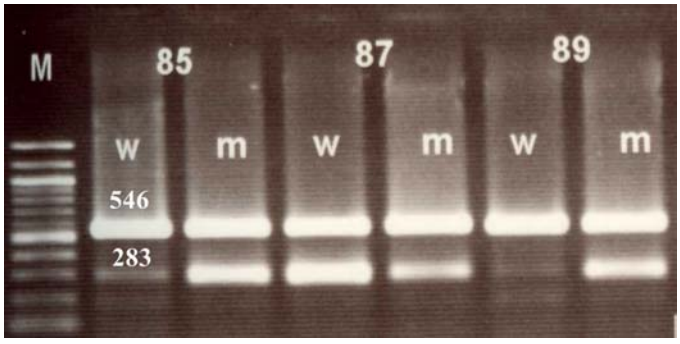

Figure 4.20 Restriction fragment length polymorphism of IVS18 + 1708C/A in *APOB* gene. Lane M is 100 bp marker. Lanes 85 and 89 are homozygous mutant. Lanes 87 are heterozygous mutant.

4.9 Allele frequencies of SNPs in *MTTP* and *APOB* genes

We had conducted a pilot study by performing genotyping *MTTP* and *APOB* polymorphisms in 100 blood donors at Ramathibodi Hospital. This was aimed to make

sure that those interested polymorphisms were able to identify in our setting population. Genotype and allele frequencies of the pilot and studied subjects were quite similar, as described in Table 4.5. Hardy-Weinberg equilibrium (HWE) was assessed for each SNP and found that all SNPs were complied with HWE rule except *APOB* IVS18+1708 polymorphism (p=0.011).

Table 4.5 Genotype distribution and allele frequencies of *MTTP* and *APOB* SNPs in regular blood donors and obese children

SNP		Blood donors (N=100)	Obese children (N=99)	HWE*
MTTP				
453T>C				
Number of individual	TT	19	23	
	TC	53	42	
	CC	28	34	
Allele frequency	T	0.45	0.44	0.16
	C	0.55	0.56	
IVS6-116A>G				
Number of individual	AA	38	48	
	AG	51	42	
	GG	11	9	
Allele frequency	A	0.64	0.70	1.000
	G	0.36	0.30	
891C>G				
Number of individual	CC	21	22	
	CG	47	41	
	GG	32	36	
Allele frequency	C	0.45	0.43	0.15
	G	0.55	0.57	

**Exact significance probability*

Table 4.5 (CONT.)

SNP		Blood donors (N=100)	Obese children (N=99)	HWE*
APOB				
T71I				
Number of individual	CC	70	70	
	CT	27	27	
	TT	3	2	
Allele frequency	C	0.84	0.84	1.000
	T	0.16	0.16	
T2488T				
Number of individual	CC	81	80	
	CT	18	17	
	TT	1	2	
Allele frequency	C	0.9	0.89	0.29
	T	0.1	0.11	
R3611Q				
Number of individual	GG	100	98	
	GA	0	1	
	AA	0	0	
Allele frequency	G	1	0.99	1.000
	A	0	0.01	
IVS18+1708C>A				
Number of individual	CC	57	71	
	CA	37	21	
	AA	5	7	
Allele frequency	C	0.76	0.82	0.011
	A	0.24	0.18	

**Exact significance probability*

4.10 Relationship between *MTTP* and *APOB* genes and LDL-C level

Relationships between genes and LDL-C were assessed as described in Table 4.6. Only *MTTP* 891C/G polymorphism was significantly correlated with LDL-C level (p=0.016). Multiple comparison using Scheffe method was applied and found that mean LDL-C levels were higher for CG and GG genotypes than CC genotype (p = 0.016 for both).

Although the genetic effect of *MTTP* 453T/C polymorphism was not statistically significant (p=0.057), there was a trend of correlation. This was mean LDL-C were higher in TC, and TT genotypes (mean = 124.3 mg/dl and 118.3 mg/dl, respectively) compared with CC genotype (mean = 108.0 mg/dl).

Table 4.6 LDL-C levels according to genotypes

SNP	N	LDL-C (mg/dl)	P-value
MTTP			
453T/C			
TT	23	108.04±27.15	0.057
TC	42	124.3±26.3	
CC	34	118.3±23.98	
IVS6 - 116A/G			
AA	48	115.6±27.98	0.585
AG	42	121.2±25.5	
GG	9	120.8±20.01	
891C/G			
CC*	22	106.5±26.73	0.016
CG*	41	126.04±25.93	
GG	36	117.1±23.8	
APOB			
T71I			
CC	70	117.2±26.8	0.485
CT/TT	29	121.3±25.1	
T2488T			
CC	80	119.1±26.2	0.634
CT/TT	19	115.8±26.7	
IVS18 + 1708C/A			
CC	71	119.6±27.2	0.653
CA	21	117±22.5	
AA	7	110.4±28.6	

** Scheffe's test, P= 0.016*

4.11 Association between *MTTP* and *APOB* polymorphisms and abnormal LDL-C

LDL-C level was classified as abnormal if the level was higher than the 95th percentile for sex and age. There were 20 and 79 subjects classified as abnormal and normal LDL-C, respectively. Most subjects in normal and abnormal groups were adolescents (70.9% and 80%, respectively). The majority of both groups were males (55.7% and 70%, respectively).

Table 4.7 Comparison of characteristics between obese children with normal and abnormal LDL-C levels

Characteristics	Normal (n=79)	Abnormal (n=20)	P-value
Age(yr)	10.9±2.6	11.6±2.8	0.35
Sex			
Male	44 (55.7%)*	14 (70%)*	
Female	35 (44.3%)*	6 (30%)*	
Weight (kg)	69.8±24.1	72.0±24.7	0.72
Height (cm)	151.2±15.6	152.5±15.8	0.72
Waist (cm)	90.7±12.2	91.4±14.4	0.84
WHR	0.9 ± 0.07	0.89±0.06	0.83

Values are mean ± SD

** N(%), number*

The association between genotype frequencies of polymorphisms and abnormal LDL-C were described (Table 4.8). Although there was no statistically significant association between polymorphisms and abnormal LDL-C, there was a trend of association for some polymorphisms. For instance, the *MTTP* 453T/C polymorphism with TC was about 1.5 times more likely to have abnormal LDL-C than TT genotypes. For the *MTTP* IVS6 -116A/G polymorphism, the AG genotype was about 33% lower risk to have abnormal LDL-C than AA genotype. For *MTTP* 891C/G polymorphism, CG and GG genotypes were 2.3 and 1.3 times more likely to have abnormal LDL-C than CC genotype. For the *APOB* at I71T and 2488T polymorphism, the CT and TT genotypes were about 1.5 times higher risk of having abnormal LDL-C

than CC genotype**.** For the *APOB* IVS18 + 1708C/A polymorphism, CC and CA genotype were 1.6 and 1.4 times more likely to have abnormal LDL-C than AA genotype.

Table 4.8 Association between SNPs and abnormal LDL-C in obesity

SNP	LDL-C		OR(95%CI)	P-value
	Normal (N=79)	Abnormal (N=20)		
MTTP				
453T/C				
TT	19	4	1	
TC	32	10	1.48(0.41-5.40)	0.54
CC	28	6	1.02(0.25-4.1)	0.98
IVS6 - 116A/G				
AA	37	11	1	
AG	35	7	0.67(0.23-1.93)	0.46
GG	7	2	0.96(0.17-5.31)	0.96
891C/G				
CC	19	3	1	
CG	30	11	2.32(0.57-9.41)	0.24
GG	30	6	1.27(0.28-5.68)	0.76
APOB				
T71I				
CC	57	13	1	
CT/TT	22	7	1.4 (0.49-3.96)	0.53
T2488T				
CC	65	15	1	
CT/TT	14	5	1.55(0.48-4.96)	0.46
IVS18 + 1708C/A				
CC	56	15	1.61(0.18-14.39)	0.67
CA	17	4	1.41(0.13-15.27)	0.78
AA	6	1	1	

Values are numbers of subjects.

CHAPTER V
DISCUSSION

The results of this study have demonstrated the effect of *MTTP* SNPs and *APOB* SNPs on plasma LDL-C level of obese children. Moreover, the results in this study indicate that one SNP in *MTTP* gene was associated with high plasma LDL-C level. However, SNPs did not affect the occurrence of dyslipidemia in obese children.

5.1 Effect of obesity on LDL-C level

In 99 obese children of this study, approximately one-fifth of them had abnormal blood lipids. However, the mean values of LDL-C were not significantly different among various classes of obesity. A case-control study in Mexican school children found that obesity was associated with abnormal values for cholesterol, triglycerides, LDL-C, and HDL-C.[85]

5.2 Effect of *MTTP* SNPs on LDL-C level

There have been few studies regarding the association between *MTTP* SNPs and cholesterol and betalipoprotein levels. Almost all of the studies were conducted in adults.

In this study, 3 SNPs of *MTTP* gene, the 453T/C (I128T) in exon 4 and the IVS6 - 116A/G, the 891C/G (H297Q) in exon 7 were tested. The results indicated that the heterozygous 891C/G had significantly higher plasma LDL-C level. The 453T/C and IVS6 - 116A/G had no significant effect on LDL-C level. However, homozygous mutant (GG) of 891C/G SNP was not associated with high LDL-C level. All the same in Sweden, study concerning stability and ligand binding properties of Ile128Thr polymorphism in *MTTP* gene found that Ile128Thr polymorphism reduced structural stability, which leads to decreased binding of *MTTP* to *APOB*.[86]

As shown in Table 5.1, it seems that the 453C (T>C) and the 891G (C>G) alleles are more prevalent in Thai population (0.56 and 0.57, respectively) than in European population (0.24 and 0.23-0.32, respectively).[69, 87]

Table 5.1 Comparison of allele frequencies of *MTTP* SNPs between Thai and other population

SNP	This study	Author/year/ study population	
		Ledmyr H et al./2002/ Swedish [69]	Talmud PJ et al./2000/ UK[87]
453C	0.56	0.24	NA
IVS6-116G	0.3	NA	NA
891G	0.57	0.23	0.32

NA, not available

A study in Canada found that 891C>G was association with visceral obesity[68]. In a British study, individual with homozygous mutation of 891C>G had higher triglyceride levels, and carriers of 493T allele tended to have higher level of apoB. Functional study of 453T>C polymorphism revealed a reduced structural stability in the N-terminal domain, leading to reduction of MTTP and LDL particles binding capacity of the mutant apoB.[88]

5.3 Effect of *APOB* SNPs on LDL-C level

There were few studies of mutation in patients with familial hypercholesterolemia. We have studied 4 SNPs of *APOB* gene, 293C/T (T71I) in exon 4, 7545C/T (T2488T) and 10913G/A (R3611Q) in exon 26 and IVS18 + 1708C/A in intron 18. The results indicate that the SNPs of *APOB* gene are not significantly associated with LDL-C level.

As shown in Table 5.2, the allele frequencies of 4 SNPs of *APOB* gene in this study were lower than those found in Danish, Senegal and thai.[81, 82, 89-91] This discrepancy may be explained by the difference in allele frequencies in each race and the smaller sample size of this study.

For IVS18 + 1708C/A of *APOB* deviated from Hardy-Weinberg expectation because of selection subject group was small.

Table 5.2: Comparison of allele frequencies of *APOB* SNPs between Thai and other population

SNP	Author/year/ study population				
	This study/ Thai	Saiphon P /2000/ Thai[91]	Chauffert M et al. /1997/ Senegal[89]	Bentzen JJT et al /2002/ Danish [81]	Marianne B et al. /2008/ Danish [82]
71 I	0.16	NA	NA	0.64	0.67
2488T	0.11	NA	0.79	0.47	0.48
3611Q	0.01	0.02	0.04	0.91	0.91
Ivs18+1708A	0.18	NA	NA	NA	0.45

NA, not available

Table 5.3 shows the comparison of *APOB* SNP effect on lipid levels between this study and the Danish study. This study found no effect of *APOB* SNPs on LDL-C level but the Danish study found that for the *APOB* I71T polymorphism, I-allele was associated with increased LDL-C level. For the *APOB* T2488T polymorphism, C-allele reduced LDL-C. For the *APOB* R3611Q polymorphism, Q-allele was associated with increased LDL-C level. For the *APOB* IVS18 + 1708C/A polymorphism, A-allele was associated with increased LDL-C level.[81, 92] This study was different from the Danish study because the sample size of this study (n=99) was much smaller than the Danish study (n=2,656 and n=9,259, respectively) and the ethnics were different between studies.

Table 5.3 Effect of single nucleotide polymorphisms on lipid level: comparison between Danish and this study

SNP	This Study	Bentzen et al./2002/ Danish [81]	Marianne et al./2008/ Danish [82]
T71I	LDL-C levels in I-allele and T-allele were not significantly different.	LDL-C and TC levels in I-allele were higher than T-allele. I-allele raised LDL-C and TC levels.	TC, LDL-C, and *APOB* of I-allele were higher than T-allele. T71I was associated with increased LDL-C level.
T2488T	LDL-C levels in C-allele and T-allele were not significantly different.	LDL-C, VLDL-C, TC, and TG of T-allele were higher than C-allele. C-allele reduced LDL-C, VLDL-C, TC, and TG level.	TC, LDL-C, and *APOB* in T-allele were higher than C-allele. T2488T was associated with increased effect LDL-C level.
R3611Q	LDL-C levels in A-allele and G-allele were not significantly different.	In Q3611Q, subjects who smoked >25 g tobacco/wk had lower BMI than those who never smoked.	TC, LDL-C, and *APOB* in Q- allele were higher than R-allele. R3611Q was associated with increased LDL-C.
IVS18+1708C>A	LDL-C levels in C-allele and A-allele were not significantly different.	NA	Ivs18+1708C>A was associated with increased LDL-C level.

Previous studies suggested that I71T polymorphism was associated with *APOB* levels but not with lipids because the region containing codon 71 of *APOB* is responsible for assembly and secretion of VLDL particles in the liver.[93, 94] Because of I71T polymorphism, MTTP protein cannot bind apoB. Thus, secretion of VLDL was reduced.

Regarding the association of T2488T polymorphism with lipid levels, recent study found that the C allele is associated with lower levels of triglyceride[95], cholesterol, and LDL-C. The heterozygous mutant is associated with the highest triglyceride but the lowest cholesterol and LDL-C.[96]

5.4 Summary

From the result of the present study, we found that 1 SNP (891C/G) of *MTTP* gene and no *APOB* SNPs were associated with elevated plasma LDL-C level in obese children. This study found no effect of *MTTP* and *APOB* SNPs on abnormal LDL-C level.

5.5 Limitation of this study

The study enrolled 99 subjects because of limited time and budget. Small numbers of participants can cause unclear association between SNP variation and abnormal LDL-C level.

5.6 Suggestion for future research

The association between these SNPs with other lipid types, i.e. triglyceride and total cholesterol, as well as with apolipoprotein B levels and metabolic syndrome should be studied.

The statistical powers in this study are shown in Table 5.4. With our sample size of 99 subjects, the statistical power ranges from 0.11-0.54 and 0.07-0.83 for the slopes of 5 and 10, respectively.

Table 5.4 Statistical power of association study for SNPs and LDL-C levels

SNPs	Statistical power	
	Slope (λ) = 5	Slope (λ) = 10
MTTP		
453T/C	0.30	0.82
IVS6 - 116A/G	0.23	0.68
891C/G	0.31	0.83
APOB		
T71I	0.13	0.40
T2488T	0.11	0.31
R3611Q	0.54	0.07
IVS18 + 1708C/A	0.20	0.62

CHAPTER VI
CONCLUSION

Conclusion

The presence of 891C/G SNP of *MTTP* gene was associated with increased plasma LDL-C level in obese children.

Limitation of this study

The study enrolled 99 subjects because of limited time and budget. Small numbers of participants can cause unclear association between SNP variation and abnormal LDL-C level.

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APPENDIX

APPENDIX

Reagents that used in this study were laboratory and molecular biological grade.

Reagents for DNA extraction from human blood sample

1. Lysis buffer
2. 0.1% Nonidet
3. Proteinase K
4. 10% SDS
5. 10X STE
6. Phenol
7. Chloroform
8. Sodium acetate
9. Absolute ethanol
10. 70% ethanol
11. TE buffer

Reagents for polymerase chain reaction (PCR)

1. Sterile water
2. 10X NH_4 buffer
3. 50mM $MgCl_2$
4. 2mM dNTPs
5. 5μM forward primer
6. 5μM reverse primer
7. 5U tag DNA polymerase
8. 100 ng/μl DNA

Reagents for restriction fragment length polymorphism (RFLP)

1. Sterile water
2. 10X buffer
3. Restriction enzyme
4. 5U primer

5. PCR solution

Reagents for Amplification-refractory mutation system (ARMS)

1. Sterile water
2. 10X NH_4 buffer
3. 25mM $MgCl_2$
4. 2mM dNTPs
5. 5μM forward primer
6. 5μM reverse primer normal
8. 5μM reverse primer mutant
9. 5μM forward primer control (I71T)
10. 5μM reverse primer control (I71T)
11. 5U tag DNA polymerase
12. 100 ng/μl DNA

Reagents for agarose gel electrophoresis

1. Agarose
2. 1× TBE
3. Ethidium bromide

แบบสอบถามการบริโภคอาหาร

ผู้สัมภาษณ์ ……. **วันที่** …….

ประวัติจาก ☐ ผู้ป่วย

☐ มารดา☐ บิดา ☐ อื่นๆระบุ

……

ชื่อ-นามสกุลผู้ป่วย ……..……. H.N. ……..

วัน/เดือน/ปี เกิด …….. อายุปัจจุบัน ……..…… ปี ……...…… เดือน

น้ำหนัก …… กก. ส่วนสูง ……... ซม.

ที่อยู่ บ้านเลขที่.....หมู่ที่.....ซอย.....ถนน .....

ตำบล/แขวง.....อำเภอ/เขต.....จังหวัด.....

รหัสไปรษณีย์.....โทรศัพท์.....

พฤติกรรมการรับประทานอาหาร

รับประทานอาหารมื้อหลักวันละ ……..…… มื้อ

ข้าวเช้า ☐ กิน ☐ ไม่ได้กิน

น้ำมันที่ใช้เป็นประจำที่บ้าน (ตอบได้มากกว่า 1 อย่าง)

☐ น้ำมันถั่วเหลือง ☐ รำข้าว ☐ มะพร้าว ☐ ปาล์ม ☐ หมู ☐ ไก่

☐ อื่นๆระบุ ……..

ปริมาณที่ใช้ต่อมื้อ.....

อาหารที่แพ้ ☐ ไม่มี ☐ มีระบุชนิดอาหารและอาการ

……

ผลิตภัณฑ์เสริมอาหาร / อาหารเสริม (เช่น ซุปไก่สกัด ชาเขียว รังนก)

☐ ไม่รับประทาน

☐ รับประทาน ระบุชนิด ……. ความถี่ …… ครั้ง/สัปดาห์ ปริมาณต่อครั้ง …….

อาหาร ฟาสต์ฟู้ด (เช่น พิซซ่า ไก่ทอด แฮมเบอร์เกอร์ โดนัท)

☐ ไม่รับประทาน

☐ รับประทาน เฉลี่ย เดือนละ …… ครั้ง ระบุชนิด ……...

การกินขนม ใน 1 สัปดาห์ก่อนมาโรงพยาบาล (หรือ 1 สัปดาห์ก่อนการเจ็บป่วย)

- ขนมถุง กรอบๆ เช่น มันฝรั่ง ครั้งละ …….. ถุง ระบุยี่ห้อที่กินบ่อย ……
  ☐ ทุกวัน ☐ 5-6 วัน/สัปดาห์ ☐ 3-4 วัน/สัปดาห์
  ☐ 1-2 วัน/สัปดาห์ ☐ ไม่กิน
- น้ำอัดลม ครั้งละ …….. ขวด/กระป๋อง ระบุยี่ห้อที่กินบ่อย ……
  ☐ ทุกวัน ☐ 5-6 วัน/สัปดาห์ ☐ 3-4 วัน/สัปดาห์
  ☐ 1-2 วัน/สัปดาห์ ☐ ไม่กิน
- น้ำชา
  ☐ ทุกวัน ☐ 5-6 วัน/สัปดาห์ ☐ 3-4 วัน/สัปดาห์
  ☐ 1-2 วัน/สัปดาห์ ☐ ไม่กิน
- กาแฟ
  ☐ ทุกวัน ☐ 5-6 วัน/สัปดาห์ ☐ 3-4 วัน/สัปดาห์
  ☐ 1-2 วัน/สัปดาห์ ☐ ไม่กิน
- น้ำผลไม้
  ☐ ทุกวัน ☐ 5-6 วัน/สัปดาห์ ☐ 3-4 วัน/สัปดาห์
  ☐ 1-2 วัน/สัปดาห์ ☐ ไม่กิน
- ไอศกรีม
  ☐ ทุกวัน ☐ 5-6 วัน/สัปดาห์ ☐ 3-4 วัน/สัปดาห์
  ☐ 1-2 วัน/สัปดาห์ ☐ ไม่กิน
- ท็อฟฟี่/ช็อคโกแลต
  ☐ ทุกวัน ☐ 5-6 วัน/สัปดาห์ ☐ 3-4 วัน/สัปดาห์
  ☐ 1-2 วัน/สัปดาห์ ☐ ไม่กิน
- ขนมหวานอื่นๆที่ชอบ ระบุ ……..
  ☐ ทุกวัน ☐ 5-6 วัน/สัปดาห์ ☐ 3-4 วัน/สัปดาห์
  ☐ 1-2 วัน/สัปดาห์ ☐ ไม่กิน

พฤติกรรมการออกกำลังกาย และกิจกรรมต่างๆ

- การออกกำลังกาย ☐ สม่ำเสมอ ☐ นานๆครั้ง ☐ ไม่ชอบออกกำลังกาย
- ระยะเวลาในการออกกำลังกาย.....
- กิจกรรมอื่นๆ.....

แบบสอบถามความถี่ในการกินอาหารในช่วง 1 เดือนที่ผ่านมา

ชื่ออาหาร	ปริมาณที่กินแต่ละครั้ง	เดือน		สัปดาห์			วันละ	
		≤ 1 ครั้ง	2-3 ครั้ง	1-2 ครั้ง	3-4 ครั้ง	5-6 ครั้ง	1 ครั้ง	2-3 ครั้ง
1. ข้าว-แป้ง 1.1 ข้าว	…… ทัพพี (1 ทัพพี = ½ ถ้วยตวง) ที่เด็กกินบ่อยคือ ☐ ข้าวเจ้า ☐ ข้าวเหนียว							
1.2 ก๋วยเตี๋ยว, บะหมี่	…… ทัพพี (1 ทัพพี = ½ ถ้วยตวง)							
2. เนื้อสัตว์	…… ช้อนโต๊ะ ที่เด็กกินบ่อยคือ (ตอบได้มากกว่า 1 อย่าง) ☐ หมู ☐ ปลา ☐ ไก่ ☐ วัว ☐ กุ้ง ☐ อื่นๆระบุ ……..							
3. ไข่	…… ฟอง - ที่เด็กกินบ่อยคือ ☐ ไข่ไก่ ☐ ไข่เป็ด - กินทั้งฟอง ☐ ใช่ ☐ ไม่ใช่ ระบุ …….							
4. ผัก	…… ทัพพี (1 ทัพพี = ½ ถ้วยตวง) ผักที่เด็กกินบ่อยคือ …… ……..							
5. ผลไม้	…… ส่วน (1 ส่วน = ½ ถ้วยตวง) ผลไม้ที่เด็กกินบ่อยคือ …… …….							
6. นม 6.1 นมกล่อง	- ปริมาณ ☐ 200 ซีซี ☐ 250 ซีซี ☐ อื่นๆระบุ …….. - ชนิดพร่องมันเนย ☐ ใช่ ☐ ไม่ใช่ - รสที่เด็กกินบ่อยคือ ☐ รสจืด ☐ ช็อคโกแลต ☐ สตรอเบอร์รี ☐ รสหวานอื่นๆ							

ชื่ออาหาร	ปริมาณที่กินแต่ละครั้ง	เดือน		สัปดาห์			วันละ	
		≤ 1 ครั้ง	2-3 ครั้ง	1-2 ครั้ง	3-4 ครั้ง	5-6 ครั้ง	1 ครั้ง	2-3 ครั้ง
6.2 นม เปรี้ยว พร้อมดื่ม	ปริมาณ ☐ 200 ซีซี ☐ 120 ซีซี ☐ อื่นๆระบุ ……							
6.3โยเกิร์ต ชนิดถ้วย	ปริมาณ ☐ 150 ซีซี ☐ อื่นๆระบุ ……..							
6.4 น้ำเต้าหู้	…… แก้ว							
6.5 นม อื่นๆ ระบุ	.....							

SNPs in this study

SNP	rs
MTTP	
453T/C	991811
Ivs6-116A/G	2306984
891C/G	2306985
APOB	
T71I	1367117
T2488T	693
R3611Q	1801701
Ivs18+1708C>A	3791980

Subject data

ID	Age (year)	Sex	Weight (kg)	Height (cm)	Waist (cm)	Hip (cm)	TG (mg/dl)	Chol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
OB-001	10	1	111.4	162	112	113	173	169	33	102
OB-002	11	1	82.1	158.5	109	109	77	163	38	109
OB-003	14	1	104.7	175.4	110	117	107	198	53	124
OB-004	7	1	46.1	132	84	83	128	168	44	99
OB-005	8	2	38.2	128.2	72	81	292	213	42	113
OB-006	6	1	33.9	119	72.5	80	60	166	46	108
OB-007	11	2	94.7	155.8	107	125	113	225	65	137
OB-008	11	1	68.8	154.5	90	99.5	92	196	46	132
OB-009	11	1	55.5	152	83	90	41	217	38	171
OB-010	12	1	55.2	142.4	86.5	94	140	197	39	130
OB-013	11	1	62.5	158.3	81.3	98.5	57	150	33	105
OB-014	15	1	82.2	174	92	106	33	163	53	104
OB-015	5	2	45.6	121	90	91	75	153	39	100
OB-016	10	2	59.4	148.8	84	94.5	39	147	39	100
OB-017	15	1	88.7	167.6	104	105	455	198	32	112
OB-018	14	2	70.4	161.4	84	101.5	152	163	35	98
OB-020	14	1	65.9	153.7	87	97	78	181	37	129
OB-021	14	2	85.9	160.9	90	115	78	217	49	153
OB-022	13	2	87.2	167.2	86.5	119	112	166	35	109
OB-023	10	1	46.8	145	79.5	84.9	106	225	44	159
OB-024	8	2	63.9	133	96.4	105	81	198	41	142
OB-025	13	1	79.5	161	99.4	105.2	68	165	38	114
OB-026	8	2	39	130	80.5	81	98	161	34	108
OB-027	16	1	100.6	175	109	119	113	219	48	149
OB-028	9	1	46.3	141	82.2	85	57	154	49	94

ID	Age (year)	Sex	Weight (kg)	Height (cm)	Waist (cm)	Hip (cm)	TG (mg/dl)	Chol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
OB-029	13	1	200	172	144	170	138	139	44	68
OB-030	9	1	52.4	142.4	90.1	91.4	150	132	41	61
OB-031	16	2	90.6	167.8	88	114	47	153	49	95
OB-032	8	1	41.9	129	75.6	85	77	159	45	98
OB-033	12	2	77.8	166	87.2	107.9	57	124	36	77
OB-034	5	2	38.5	114.5	77.6	81.5	123	184	37	122
OB-035	10	2	43.5	137.4	72.4	82.7	190	201	37	127
OB-036	17	2	90.7	167	105.5	117	79	183	47	120
OB-037	5	1	33.3	112.7	79	78	79	164	43	106
OB-038	13	2	88.7	158.2	91.5	116.5	81	175	41	118
OB-039	10	1	52.7	148	84.5	88	73	166	46	105
OB-040	9	1	45.1	135	68.9	87.8	97	184	51	114
OB-041	14	2	83.7	168.4	88.5	105.9	67	152	46	93
OB-043	9	2	48.8	142.3	77.9	87.4	66	162	40	109
OB-044	10	1	54.5	142	81.7	93.5	36	159	59	92
OB-045	11	1	69.5	145	95.6	102	115	203	45	136
OB-046	11	1	72.6	150	95.4	100.5	79	187	46	125
OB-047	8	1	69.5	137.3	105.2	106.9	206	188	36	111
OB-048	12	1	81.6	160.2	94.5	107.8	51	172	38	124
OB-049	7	2	34.5	125.9	67.2	79.8	72	225	55	156
OB-050	12	2	76.6	162.2	86.5	106.4	116	165	30	112
OB-051	15	1	97.5	173.8	103.8	113	66	180	45	122
OB-052	8	2	58.7	149.3	92.5	98	127	235	56	159
OB-053	7	2	49.1	135	84.7	88.9	48	151	37	105
OB-054	8	1	46.3	139.7	78.5	87.2	51	188	53	125
OB-055	8	1	39.3	127.2	77.5	82.2	55	134	32	91

ID	Age (year)	Sex	Weight (kg)	Height (cm)	Waist (cm)	Hip (cm)	TG (mg/dl)	Chol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
OB-056	8	2	34.1	125.2	69.2	78.8	104	174	47	107
OB-057	6	1	31.5	117.3	70.7	76.2	91	211	42	151
OB-058	13	1	71.8	164	88.7	103.6	138	129	34	68
OB-059	8	1	54.5	134.4	86.4	93.5	80	169	61	92
OB-060	12	1	90.9	166	106	112	119	196	53	119
OB-061	12	1	95	169.6	102.7	116.6	107	202	38	143
OB-062	6	1	32.2	123.7	66.9	74.5	55	246	48	187
OB-063	11	1	78.4	164	101.4	100	89	189	39	132
OB-064	13	1	64.8	148	91.4	98.3	73	217	53	150
OB-065	7	2	38.8	129.8	76	84.5	93	186	55	113
OB-066	11	1	61.7	149.4	95.2	96.4	91	129	40	70
OB-067	12	1	71.2	168	89	102.5	70	192	39	139
OB-068	7	1	43.2	126.6	83.3	88	113	189	34	132
OB-069	11	1	59.5	151.5	90	92	82	171	47	107
OB-070	12	2	82.3	156.6	99.5	112	58	145	43	91
OB-071	11	2	88.7	159.1	110	113	202	163	52	71
OB-072	9	2	74.8	145.5	104.5	107	239	202	41	114
OB-073	13	2	87.9	159.2	97.2	112.8	99	138	28	91
OB-074	11	1	68.5	156.2	97	96.2	329	181	42	74
OB-075	11	1	91.4	171.8	96	115.2	61	140	44	84
OB-076	10	2	55.8	143.2	80.4	97.8	74	139	47	78
OB-077	11	1	67.8	157.6	90	99.5	136	207	44	136
OB-078	11	1	58.3	141.5	94	97.4	86	209	43	149
OB-079	14	1	125	172.4	123.5	134.2	66	208	44	152
OB-080	8	1	57.6	145	86.3	95.4	42	195	53	133
OB-081	11	2	72.7	163.8	83.4	102	104	197	46	131

ID	Age (year)	Sex	Weight (kg)	Height (cm)	Waist (cm)	Hip (cm)	TG (mg/dl)	Chol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
OB-082	12	2	78.7	158.6	90	107	50	243	44	189
OB-083	12	2	73.5	156.4	91	101	89	194	49	127
OB-084	12	2	74.8	155	85	107	62	205	53	139
OB-085	13	2	89.6	156.9	98	118.4	184	202	36	129
OB-086	13	2	69.5	153	82	103.8	95	125	36	70
OB-087	12	1	67.3	160	85.7	99	37	198	57	133
OB-088	12	2	78.3	160.8	91.5	107.5	86	192	49	125
OB-089	13	2	99.3	162.8	111	116.5	202	244	67	137
OB-090	12	2	66.7	149	81	99.5	75	181	44	122
OB-091	14	1	70.3	159	91.6	98.5	84	182	34	132
OB-092	13	1	89.2	163.6	98	111	181	230	46	148
OB-093	11	2	66.6	152	87	99.5	79	193	44	134
OB-094	13	1	77	172.8	99	105.2	113	192	46	124
OB-095	13	2	85.8	150	98	115	105	226	49	156
OB-096	14	1	77.3	158	95.7	104	100	231	70	142
OB-097	13	1	81.9	166.4	100	108.2	86	156	52	88
OB-098	13	2	70.4	155.4	83.5	103.4	115	206	53	131
OB-099	14	1	106.6	178.8	105	120.5	112	148	36	90
OB-100	14	1	71.6	160.4	84	104	146	222	56	138
OB-101	13	2	79.8	156.7	88.8	105.5	96	214	43	151
OB-102	12	1	97.2	155	113.2	107	256	242	54	137
OB-103	14	1	71.4	162	90	103	151	181	54	97

ID: identify number, Sex 1: boy, Sex 2: girl

BIOGRAPHY

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