SIMPLE METHOD FOR QUANTIFICATION OF SERUM SMALL DENSE LDL PARTICLES

NATTAPOL NIYOMPONG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(MEDICAL TECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2008

Thesis
Entitled

SIMPLE METHOD FOR QUANTIFICATION OF SERUM SMALL DENSE LDL PARTICLES

……
Mr. Nattapol Niyompong
Candidate

……
Asst.Prof. Pairoj Leelahakul,
D.Sc. (Nutrition)
Major-Advisor

……
Assoc.Prof. Kulnaree Sirisali,
M.Sc. (Clinical Pathology)
Co-Advisor

…….....
Assoc.Prof. Sudarat Manochiopinij,
Ph.D. (Biochemistry)
Co-Advisor

……
Asst.Prof. Auemphorn Mutchimwong,
Ph.D.
Acting Dean
Faculty of Graduate Studies

……
Lect. Ratana Lawung,
Ph.D. (Pure and Applied Biochemistry)
Chair
Master of Science Programme in
Medical Technology
Faculty of Medical Technology

Thesis
Entitled

SIMPLE METHOD FOR QUANTIFICATION OF SERUM SMALL DENSE LDL PARTICLES

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Medical Technology)
on
29 October, 2008

……...
Mr. Nattapol Niyompong
Candidate

……...
Assoc. Prof. Col. Thaval Rerk-Ngam,
Ph.D. (Higher Education)
Chair

……...
Asst.Prof. Pairoj Leelahakul,
D.Sc. (Nutrition)
Member

……
Assoc.Prof. Kulnaree Sirisali,
M.Sc. (Clinical Pathology)
Member

……...
Assoc.Prof. Sudarat Manochiopinij,
Ph.D. (Biochemistry)
Member

……
Asst.Prof. Auemphorn Mutchimwong,
Ph.D.
Acting Dean
Faculty of Graduate Studies
Mahidol University

……...
Assoc.Prof. Virapong Prachayasittikul,
Ph.D. (Microbiology)
Dean
Faculty of Medical Technology
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my major advisor Asst.Prof. Dr. Pairoj Leelahakul, Department of Clinical Chemistry, Faculty of Medical Technology, Mahidol University. I am deeply grateful to his extensive support, kindness attention and guidance in this thesis.

I would like to express my sincere gratitude and appreciation to my co-advisors Assoc.Prof. Kulnaree Sirisali and Assoc.Prof. Dr. Sudarat Manochiopinij, Department of Clinical Chemistry, Faculty of Medical Technology, Mahidol University for their kindly supervision, valuable suggestion and guidance throughout this research.

I am also sincerely grateful to Assoc. Prof. Phanee Pidetcha, Lect. Rattana Lawung and all staffs of Center of Medical Laboratory Services, Faculty of Medical Technology, Mahidol University for their kindness attention and obtaining the samples.

I would like to thank all of the lecturers and staffs in the Faculty of Medical Technology, Mahidol University, especially Department of Clinical Chemistry, Asst. Prof. Sureerut Porntadavity, and Mr. Lerson Suwannaton for their kindness support during my study. I also thank for friendship and kind suggestion of my friends.

I am grateful for the Graduate Scholarship of the Faculty of Medical Technology, Mahidol University for the partly supported during the time of my study.

I would like to thank the Faculty of Medical Technology, Mahidol University for supporting all the expenses and other utilities.

Last of all, but most of my mind, I wish to express my gratitude and deepest appreciation to my father, mother, members of my family for their infinite love, great attention, understanding and full encouragement which never be forgotten

Nattapol Niyompong

SIMPLE METHOD FOR QUANTIFICATION OF SERUM SMALL DENSE LDL PARTICLES

NATTAPOL NIYOMPONG 4838737 MTMT/M

M.Sc. (MEDICAL TECHNOLOGY)

THESIS ADVISORS: PAIROJ LEELAHAKUL, D.Sc., KULNAREE SIRISALI, M.Sc., SUDARAT MANOCHIOPINIJ, Ph.D.

ABSTRACT

Cardiovascular disease is a leading cause of global morbidity and mortality and is responsible for one-in-three deaths. It is very important to monitor LDL particle levels, especially small dense LDL particle levels which play an important role in cardiovascular disease. A modified method that can detect the small dense LDL particles and report in term of particle number is presented here. The samples were collected from the Center of Medical Laboratory Services, Faculty of Medical Technology, Mahidol University, during July to August 2008. They were categorized into 3 groups according to their LDL-C levels. These 3 groups of samples were subjected to separation of small dense LDL particles by ultracentrifugation and precipitation methods. The method validation was done by polyacrylamide gel electrophoresis (PAGE) and apo B-100 analysis. PAGE showed the good accuracy of separation of small dense LDL particles by precipitation method. Apo B-100 analysis demonstrated the good precision of this method. The %CV of each sample group was 3.91, 3.54, and 3.89, respectively. The total error of this quantification method was 12% that lay within the allowable total error of the apo B-100 determination. These results showed that the quantification method can be used in routine clinical laboratory with linearity of 100 – 180 mg/dL of LCL-C. The results from apo B-100 analysis were converted to small dense LDL particle number which is one of the crucial cardiovascular risk indicators. Mean of the apo B-100 levels in term of molecule number of each sample group was 2.51×10^{16} molecule/dL, 2.90×10^{16} molecule/dL, and 3.20×10^{16} molecule/dL respectively. Further study of the others parameters of method validation will be done to complete the simple method for quantification of serum small dense LDL particles.

KEY WORDS: SMALL DENSE LDL PARTICLE/ PRECIPITATION/ ULTRACENTRIFUGATION/ APO B-100 ANALYSIS

66 pp.

การตรวจวัดปริมาณโมเลกุล small dense LDL ในซีรั่มด้วยวิธีการอย่างง่าย
(SIMPLE METHOD FOR QUANTIFICATION OF SERUM SMALL DENSE LDL PARTICLES)

นัฐพล นิยมพงษ์ 4838737 MTMT/M

วท.ม. (เทคนิคการแพทย์)

คณะกรรมการควบคุมวิทยานิพนธ์: ไพโรจน์ ลีพหกุล, วท.ด., กุลนารี สิริสาลี, วท.ม., สุดารัตน์ มโนเชี่ยวพินิจ, พีเอช.ดี.

บทคัดย่อ

โรคหัวใจและหลอดเลือด เป็นสาเหตุสำคัญของการเสียชีวิตถึงหนึ่งในสามของประชากรทั่วโลก การตรวจวัดปริมาณของ small dense LDL particle จึงมีความสำคัญมาก เนื่องจาก small dense LDL particle เป็นสาเหตุหนึ่งในการก่อให้เกิดโรคหัวใจและหลอดเลือด การศึกษาครั้งนี้ ได้ทำการศึกษาวิธีตรวจวัดปริมาณ serum small dense LDL และทำการรายงานผลในรูปแบบของจำนวนโมเลกุล ไม่ใช่ในรูปแบบของปริมาณคลอเลสเตอรอลในโมเลกุลของ LDL อีกต่อไป โดยตัวอย่างเลือดทั้งหมด นำมาจากสถานเวชศาสตร์ชัณสูตร คณะเทคนิคการแพทย์ มหาวิทยาลัยมหิดล ตั้งแต่เดือนกรกฎาคม ถึงเดือนสิงหาคม พ.ศ.2551 และถูกแบ่งออกเป็น 3 กลุ่ม ตามระดับของ LDL-C หลังจากนั้น ตัวอย่างเลือดทั้ง 3 กลุ่ม จะถูกนำไปใช้ในการแยก serum small dense LDL particle ด้วยวิธี ultracentrifugation และวิธีการตกตะกอน หลังจากนั้นจึงทำ method validation เพื่อประเมินความน่าเชื่อถือของวิธีการตกตะกอน ซึ่งจากการวิเคราะห์ด้วยวิธี PAGE พบว่า วิธีการตกตะกอนมีความสามารถในการแยก serum small dense LDL particle ได้ถูกต้อง เมื่อเทียบกับวิธี ultracentrifugation ซึ่งเป็นวิธีมาตรฐาน และจากการวิเคราะห์ด้วยวิธี apo B-100 analysis พบว่าการวัดปริมาณ small dense LDL particle ด้วยวิธีการตกตะกอนนั้น มีความแม่นยำสูง โดยมีค่า %CV เท่ากับ 3.91, 3.54 และ 3.89 ตามลำดับ ในตัวอย่างตรวจที่มีความเข้มข้นของ LDL-C ระหว่าง 100 – 180 mg/dL และค่าที่ได้จากการวัดปริมาณ apo B-100 ได้ถูกแปลงให้เป็นหน่วยจำนวนโมเลกุลของ small dense LDL ซึ่งเป็นปัจจัยเสี่ยงที่สำคัญตัวหนึ่งของโรคหัวใจและหลอดเลือด โดยมีค่าเฉลี่ยจำนวนโมเลกุลของ small dense LDL ของแต่ละตัวอย่างเท่ากับ 2.51×10^{16} molecule/dL, 2.90×10^{16} molecule/dL, and 3.20×10^{16} molecule/dL ตามลำดับ จากการศึกษาครั้งนี้ พบว่า การแยก small dense LDL particle ด้วยวิธีการตกตะกอนนั้น สามารถนำมาใช้แทนวิธีการตรวจวิเคราะห์ดั้งเดิมได้ เนื่องจากค่า total error ของวิธีการตกตะกอนนี้ อยู่ในช่วง allowable total error ของวิธีการตรวจวัดปริมาณ apo B-100 สำหรับตัวอย่างตรวจที่มีความเข้มข้นในระดับอื่น และพารามิเตอร์ตัวอื่นๆ ในการทำ method validation นั้น จะได้มีการศึกษาในลำดับต่อไป

66 หน้า

CONTENTS

Page

CONTENTS (CONTINUED)

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AHA	American Heart Association
AMI	Acute myocardial infarction
Apo	Apolipoprotein
CAD	Coronary artery disease
CCR-2	Coordinate expression of chemokine receptor-2
CD36	Cluster differentiation 36 surface molecules
CE	Cholesterol esterase
CHD	Coronary heart disease
CIF	Center of instrument facility
CO	Cholesterol oxidase
CRP	C-reactive protein
DHAP	Dihydroxyacetone phosphate
dl	deciliter
EDTA	Ethylenediaminetetraacetic acid
g	gram
GK	Glycerol kinase
GPO	Glycerol phosphate oxidase
HDL	High-density lipoprotein
HL	Hepatic lipase
HRP	Horseradish peroxidase
ICAM-1	Intracellular adhesion molecule-1
IDL	Intermediate-density lipoprotein
IL-8	Interleukin-8
l	liter
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LPE	Lipoprotein electrophoresis

LIST OF ABBREVIATIONS (CONTINUED)

LPL	Lipoprotein lipase
LRP	LDL receptor-related protein
MCP-1	Macrophage chemotactic protein-1
mg	milligram
mmol	millimol
MMP	Matrix metalloproteinase
NCEP	National Cholesterol Education Program Expert Panel
NMR	Nuclear magnetic resonance
NO	Nitric oxide
eNOS	endothelial Nitric oxide synthase
OPD	Out patient department
PDGF	Platelet-derived growth factor
rpm	round per minute
sdLDL	small dense low-density lipoprotein
SMCs	Smooth muscle cells
TEMED	Tetramethylethylenediamine
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
VLDL	Very low-density lipoprote

CHAPTER I
INTRODUCTION

Cardiovascular disease is a leading cause of global morbidity and mortality and is responsible for one-in-three deaths. Every year, about 17 million people throughout the world die of a heart attack or a stroke. Those who survive a heart attack or stroke often need to take long-term medical treatment. These diseases affect the poor as well as the rich. Most people think that they are diseases of middle-aged men. The truth is that both men and women suffer from heart attacks and strokes (1).

In Thailand, cardiovascular disease including coronary heart disease, congestive heart failure, and strokes is also a leading cause of morbidity and mortality. 32,896 people died in 2002 and reached 36,771 people in 2005. 75,931 and 98,895 people suffered from coronary disease in 2002 and 2005 respectively.

In the United States and most other Western countries, atherosclerosis is the leading cause of illness and death. In the United States alone, it caused almost 1 million deaths in 1996 – twice as many as cancer caused and 10 times as many as accidents caused. Despite significant medical advances, heart attack due to coronary artery disease and strokes are responsible for more deaths than all other causes combined.

It is very important to monitor LDL particle levels especially small dense LDLs particle levels which play an important role in cardiovascular disease. Unfortunately, current routine clinical laboratory service cannot provide the information about LDL size and number. They can provide only LDL cholesterol concentration which is the estimation of LDL cholesterol level in plasma. They cannot report the exact number of LDL particles that is more precise and useful than report in LDL cholesterol concentration format. If the laboratory can report the exact atherogenic LDL particle number or small dense LDL particle number, the doctor will know how risky of each patients to develop cardiovascular disease and give them the appropriate advice and treatment early. Therefore, my study tries to modify the method that can detect the

small dense LDL particles and can report in term of particle number. All of these will help protecting people from the leading cause of global morbidity and mortality, cardiovascular disease.

The objective of the study is to modify the method that can detect the small dense LDL particles and can report in term of particle number. All of these will help protecting people from the leading cause of global morbidity and mortality, cardiovascular disease.

CHAPTER II
OBJECTIVES

The determination of LDL level in current routine clinical laboratory is the determination of cholesterol that contain within LDL molecules which is just the indirect measurement of the LDL level and this method cannot provide any information about small dense LDL particles which are the true atherogenic molecules. The current methods that directly detect and determine small dense LDL particles are proton nuclear magnetic resonance (NMR), analytical ultracentrifugation, gradient gel electrophoresis. But these method are not suitable for routine clinical laboratory. The expensive instrument, well-trained technician, and time consuming are needed by these methods.

From the above problem, this study tries to solve this problem by development the precipitation method that is suitable for routine clinical laboratory, directly detectable the small dense LDL particles, and reportable in molecule number. This method will not only improve the routine clinical laboratory's efficiency in determination of atherogenic parameters, but it also benefit for coronary heart disease prevention and treatment.

CHAPTER III
LITERATURE REVIEW

1. The vascular biology of atherosclerosis

Cardiovascular disease is the leading cause of mortality in the United States, Europe, the vast majority of Asia, and is likely to be the greatest threat to overall health worldwide (2,3). As a major cause of cardiovascular disease, the development of atherosclerosis starts early in childhood (4). Despite this fact, most individuals are asymptomatic until many decades later. Autopsy studies of coronary arteries from healthy, young American soldiers killed during the Korean conflict revealed surprisingly advanced atherosclerotic lesions (5). Intimal lesions were discovered in more than 50% of the right coronary arteries of the youngest group (15-19 years of age). More recently, fatty streaks, an early marker of atherosclerosis, have been found in the intima of infants (6). More advanced atherosclerotic lesions are first identified in the intima of three primary target vessels: the carotid and coronary arteries and the aorta (7,8). Figure 1 illustrates the progressive narrowing of the artery during atherosclerosis. Although there is significant disparity in the evolution of lesion formation, ischemic coronary disease, stroke, peripheral artery disease, and transient ischemic attacks are among the clinical presentations of matured lesions and ruptured plaques (9,10).

Emerging epidemiologic studies (11) have shown that elevated LDL, male gender, increased homocysteine, and ethnicity are among the many risk factors and markers involved in the pathogenesis of atherosclerosis. In a recent study of 557 first-generation immigrants, it was concluded that acculturation into western societies may also be an independent risk factor for coronary artery disease and atherosclerotic lesion development (12). Nevertheless, among the consequences of acculturation are stress, dietary patterns, and physical inactivity which also have been identified as major risk factors for atherosclerosis and cardiovascular disease.

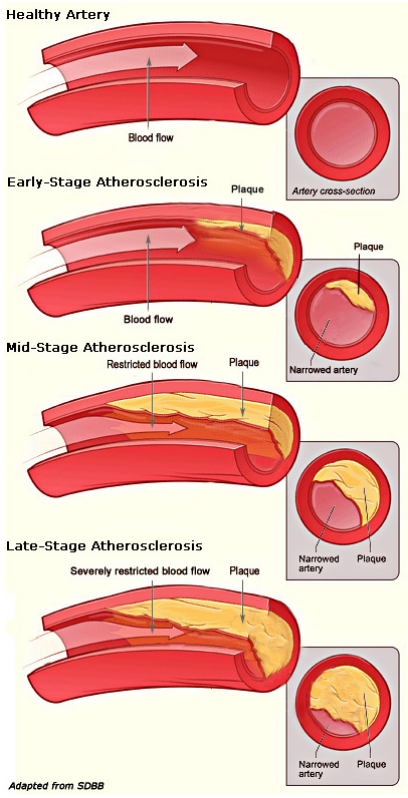

Figure 1. Progression of atherosclerosis

Source : http://www.resverlogix.com/upload/body_image/46/03/artery-for-web-final.jpg

Access date : 24/05/2008

1.1 Anatomical structure of the normal human artery

The structure of the normal artery consists of three layers: the intima, the media, and the adventitia (Figure 2). The intima, the innermost layer, is composed of an endothelial monolayer lying on the basement membrane with elastic fibers comprised of type IV collagen, laminin, and heparin sulfate proteoglycans (13). This layer also contains smooth muscle cells (SMCs) embedded in sulfated polysaccharide, hyaluronic acid intimal thickening (14).

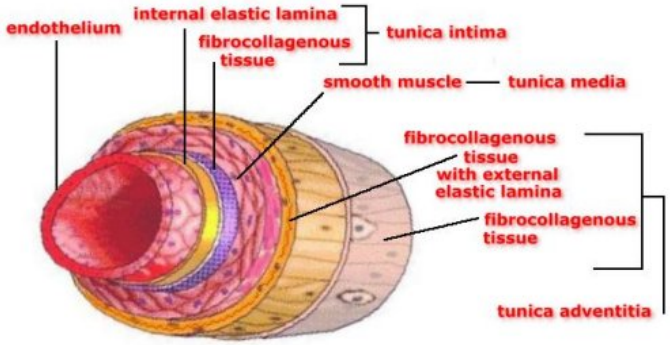

Figure 2. Normal artery

Source : http://greenfield.fortunecity.com/rattler/46/images/artery1.JPG

Access date : 24/05/2008

The endothelium of a normal, healthy artery functions as a non-thrombogenic surface and serves as a selectively permeable barrier, which regulates the transport of solutes across the arterial wall. Importantly, the vascular endothelium is also essential in the regulation of vascular tone, coagulation, and inflammatory responses (15-17). Changes in shear stress and blood flow lead to phosphorylation of endothelial nitric oxide synthase (eNOS), which generates nitric oxide (NO), which then produces vasodilation (18). The intima is separated from the media by an internal elastic lamina comprised primarily of the protein polymer elastin.

The tunica media, the middle layer, is primarily comprised of SMCs surrounded by its own basement membrane. The media's basement membrane is anchored within an interstitial matrix composed of type I collagen, fibronectin, dermatan, and chondroitin sulfate proteoglycans (19). This interstitial matrix is intertwined with perforated sheets of elastic fibers.

The adventitia attaching the vessel to the surrounding tissue is made up of capillaries, fibroblasts, fat cells, proteoglycans, connective tissue, and elastic and collagen bundles. The adventitia is separated from the tunica media by the external elastic lamina. The connective tissue in the adventitia is very compressed where it borders the tunica media, but it changes to loose connective tissue near the periphery of the vessel (20).

1.2 Endothelial dysfunction

In humans, the normal endothelium has many unique anti-atherosclerotic properties, including vasoregulation of conductive and resistance vessels, monocyte disadhesion, and vessel growth (21). The pathophysiological consequences of disruption of these factors serve as hallmarks of endothelial dysfunction. Endothelial dysfunction as a result of injury leads to compensatory responses that modify the normal physiological characteristics of the endothelium and become the foundation for the disease process.

Endothelial dysfunction is characterized as a systemic, reversible disorder and is associated with an impairment in endothelium-dependent vasodilation and recruitment of inflammatory cells to the vessel wall (22). Potential causes of endothelial dysfunction include hypercholesterolemia, diabetes (23), smoking (24), hypertension (25), and infectious microorganisms (26) such as *Chlamydia pneumoniae* (27), cytomegaloviral infection, *Helicobacter pylori* infection, and herpes virus infection (28), many of which are associated with a reduction in availability of vasodilators such as NO, decreased flow-induced vasodilation, and increased endothelium-derived contracting factors. Lipid and cell permeability, lipoprotein oxidation, inflammation, platelet activation, and thrombus formation are all promoted by endothelial dysfunction (29,30).

Endothelium dysfunction is also involved in the activation of endothelial-leukocyte adhesion molecules (31,32). Specifically, P-selectin, E-selectin, intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule (VCAM-1) are adhesion molecules known to be involved with the recruitment of leukocytes (33). VCAM-1 plays a role in the binding of both monocytes and leukocytes to endothelial cells. In lesion-prone areas (e.g., endothelial cells exposed to long duration, high shear stress), VCAM1 is up-regulated and occurs in response to inflammatory cytokines. Increased expression of ICAM-1 on endothelial cells has been detected in both lesion-prone areas as well as on endothelial cells exposed to normal shear stress (34). In humans, E-selectin is only up-regulated on injured endothelial cells and is important in the regulation of adhesive interactions between certain blood cells and the endothelium (35), whereas P-selectin is involved in adhesion of certain leukocytes and platelets to the endothelium (36-38). The

importance of P-selectin during atherosclerosis has also been demonstrated in animal models (39). For example, P-selectin is expressed on endothelial cells overlying active atherosclerotic plaques, and inactive atherosclerotic plaques lacking in P-selectin expression. Furthermore, animals lacking P-selectin have a decreased tendency to form atherosclerotic plaques.

1.3 Stages of atherosclerosis

Initiation of LDL-mediated atherosclerosis (lipid accumulation)

Atherosclerosis lesion development begins with the accumulation of LDL cholesterol levels within the circulation. Under pathologic conditions where LDL levels are elevated, lipid accumulation is noticeable along the lining of the arterial wall termed the tunica lamina. The aggregates of lipid particles form intimate associations with epithelia moieties such as proteoglycans and become embedded in the tunica lamina structure. In defense, the arterial epithelium fortifies itself with self-protective structural and biochemical mechanisms that maintain a homeostatic environment in the presence of lipid accumulation. The expression of molecules such as heparin sulfate constituents, which provide arterial integrity and blood fluidity and the expression of many antithrombin molecules, are instrumental in protection against atherosclerosis (40). However, under hypercholesterolemic conditions, the protective integrity of the epithelium falls prey to initiation of lesion development.

LDL oxidative modification and fatty streak formation

Atherosclerotic lesions present initially in the form of fatty streaks forming along the endothelium of arteries. The major contributing event believed to be responsible in fatty streak development is oxidative modifications of the lipid and apolipoprotein B (apo B) components of LDL (41).

The precise molecular mechanisms responsible for LDL oxidation are largely unknown. Studies have identified several plausible mechanisms supportive of LDL modification. The enzymatic activity of nitric oxide synthase, 15-lipoxengenase activity (42), as well as nitric oxide production by epithelial cells and macrophages (43) have been shown to be capable of LDL modification. Recent findings supporting their proatherogenic role have been documented using gene knockout models (44-46).

Despite formidable evidence that LDL oxidation confers lesion formation, data regarding antioxidant therapy to date have not shown promise (47). In broad terms, atherosclerosis can be characterized as a chronic inflammatory condition. As such, cellular responses such as cellular adhesion and recruitment during lesion development are central components as in other chronic inflammatory diseases.

The recruitment of monocytes occurs at the sites of lipid accumulation and function in uptake of various lipids and apolipoprotein components produced from oxidative stress and other biochemical breakdown products of LDL. Such recruitment is known to be regulated by chemotactic factors as well as being attracted by oxidative-LDL species. Chemokines are small proteins of the first two cysteines at the amino terminus of the molecule (48-50). Chemokines stimulate the migration and activation of cells, especially phagocytic cells and lymphocytes. Most notable is the release of macrophage chemotactic protein 1 (MCP-1) found to be produced locally by endothelial cells and the coordinate expression of chemokine receptor 2 (CCR2), the receptor for MCP-1 by monocytes. In fact, it has been shown that hypercholesterolemia patients exhibit increased MCP-1 production. Furthermore, disruption of MCP-1 and its receptor CCR2 genes was shown to reduce the development of atherosclerosis in mice (51). Other chemokines such as interleukin-8 (IL-8), RANTES, and IP-10 have also been implicated in monocyte recruitment. Current research in this area offers the potential for therapeutic use in deterring atherogenic processes by impairing leukocyte trafficking.

It has been speculated that macrophage-mediated uptake of modified LDL species may be an initial attempt to dampen the inflammatory environment produced by oxidative LDL species. Ultimately, however, the response and uptake of oxidized LDL species leads to progressive inflammation and atherosclerotic lesions. The uptake of LDL occurs mainly via macrophage LDL receptors or by scavenger receptor-mediated uptake. The mode of LDL uptake is determined by the nature of LDL modification. Studies show that while native LDL is normally endocytosed via specific LDL receptors, highly modified LDL, such as certain apolipoproteins, are not recognizable by the LDL receptors and are relegated to uptake by scavenger receptors. The latter is most associated with macrophage foam cell formation, a topic to be discussed in a subsequent section of this chapter. As a result of macrophage

recruitment and uptake of LDL constituents, fatty streaks form and become what is the initial site of atherosclerotic lesions.

Another mechanism responsible for the initiation of atherosclerotic lesions is the increase in adhesion molecules present on endothelial cells. Under normal circumstances, the arterial endothelium is highly resistant toward cellular adhesion. However, studies have shown that hypercholesterolemia induces leukocyte adherence to the endothelium allowing diapedesis between the endothelial cell and entry into the lamina. Several adhesion molecules have been implicated to significantly foster translocation of leukocytes across the endothelium. Vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin superfamily, is expressed by endothelial cells and regulates the adherence of monocytes and T cells. VCAM-1 has been found to interact with very late antigen-4 (VLA-4) and influence monocyte adherence during the initial stages of atheroma formation (52). Selectins P and E have also been implicated in monocyte adhesiveness to the endothelium. Quantitative decreases in atherosclerosis were shown in apo E mice lacking their respective genes (53).

Foam cell formation (intracellular lipid accumulation by macrophages)

Macrophages play an important role in LDL metabolism by uptake of native LDL cholesterol and modified species of LDL via two major receptor mechanisms, LDL-specific receptor and scavenger receptor endocytosis, respectively. As the accumulation and modification of LDL ensues, macrophages within the subendothelium begin to incorporate large amounts of oxidized LDL species via scavenger receptor uptake, resulting in a phenotype given in term “foam cell”. The most notable scavenger receptors identified to date that have been demonstrated to have a significant impact on atherosclerotic development are the scavenger receptor A (SR-A) and the receptors of the cluster differentiation 36 surface molecules (CD36) receptors (54). In particular, it was shown that in apo E-deficient murine models deficient in SR-A or CD36, gene receptor expression resulted in a significant reduction in lesion formation (54,55).

Homeostatic control of cholesterol uptake is under strict mediation through LDL-specific receptor feedback mechanisms regulated by the SREBP transcription factors required for LDL receptor expression. In the presence of elevated membrane-bound cholesterol, inactivation of SREBP occurs, inhibiting LDL receptor expression. In contrast, however, uptake of oxidative LDL species via scavenger receptors, SR-A or CD36 or by macrophage-mediated phagocytosis is not under such regulatory control. Instead, prevention of cholesterol intracellular overload is dependent on mechanisms of active efflux out of the cell. The vast majority of oxidized LDL entering macrophages via the scavenger receptors consists of free cholesterol or esterified cholesterol. There are several fates of native cholesterol metabolism, which include Acyl CoA esterification and the storage of lipid droplets containing cholesterol esters that characterize the phenotype of foam cells.

A major pathway of cholesterol efflux is called reverse cholesterol transport pathway that involves HDL as an acceptor molecule. The HDL-reverse cholesterol transport mechanism received much attention when studies found an inverse relationship between risk for atherosclerosis and HDL content (56). A genetic basis for HDL-mediated cholesterol transport is shown in patients afflicted with Tangier disease, which is characterized by extremely low levels of HDL and accumulation of cholesterol within macrophages. Under normal conditions, HDL-cholesterol is esterified via lecithin-cholesterol acyltransferase (LCAT) or is directly transported to the liver via SR-B1 binding.

Immigration of smooth muscle cells

A hallmark of advanced lesion development is the immigration of smooth muscles cells from the arterial wall into the subepithelial space. The factors that lead to the mobilization of smooth muscle cells are not well understood, but it is believed to be due to preexisting stimuli. For example, macrophages have been shown to secrete the chemokine platelet-derived growth factor (PDGF), which is a chemoattractant for smooth muscle (57). In fact, studies have demonstrated PDGF expression to be elevated in individuals with atherosclerosis (58). Smooth muscle cells found within the atherosclerotic region were found to have distinct characteristics from normal smooth muscle cells. These cells exhibit characteristics of

clonal expansion. Studies have demonstrated that the slow but steady proliferation can be attributed to a single cell (59). Smooth muscle cells in developing atheroma also are capable of taking up modified lipoproteins (58). Not only does their proliferative capacity augment atheroma development, but apoptotic cell death of smooth muscle cells also participates in lesion progression. Apoptosis of smooth muscle cells is believed to be associated with the presence of inflammatory cytokines at the lesion site (60). Thus, smooth muscle cell immigration plays a significant role in progression of atheroma. Current research is aimed at developing molecular strategies targeting both proliferative and apoptotic pathways.

Plaque formation

Plaques develop from initial fatty streaks that progress into advanced lesions comprised of inflammatory cells, extracellular lipid, and fibrous tissue. Their continued accumulation proliferation and activation within the lesion leads to plaque expansion. Consistent with the earlier events of atherosclerotic lesion development, plaque formation involves the participation of cytokines, chemokines, hydrolytic enzymes, and growth factors in this process (61).

While the initial events of atherogenesis involve mainly the disruption of the endothelium and leukocyte accumulation, the formation of the more advanced plaque includes smooth muscle cells. Smooth muscle cells migrate via chemotactic regulation into the arterial intimal lesion site and become active participants in atheroma development. The smooth muscle cells involved in atheroma exhibit an altered phenotype in comparison to normal arterial tunica media smooth muscle cells. These smooth muscle cells proliferate at a higher rate within atherosclerotic plaques versus normal intimal regions of the aorta (62). It is still unclear, however, what initiates media smooth muscle proliferation versus normal smooth muscle cells. It is believed that growth factors in conjunction with additional stimuli promote the proliferative response by smooth muscle cells at the lesion site.

A large proportion of the developing atheroma includes connective tissue consisting of extracellular matrix macromolecules. Among the matrix proteins, the class collagens and proteoglycans are commonly associated with plaque development. Matrix proteins are produced by vascular smooth muscle cells and can accumulate

within the developing plaque upon stimulation by transforming growth factor-β and platelet-derived growth factor (63). Matrix molecules have an important regulatory function. For example, fibronectin and heparin sulfate are found to inhibit cell cycle and cell-matrix interactions and influence chemokine expression by macrophages (64-67). Matrix accumulation within the intima is under control of matrix metalloproteinase (MMPs) (65). MMPs act in degradation of matrix molecules and therefore control lesion accumulation. Matrix molecules also contribute to the outward growth of the lumina. Thus, the extracellular matrix is a key component in plaque development.

1.4 Atherosclerosis risk factors

An American Heart Association (AHA) Prevention Conference statement in 1999 classified risk factors into 3 categories (Table 1). The conventional risk factors appear to have a direct causal role in atherosclerosis. Predisposing factors, including obesity, family history of early-onset CHD, and sedentary lifestyle, mediate some risk through the causal factors but may also have independent effects. The term "conditional" risk factors was used for factors that have an association with increased risk for coronary artery disease although their causative, independent, and quantitative contributions to coronary artery disease are not well documented. The conditional risk factors are homocysteine, fibrinogen, Lp(a), low-density lipoprotein (LDL) particle size, and C-reactive protein (CRP). The fourth category that can be added is that of emerging risk factors: lipoprotein-associated phospholipase A_2, pregnancy-associated plasma phosphatase, asymmetric dimethylarginine, myeloperoxidase, nitrotyrosine, and markers of oxidative stress. The reported association of these factors to CHD needs further confirmatory studies (68,69).

Table 1. Categories of risk factors for atherosclerosis

Conventional	Predisposing	Conditional	Emerging
Cigarette smoking	Overweight and obesity	Homocysteine	Lipoprotein-associated phospholipase A_2
Elevated blood pressure	Physical inactivity	Fibrinogen	Pregnancy-associated plasma phosphatase
Elevated serum cholesterol	Male sex	Lipoprotein(a)	Asymmetric dimethylarginine
Low HDL cholesterol	Family history of early-onset CHD	Small LDL particle size	Myeloperoxidase
Diabetes mellitus	Socioeconomic factors	C-reactive protein	Nitrotyrosine
	Behavioral factors		Measures of oxidative stress
	Insulin resistance		Candidate gene polymorphisms

Cigarette smoking

Smoking contributes to the risk of developing heart disease. All smoke contains very fine particles that are able to penetrate the alveolar wall into the blood and exert their effects on the heart in the short time.

Inhalation of smoke causes several immediate responses within the heart and blood vessels. Within one minute the heart rate begins to rise, increasing as much as 30 percent during the first 10 minutes of smoking. Carbon monoxide in smoke exerts its negative effects by reducing the blood's ability to carry oxygen.

Smoking tends to increase blood cholesterol levels. Furthermore, the ratio of high-density lipoprotein to low-density lipoprotein tends to be lower in smokers compared to non-smokers. Smoking also raises the levels of fibrinogen and increases platelet production with makes the blood viscous. Carbon monoxide binds to haemoglobin, resulting in a much more stable complex than haemoglobin bound with oxygen or carbon dioxide and lead to permanent loss of blood cell functionality.

Blood cells are naturally recycled after a certain period of time, allowing for the creation of new, functional erythrocytes. However, if carbon monoxide exposure reaches a certain point before they can be recycled, hypoxia occurs. All these factors make smokers more at risk of developing various forms of atherosclerosis. As the atherosclerosis progresses, blood flows less easily through rigid and narrowed blood vessels, making the blood more likely to form a thrombosis. Sudden blockage of a blood vessel may lead to an infarction.

Smoking causes endothelial dysfunction, possibly through increased oxidative stress, and this is also true for passive smoking. A 30-minute passive smoking exposure was found to affect coronary flow velocity reserve in non-smokers. Light and heavy smoking have similar detrimental effects on endothelium-dependent vasodilation and the nitric oxide biosynthetic pathway.

Hypertension

The renin-angiotensin system contributes to the pathogenesis of atherosclerosis. Angiotensin II may elicit inflammatory signals in vascular smooth muscle cells and is responsible for activating expression of cytokine gene networks in vascular smooth muscle cells. It can also promote long-term changes in vascular smooth muscle cell function by its ability to induce cellular hypertrophy, extracellular matrix production, and early gene expression. Angiotensin II also activates inflammatory pathways in human monocytes.

Homocysteine

Homocysteine is a highly reactive, sulfur-containing amino acid form as a by-product of the metabolism of the essential amino acid methionine. Cells remetabolize homocysteine by a number of possible pathways involving several different enzymes; these enzymes variously use B vitamins as substrates or cofactors, namely folate, cobalamin (vitamin B_{12}), and pyridoxine (vitamin B_6).

Homozygous homocystinurias are extremely high levels of homocysteine in the blood and urine of individuals homozygous for these mutations; half to effected individuals develop arterial or venous thrombosis by 30 years of age. This risk can be

substantially ameliorated by the provision of high-dose B vitamins, which partially lower homocysteine levels back toward the normal range.

It has been postulated that mild to moderate elevations of homocysteine in the general population predispose to atherosclerosis in a manner akin to the classic risk factors. This is important because of the availability of an inexpensive, safe, and effective therapy for lowering homocysteine. Mechanistic studies have demonstrated that homocysteine may induce vascular damage by promoting platelet activation, oxidative stress, endothelial dysfunction, hypercoagulability, vascular smooth muscle cell proliferation, and endoplasmic reticulum stress (70-74).

Fibrinogen

Fibrinogen is a circulating glycoprotein that acts as the final step in coagulation response to vascular and tissue injury. Cleavage by thrombin produces soluble fibrin fragments, which are the most abundant component of blood clots. Aside from its role in thrombosis, fibrinogen has a number of other functions that lend its biological plausibility as a possible participant in vascular disease, including the following: (1) regulation of cell adhesion, chemotaxis, and proliferation; (2) vasoconstriction at sites of vessel wall injury; (3) stimulation of platelet aggregation; and (4) determination of blood viscosity. Fibrinogen, like CRP, is an acute-phase reactant. Hepatic synthesis of fibrinogen can increase up to 4-fold in response to inflammatory or infectious triggers.

Several factors other than inflammation have been shown to modulate fibrinogen levels. Smoking and smoking cessation are associated with an increase or decrease, respectively, of approximately 0.15 g/L in plasma fibrinogen. Furthermore, there is a dose-response relationship between number of cigarettes smoked and fibrinogen level. Fibrinogen levels tend to be higher in patients with diabetes, hypertension, obesity, and those with sedentary lifestyles. Fibrates and niacin lower fibrinogen levels, whereas statins and aspirin do not.

Lipoprotein(a)

Lipoprotein(a) is an LDL-like particle in which an apolipoprotein(a) moiety is linked via a disulfide bond to apo B-100. Concentration of Lp(a) are largely under

genetic control and vary substantially between individuals depending on the size of the apo(a) isoform present; conversely, Lp(a) levels vary little with diet or exercise, unlike other lipoproteins such as LDL and HDL. The wide range of Lp(a) in plasma within a population is due in large part to a variable number of plasminogen-like kringle IV repeats, and an inverse correlation between the number of kringle IV type 2 repeats in the apo(a) gene and Lp(a) plasma concentration exists. The biological function of Lp(a) is still unclear, but there is strong evidence that its phylogenetic role may have been to response to tissue injury and vascular lesions, prevent infectious pathogens from invading cells, and promote wound healing.

Lipoprotein(a) is an acute-phase reactant, more than doubling in concentration in response to the proinflammatory cytokine IL-6. Lipoprotein(a) binds avidly to endothelial cells, macrophages, fibroblasts, and platelets, as well as to the subendothelial matrix; there, it may promote proliferation of vascular smooth muscle cells and chemotaxis of human monocytes. However, its most important putative role in atherothrombosis may be to inhibit clot fibrinolysis at sites of tissue injury. By virtue of its unique structural homology to plasminogen, Lp(a) is thought to compete with plasminogen for binding to plasminogen receptors, fibrinogen, and fibrin. Lp(a) may also induce the production of plasminogen activator inhibitor 1 (the main inhibitor of the fibrinolytic system) and may inhibit the secretion of tissue-plasminogen activator by endothelial cells.

LDL particle size

LDL particles differ in size and density. Two distinct phenotypes have been described: pattern B with a predominance of small dense LDL particles and pattern A with a higher proportion of large, more buoyant LDL particles. Small dense LDL particles tend to coexist with elevated triglyceride and low HDL cholesterol levels. This trait has been called “atherogenic dyslipidemia” and appears to be highly heritable. Small dense LDL particle size is associated with several other cardiovascular risk factors, including metabolic syndrome, type 2 diabetes mellitus, and postprandial hypertriglyceridemia.

Whether measurement of LDL particle size will add to the prediction of CHD in an individual patient needs to be clarified by further clinical and epidemiological

studies. Although particle size is significantly correlated with plasma triglyceride levels, a proportion of subjects with small dense LDL particle phenotype may not have concomitant elevation of triglycerides. A potential use of measuring LDL particle size may be in aiding decision making regarding the choice of a lipid-lowering agent. Fibrates and niacin have been shown to increase LDL particle size, but whether adding one of these to a statin in high-risk patients with small dense LDL particle size is beneficial remains unproved. Statins have little effect on LDL particle size and primarily reduce the number of LDL particles.

A predominance of small dense LDL is associated with a 2- to 3-fold increase in risk for coronary heart disease. There is the incidence of developing coronary heart disease even if the total cholesterol level is normal. Several reasons have been suggested for atherogenicity of small dense LDL. Smaller and denser LDLs are taken up more easily by arterial tissue than larger LDLs, decreased binding to LDL receptor and it has been shown that oxidative susceptibility increases and an antioxidant concentration decreases with decreasing LDL size.

C-reactive protein

Because markers specific for atherosclerotic plaque inflammation are currently not available, circulating markers of systemic inflammation have been studied as surrogates. These include “proximal” markers such as cytokines, “distal” markers such as cell adhesion molecules (expressed on endothelial cells “activated” by cytokines), and acute phase reactants such as CRP and serum amyloid A. Cytokines and cell adhesion molecules are directly involved in the immune response, but assays have suboptimal reliability. C-reactive protein is the most widely studied of the inflammatory markers and has been shown to be an independent predictor of cardiovascular events in otherwise asymptomatic patients. C-reactive protein is present in low concentrations (about 1 mg/L) in healthy subjects, increases early in the acute phase response, peaks in the first few days (levels can increase by 1000-fold), and decreases to baseline levels in 8 to 10 days.

The pathways that link CRP to CHD events remain unclear. Recently, several atherogenic properties were attributed to CRP. However, CRP levels are not

significantly associated with extent of coronary atherosclerosis and may therefore reflect plaque inflammation and instability rather than plaque burden.

Diabetes mellitus

Diabetes mellitus is associated with earlier and more extensive development of atherosclerosis as part of a widespread metabolic disorder. Diabetes is a particularly strong risk factor in women and effectively negates the protective effect of female hormones. The myocardium is especially sensitive to oxidative stress in hyperglycemia while hyperinsulinemia damages vascular endothelium (75,76).

Obesity

Obesity is an independent risk factor for CAD. Hypertriglyceridemia is commonly associated with obesity and may presage diabetes mellitus by increasing specific lipids. Not all triglyceride elevations are likely to be atherogenic; LDL is associated with greater risk. Diet plays a crucial role in obesity. A diet that is high in saturated fatty acids or calories will contribute to hypertriglyceridemia.

Exercise

Physical inactivity and a sedentary lifestyle are linked with increased risk of CAD and studies have shown that regular exercise is protective and reduces such risk.

Oxidative stress

Oxidative stress can arise through the increased production of reactive oxygen species or a deficiency of antioxidant defenses. Oxidative stress appears to amplify the other risk factors identified with development of coronary artery diseases.

2. The relationship between LDL and atherosclerosis

The relationship between plasma lipoprotein concentration and the development of atherosclerosis has been known for many years. Atherosclerosis is caused by increased concentrations of low-density lipoproteins and the catabolic remnants of the triglyceride-rich lipoproteins (chylomicrons and very low-density

lipoproteins). Lowering the plasma levels of these lipoproteins can reduce of even reverse the disease (77-81).

Chylomicrons and VLDL transport triglyceride and fatty acids from the liver to peripheral tissues. These lipoproteins are hydrolyzed with lipoprotein lipase (LPL) and release fatty acids for tissue utilization. After release the fatty acids, VLDL becomes VLDL remnant. Two-thirds of remnants is removed from the circulation by liver and one-third goes to LDL. If liver secretes more VLDL particles, the number of LDL will also increase.

The amount of cell-surface receptors for LDL or LDL receptors is also the factor that affects the LDL concentrations by removing LDL particles from circulation. 75% of circulating LDL is removed at the liver whereas the remainder is taken up by other tissues. The control of LDL concentration depends on rates of conversion of VLDL to LDL and the clearance rate of LDL receptor (82).

Many investigations reveal a positive correlation between concentration of total cholesterol and coronary heart disease events (83,84). Because LDL is major cholesterol transporter in serum, the concentration of LDL cholesterol is correlated with total cholesterol. The studies of atherosclerotic plaques show the detection of apo B-100 in atherosclerotic lesions (85) and LDL particles have been identified in plaques (86). 2 forms of hypercholesterolemia also show evidence that LDL is atherogenic molecule. Familial hypercholesterolemia which the LDL receptors are deficient on a genetic basis shows great increase of LDL cholesterol concentrations and premature coronary heart disease is common in this disease (87). Another form is familial defective apo B-100. This form has a mutation in the apo B-100 molecule that plays an important role in recognition by the LDL receptors. Consequently, LDL particles are remained longer in plasma. Patients are prone to premature coronary heart disease (88-90).

These investigations about the relationship of LDL particles to the development of atherosclerosis are not in argument, but the precise mechanisms of atherogenesis are still not clear.

One theory holds that endothelial damage is the first step in atherogenesis (91). Platelets aggregate at the injury site of endothelium and release growth factors that stimulate the proliferation of smooth muscle cells. The high concentrations of

LDL may have toxicity with endothelial cells, resulting in the initiation of atherosclerosis.

From the above theory, LDLs promote atherosclerosis without entering arterial wall. But, there are evidence that atherosclerotic plaques contain cholesterol deriving from circulating LDL cholesterol. LDL particles penetrate into the arterial wall by passing the endothelial cells. The rate of penetration depends on plasma concentration of LDL (92). The first stage of atherosclerosis is the formation of fatty streak that is characterized by the large amounts of cholesterol in the intima (93). The interaction between LDL and proteoglycans (94-96) and elastin (97-100) may cause the deposition of the cholesterol in the arterial wall. These accumulating LDLs are modified by either oxidation or by aggregation (101). The modified LDLs initiate a sequence of events, development and progression of atherosclerosis (102-103).

Modified LDLs stimulate endothelial cells to express monocyte chemotactic protein-1 or MCP-1. This MCP-1 recruits monocytes from circulation to the artery wall (104-105). Modified LDLs also promote the differentiation of monocytes into macrophages (106). Macrophages express scavenger receptors that have capacity to take up modified LDL, converting the macrophages into a cholesterol-filled foam cells (107-108). Macrophages also secrete tumour necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) that stimulate endothelial cells to express adhesion proteins (109). These adhesion proteins bind blood monocytes to the endothelium, making them available for recruitment by MCP-1. The macrophages and foam cells also secrete growth factors and metalloproteinase that lead to cell proliferation and matrix degradation (102,110-111). All processes lead to the development of unstable and vulnerable plaque that cause the acute coronary syndrome, acute myocardial infarction or even sudden death.

An increased concentration of LDL cholesterol is a major risk factor for atherosclerosis (84,112). However, there is heterogeneity in size, density, and composition of LDL particles (113,114). Peak size of LDL particles in human shows a bimodal distribution and can be separated into a buoyant and a dense phenotype (115-118). LDL size correlates positively with plasma HDL levels and negatively with plasma triglyceride concentration. The combination of small dense LDL,

decreased HDL cholesterol, and increased triglycerides has been called the atherogenic lipoprotein phenotype (119).

The prevalence of small dense LDL is approximately 30% in adult men, 5-10% in young men and women under 20 years, and approximately 15-25% in postmenopausal women (119-121). LDL size is genetically influenced with an inheritability ranging from 35-45% based on an autosomal dominant or codominant model with varying additive and polygenic effects (122). Thus, non-genetic and environmental factors also influence the expression of this phenotype (123-126). The predominance of small dense LDL is commonly found in familial combined hyperlipidemia (127), hyperapobetalipoproteinaemia (128), and hypoalphalipo-proteinaemia (129).

Particle size distribution of plasma LDL subfractions may be measured by different laboratory techniques (130-132), the most common procedure is 2-16% gradient gel electrophoresis (115). The molecular diameters are determined by migration distance comparing with standards of known diameter (117,123,133). The assignment of LDL subclass phenotype is based on particles diameter of the major plasma LDL peak. Phenotype A (large buoyant LDL) is defined when particle diameter is 258 Å or greater, whereas phenotype B (small dense LDL) is defined when particle diameter is less than 258 Å (115,123,134).

There are several reasons have been suggested for the atherogenicity of small dense LDL. Smaller and denser LDLs are taken up more easily by arterial tissue than larger LDLs (135). The smaller LDLs the greater transendothelial transport. Smaller LDL particles have decreased receptor-mediated uptake (136). This makes more available small dense LDL particle in circulation and more chance for development of atherosclerosis. With decreasing LDL size, the oxidative susceptibility increases and antioxidant concentration decreases (137). The altered content of free cholesterol and polyunsaturated fatty acids contribute to enhanced oxidative susceptibility of small dense LDL.

The predominance of atherogenic small dense LDL has been associated with an approximately 3-fold increased risk of coronary artery disease. Small dense LDL is also associated even with non-coronary forms of atherosclerosis. Small LDL

particles represent a risk factor for peripheral arterial disease in the absence or presence of diabetes (138).

3. Relationship between apolipoprotein B-100 and LDL particle

The only protein component of LDL is a single molecule of apo B-100 per particle (139-141). Apolipoprotein B-100 is the recognition site of the LDL receptors. It is the first step of receptor-mediated catabolism of LDL (142). Monoclonal antibodies are used to determine the general structure, the binding region to the LDL receptor, and genetic variants of the molecule (143-147). The binding region of apo B-100 to the LDL receptor consists of a cluster of positive-charged amino acids and appears to be placed in the aqueous face of the apo B-100 molecule (139). The size of LDL particle is an important determinant of the conformation of the apo B-100 molecule and its capacity to binding with the LDL receptor (139,147-149).

4. Method for separation and quantification of small dense LDL particle

Ultracentrifugation

The gold standard for measurement the density of LDL particle is analytical ultracentrifugation, but it is difficult, time-consuming, and expensive method. The other methods are used more commonly.

Analytical ultracentrifugation has been used to obtain quantitative data and study such parameters as the diffusion coefficient (*D*), the molecular weight (M), and the sedimentation coefficient (S). Analytical density gradient ultracentrifugation is a useful tool for determining such lipoprotein characteristics as density and particle size distribution (150-156).

Differential ultracentrifugation is a preparative method for separating lipoproteins on the basis of their hydrated density. At solvent densities higher than the lipoprotein density, lipoproteins float at rates dependent on their densities, sizes, and shapes (150,157-158).

Polyacrylamide gel electrophoresis (PAGE)

Lipoproteins are separated by PAGE on the basis of their size and electrostatic charge. Pore size of the gel depends on their concentration. Due to the widely use in lipoproteins research, the gradient gel electrophoresis are developed. This technique provides various concentrations in one gel. PAGE requires staining and densitometry for quantification of separating samples. This technique is often used in qualitative investigation rather than quantitative due to unreliable instructions to evaluate the scan and lot-to-lot inconsistencies of the staining (114,131,159-162).

Proton nuclear magnetic resonance spectroscopy (NMR)

NMR is the gold standard method for determination of LDL particle size. Quantification of small dense LDL particles by NMR does not require physical separation (163). This method is based on curve-fitting of the plasma methyl lipid resonance envelop, the amplitude and shape of which depend directly on the amplitudes of the superimposed methyl resonance of the lipoprotein components. But this method requires an expensive instrument and well-trained technician resulting not suitable for routine clinical laboratory (164).

Precipitation

Certain polyanions such as heparin combined with divalent cations such as Mg^{2+} has capacity to selectively aggregate the positive-charged apo B containing. But Hirano found that the not all apo B containing lipoproteins are precipitated. The small dense LDL part remains in the supernatant, although the exact mechanisms remain unknown. The supernatant is analyzed for the concentration of small dense LDL by the reliable methods. The precipitation method shows markedly high correlation with ultracentrifugation and gradient gel electrophoresis. This method is suitable for routine clinical laboratory due to its simplicity (165,192).

CHAPTER IV
MATERIALS AND METHODS

Materials

1. Samples

1.1 Samples collection

All samples were collected from the Center of Medical Laboratory Services, Faculty of Medical Technology, Mahidol University located on the fourth floor of the Out Patient Department (OPD) within the compound of Siriraj hospital during July to August 2008. The samples were categorized by LDL-C levels into 3 groups – 100 mg/dL (80 – 120 mg/dL), 140 mg/dL (120 – 160 mg/dL), and 180 mg/dL (160 – 200 mg/dL) respectively and were collected 1 ml. for each sample. The each group of samples was pooled together and was analyzed for LDL-C levels.

2. Chemicals

2.1 Source of chemicals

2.1.1 Ultracentrifugation

Sodium chloride (MERCK, Germany)

Potassium bromide (Baker Chemical, USA)

2.1.2 Precipitation

Heparin-sodium salt (Sigma company, USA)

Magnesium chloride (MERCK, Germany)

2.1.3 Polyacrylamide gel electrophoresis

Acrylamide (Sigma company, USA)

Bis-acrylamide (Sigma company, USA)

Sudan Black B (Fluka Chemika, Switzerland)

Boric acid powder (BDH, England)

Thyroglobulin powder (Sigma company, USA)

Sodium chloride (MERCK, Germany)
Potassium chloride (MERCK, Germany)
Trizma base (Sigma company, USA)
Coomassie blue R250 (BDH, England)
99.9% ethanol (MERCK, Germany)
Gracial acetic acid (MERCK, Germany)
TEMED (Sigma company, USA)
Ammonium persulfate (Sigma company, USA)

2.2 Solutions and reagents preparation

1. Stock salt solution (for ultracentrifugation)

NaCl	76.500	g
KBr	177.000	g

Adjust volume to 500 ml with distilled water

2. 2.0% acrylamide

30% acrylamide/0.8% bisacrylamide	6.7	ml
Distilled water	93.3	ml

3. 30% acrylamide/0.8% bisacrylamide

Acrylamide	30	g
Bisacrylamide	0.8	g

Adjust volume to 100 ml with distilled water, adjust pH to 7.0 and filter by Whatman No.1

4. 10% ammonium persulfate

Ammonium persulfate	10	g

Adjust volume to 100 ml with distilled water

5. Separating buffer pH 9.0

Trisma base	36.3	g

Adjust volume to 160 ml, adjust pH to 9.0, and adjust volume to 200 ml

6. 3.5% separating gel

30% acrylamide/0.8% bisacrylamide	1.75	ml
Separating buffer pH 9.0	13.25	ml

7. Stacking buffer pH 7.0

Trisma base	3.0	g

Adjust volume to 40 ml, adjust pH to 7.0, and adjust volume to 50 ml

8. 3.0% stacking gel

30% acrylamide/0.8% bisacrylamide	1.0	ml
Stacking buffer pH 7.0	9.0	ml

9. Tank buffer pH 7.4

Trisma base	6.055	g
Boric acid powder	3.094	g

Adjust volume to 1000 ml and adjust pH to 7.4

10. Precipitation reagent

Heparin-sodium salt	8.242	g
$MgCl_2$	90	mmol/l

Adjust volume to 10 ml with distilled water

3. Instruments

3.1 Analysis for LDL-C levels of pooled serum

The LDL-C determination of pooled serum was done by Hitachi 902.

3.2 Ultracentrifugation

The ultracentrifugation was done by Beckman Optima™ L-90 K Preparative Ultracentrifuge (Figure 3) and OptiSeal™ Ultracentrifuge tube (Figure 4).

Figure 3. Beckman Optima™ L-90 K Preparative Ultracentrifuge

Source :

http://www.beckmancoulter.com/products/instrument/centrifuges/ultracentrifuges/optima90k_inst_dcr.asp

Access date : 3/9/2008

Figure 4. OptiSeal™ Ultracentrifuge tube

Source : http://www.beckman.com/eCatalog/CatalogItemDetails.do?productId=12691

Access date : 3/9/2008

3.3 Precipitation

The incubation was done by water bath system, Mermmert, Germany and high-speed centrifugation was done by Micromax RF refrigerated Microcentrifuge, IEC, Germany.

3.4 Polyacrylamide gel electrophoresis

The polyacrylamide gel electrophoresis was done by Mini-PROTEAN Tetra Electrophoresis System, Bio-Rad Laboratory.

3.5 Apo B-100 analysis

The apo B-100 analysis was done by Cobas Integra® 400 *plus* (Figure 5). The principle of the test is immunotubidity.

Figure 5. Cobas Integra® 400 *plus*

Source :

http://labsystems.roche.com/content/products/integra_400plus/introduction.html

Access date : 3/9/2008

Methods

1. Analysis for LDL-C levels of pooled serum

Analysis for LDL-C levels of pooled serum was done by Hitachi 902. Each groups of pooled serum was used in separation of serum small dense LDL particles by ultracentrifugation and precipitation method.

2. Separation of serum small dense LDL particles

2.1 Ultracentrifugation (190-191)

The 7.905 ml. of each serum levels and 0.995 ml. of stock salt solution (total volume was 8.9 ml.) were mixed together in each OptiSeal™ Ultracentrifuge tube. Each group of samples was duplicated. Each tube were put into 80Ti fixed angle rotor and the ultracentrifuge was set up to 80,000 rpm, 25.5 °C, and run time for 6 hours. After finish running, the middle band (Figure 6) of each tube was collected by needle for polyacrylamide gel electrophoresis and apo B-100 analysis.

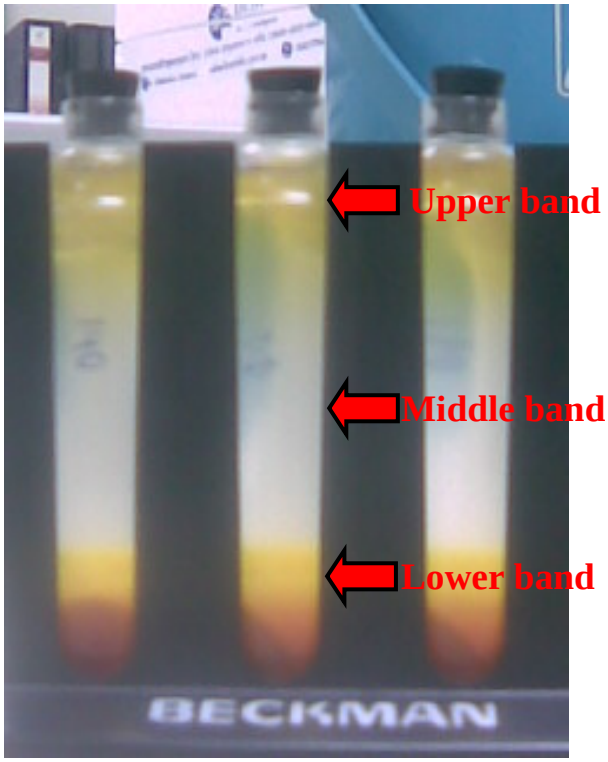

Figure 6. Tubes after centrifuged

2.2 Precipitation (192)

The precipitation method was conducted following the procedure of Hirano *et al*. 0.2 ml of the precipitation reagent was added to 0.2 ml of each serum sample levels, mixed, and incubated for 10 minutes at 37 °C. The samples were placed in an ice bath and allowed to stand for 15 minutes, and then the precipitate was collected by centrifuging at 15,000 rpm for 15 minutes at 4 °C. The supernatant was clear. An aliquot of the supernatant was collected for polyacrylamide gel electrophoresis and apo B-100 analysis.

3. Polyacrylamide gel electrophoresis (193)

3.1 Preparation of samples

200 µl of 2% acrylamide and 30 µl of Sudan black B were added to 40 µl of each serum sample levels, mixed, and stored under fluorescent lamp for 30 minutes.

3.2 Preparation of standard marker

1 g of thyroglobulin powder was added to 1 ml of Tris buffered saline solution, mixed, and this solution was pre-stained by adding 40 µl of Coomassie blue and mixed.

3.3 Casting the gel

Electrophoresis apparatus was assembled according to manufacturer's instructions and was locked to the casting stand. The 3.5% separating gel was prepared by adding 1.75 ml of 30% acrylamide/ 0.8% bisacrylamide to 13.25 ml of separating buffer pH 9.0, mixed, added 250 µl of ammonium persulfate and 25 µl of TEMED. This solution was mixed and immediately applied the separating gel solution to the sandwich along an edge of one of the spacers until the height of the solution between the glass plates was 11 cm. The separating gel was allowed to polymerize for 30 to 60 minutes at room temperature. After separating gel polymerized, stacking gel was prepared by adding 1.0 ml of 30% acrylamide/ 0.8% bisacrylamide to 9.0 ml of stacking buffer pH 7.0, mixed, added 100 µl of ammonium persulfate and 10 µl of TEMED. This solution was mixed and immediately applied the stacking gel solution to lay over the separating gel along an edge of one of the spacers until the height of the

solution in the sandwich is 1 cm from the top of the glass plates. Comb was inserted into the layer of stacking gel solution and stacking gel solution was allowed to polymerize for 30 to 45 minutes at room temperature. After stacking gel polymerized, comb was carefully removed without tearing the edge of the polyacrylamide wells.

3.4 Loading the samples

Each sample was loaded into the polyacrylamide wells follow Table 5.

Table 2. Loading of samples into the polyacrylamide wells

Well No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sample	marker	U180	P180	U140	P140	U100	P100	marker

U180, U140, U100 = sample from 180,140,100 mg/dL LDL-C pooled serum which small dense LDLs were separated by ultracentrifugation method

P180, P140, P100 = sample from 180, 140, 100 mg/dL LDL-C pooled serum which small dense LDLs were separated by precipitation method

Marker = thyroglobulin dimer pre-stained with Coomassi blue

3.5 Running the gel

After loading the samples, the polyacrylamide gel was attached to the electrophoresis chamber. The chamber was slowly filled with tank buffer until the electrode was completely covered bewaring the samples from sweep to adjacent wells. The chamber was connected to the power supply and run at 150V for 30 minutes. After finish running, the polyacrylamide gel was removed from chamber. The glass plates were carefully removed from sandwich and laid on a sheet of absorbent paper or paper towels. After the polyacrylamide gel was dry, it was taken the photograph.

4. Apo B-100 analysis

4.1 Samples preparation

The samples which were prepared by ultracentrifugation and precipitation method were used for apo B-100 analysis. Analysis for apo B-100 of samples was done by Cobas Integra® 400 *plus*.

4.2 Loading the samples

200 µl of each sample was loaded to sample cups and they were inserted to the machine by sample trays.

4.3 Ordering the test

Apo B-100 tests were ordered via user panel and the results were printed out via printer.

5. Conversion to molecule unit

Due to apo B-100 molecule weighted 515 kDa or 8.55×10^{-16} mg (1 Da = 1.66×10^{-24} g), the results with mg/dL unit were converted into molecule unit by following equation:

$$\frac{\text{mg/dL}}{8.55 \times 10^{-16}} = \text{molecule number/dL}$$

There is single apo B-100 molecule per one molecule of LDL, so the results that were reported in molecule number of apo B-100 can also be reported in molecule number of LDL.

6. Data analysis

The data analysis was divided into 2 sections

6.1 The simplicity of precipitation method

This section studied the simplicity of precipitation method compared with others separation methods.

6.2 The reliable of precipitation method

This section studied the reliable of precipitation method compared with the reference material from ultracentrifugation and evaluated the possibility to apply precipitation method to routine clinical laboratory.

The overall flowchart of the methods was shown in Figure 7.

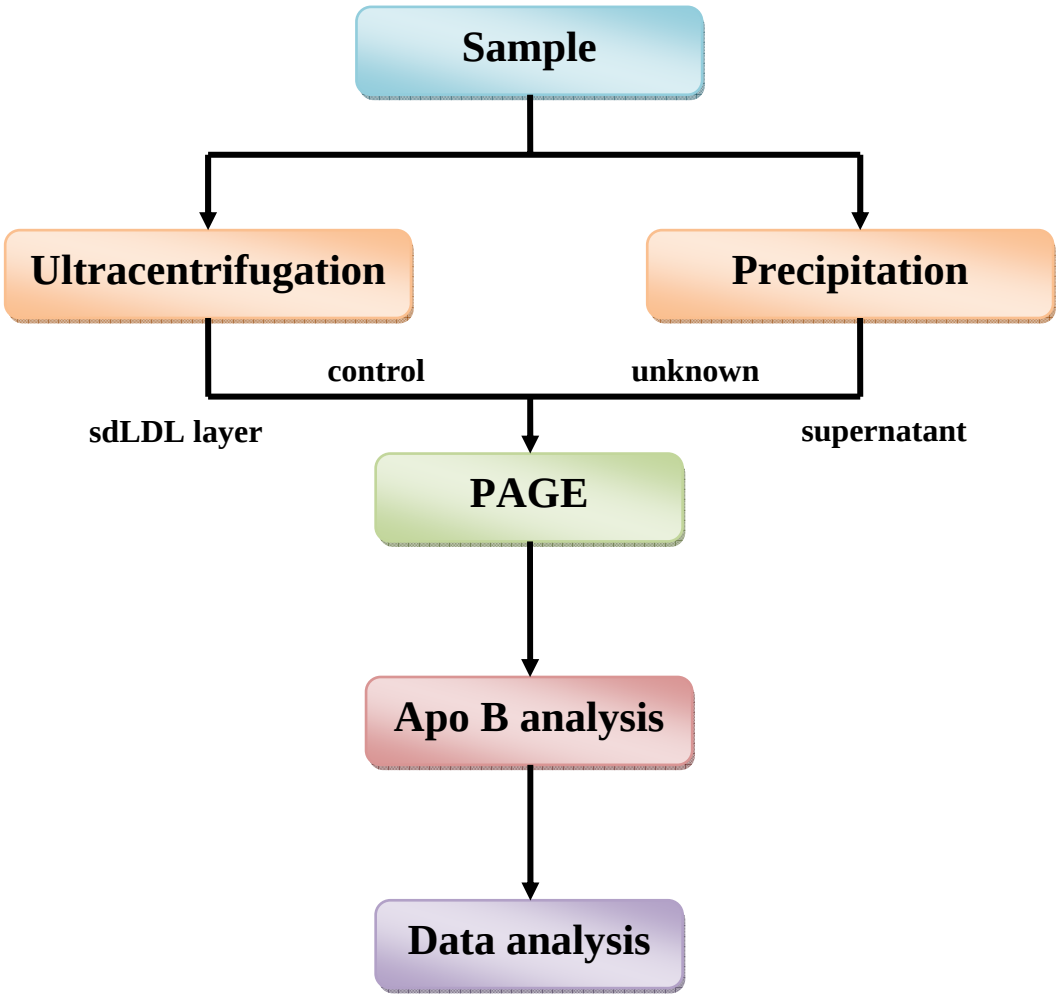

Figure 7. The schematic diagram of the scope of this study

CHAPTER V
RESULTS

1. The simplicity of precipitation method

The separation of small dense LDL particles is very simple compared with others separation methods. Precipitation method requires only simple instruments such as high-speed centrifuge and general routine clinical laboratory apparatus such as water bath, whereas either analytical centrifugation or proton nuclear magnetic resonance requires expensive and complex instrument that is not suitable in routine clinical laboratory. Precipitation method can be done within 1 hour whereas analytical centrifugation requires at least 2.5 hours depend on the rotor. Both analytical centrifugation and proton nuclear magnetic resonance require well-trained staffs, whereas precipitation method can be performed by general clinical laboratory staffs. All precipitation method processes can be performed in routine clinical laboratory. The overall schematic process of precipitation method is shown in Figure 8.

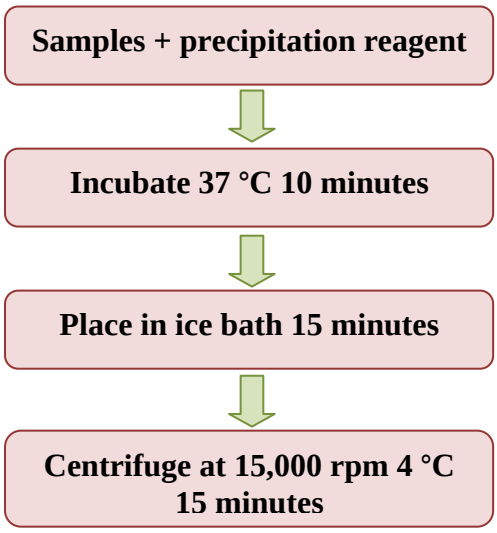

Figure 8. The overall schematic process of precipitation method

2. The reliable of precipitation method

2.1 Accuracy

The accuracy of precipitation method in separation of serum small dense LDL particles was determined by comparing with the middle band from analytical ultracentrifugation (Figure 9).

Figure 9. The middle band after running the ultracentrifugation

Samples from precipitation and ultracentrifugation were subjected to polyacrylamide gel electrophoresis. Thyroglobulin dimer was used as small dense LDL particle size marker in PAGE. Samples were pre-stained by Sudan Black B and thyroglobulin dimer were pre-stained by Coomassie Blue R250.

After performing the PAGE, bands of samples (arrow 2 – 7) and markers (arrow 1 and 8) moved and arranged in almost the same line as shown in Figure 10. This result proves that both samples from ultracentrifugation and precipitation method

contain small dense LDL particles by comparing size with standard known diameter (thyroglobulin dimer).

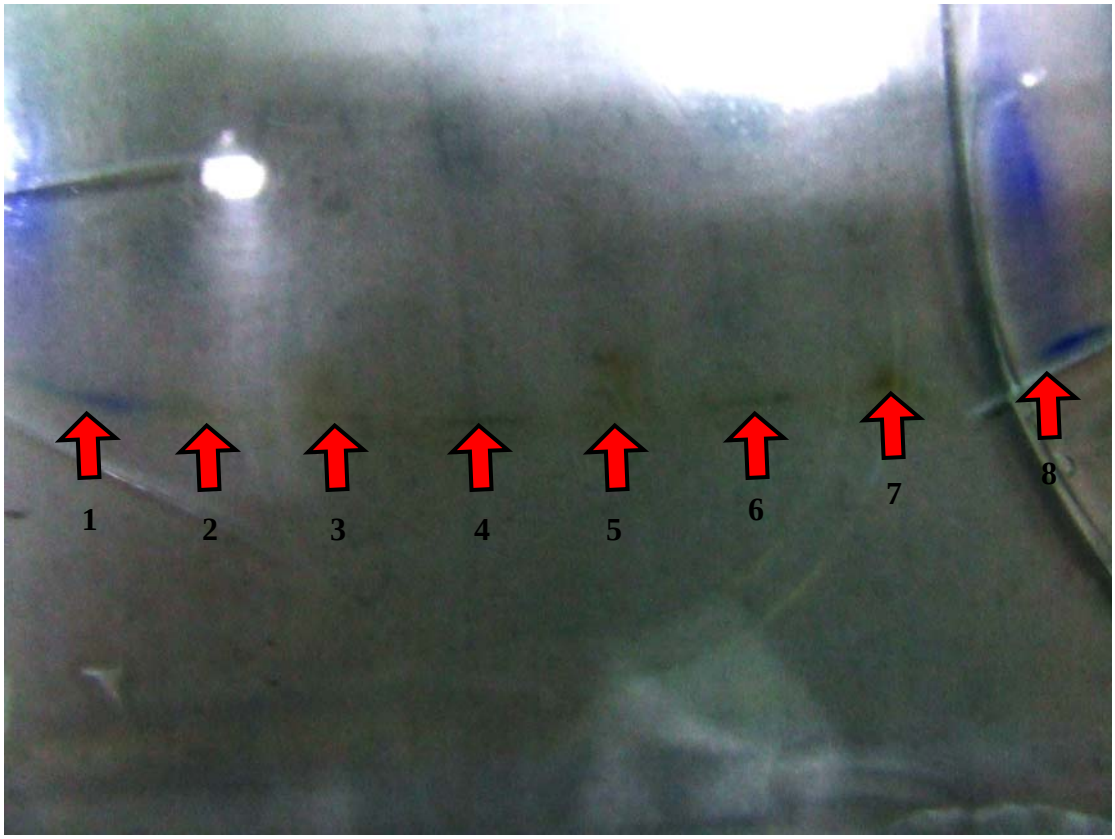

Figure 10. Bands of samples (arrow 2 – 7) and marker (arrow 1 and 8) in polyacrylamide gel electrophoresis

2.2 Precision

The precision of the precipitation method was determined by repeating the precipitation process 20 times and calculating the coefficient of variation (%CV) of the results. The results and %CV of precipitation method were shown in Table 3.

Table 3. Apo B-100 levels (mg/dL) of samples of precipitation method

Tube number	LDL-C levels (mg/dL)		
	100	**140**	**180**
1	22.17	24.77	28.39
2	22.81	24.96	28.24
3	22.24	25.07	27.07
4	20.22	23.43	26.71
5	22.41	25.29	27.68
6	21.72	24.93	29.26
7	20.58	23.79	25.90
8	20.67	24.81	26.38
9	21.32	25.37	27.70
10	21.68	25.72	27.26
11	20.53	24.72	28.26
12	21.34	24.37	28.70
13	22.67	25.81	27.38
14	21.56	23.79	28.90
15	20.63	25.93	25.26
16	20.47	24.93	25.68
17	21.38	23.29	27.71
18	22.63	24.43	27.08
19	20.53	23.07	27.24
20	21.02	25.96	27.39
Mean	21.43	24.72	27.41
SD	0.84	0.88	1.07
%CV	3.91	3.54	3.89

From the above results, the precipitation method shows good precision at all levels of samples. The %CV of each sample level was 3.91, 3.54, and 3.89 respectively.

2.3 %bias

The %bias of precipitation method was calculated by the following equation:

$$\%bias = \frac{\text{mean} - \text{reference value}}{\text{reference value}} \times 100$$

The %bias of each group of sample was shown in Table 4.

Table 4. The %bias of each group of sample

LDL-C level (mg/dL)	%bias
100	4.292
140	4.141
180	3.942

2.4 Total error of quantification of serum small dense LDL particles by precipitation method

The allowable total error (TE_a) of apo B-100 determination is 25%. The total error of precipitation method was calculated by the following equation:

$$\text{Total error} = \%bias + (Z \times \%CV) \; ; \; Z = 1.96$$

The total error of each group of samples was shown in Table 5.

Table 5. The total error of each group of samples

LDL-C level (mg/dL)	Total error (%)
100	11.96
140	11.08
180	11.57

From the above data, the total error of quantification of serum small dense LDL particles by precipitation method did not exceed the allowable total error value.

This means that the precipitation method can be used to quantify the serum small dense LDL particles in routine clinical laboratory with reliable results.

3. The conversion to molecule unit

After performed the apo B-100 analysis, the results were converted to molecule unit. The results from ultracentrifugation and precipitation method in term of molecule unit were shown in Table 6 and Table 7 respectively.

Table 6. Apo B-100 levels (molecule/dL) of samples from ultracentrifugation

Tube	LDL-C levels (mg/dL)		
	100	**140**	**180**
1	2.67	2.98	3.32
2	2.57	3.05	3.35
Average	2.62	3.02	3.34

Table 7. Apo B-100 levels (x 10^{16} molecule/dL) of samples from precipitation method

Tube	LDL-C levels (mg/dL)		
	100	**140**	**180**
1	2.59	2.90	3.32
2	2.67	2.92	3.30
3	2.60	2.93	3.17
4	2.36	2.74	3.12
5	2.62	2.96	3.24
6	2.54	2.92	3.42
7	2.41	2.78	3.03
8	2.42	2.90	3.09
9	2.49	2.97	3.24
10	2.54	3.01	3.19

Table 7. (continued) Apo B-100 levels (x 10^{16} molecule/dL) of samples from precipitation method

Tube	LDL-C levels (mg/dL)		
	100	**140**	**180**
11	2.40	2.89	3.31
12	2.50	2.85	3.36
13	2.65	3.02	3.20
14	2.52	2.78	3.38
15	2.41	3.03	2.95
16	2.39	2.92	3.00
17	2.50	2.72	3.24
18	2.65	2.86	3.17
19	2.40	2.70	3.19
20	2.46	3.04	3.20
Mean	2.51	2.90	3.20
SD	0.10	0.10	0.12
%CV	4.05	3.55	3.81

CHAPTER VI
DISCUSSION

According to the American Association of Bioanalysts, the allowable total error of apo B-100 determination is 25%. The total error of the quantification of serum small dense LDL particles by precipitation method is 12%. It is within the acceptable range of the method, so this method can be used in routine clinical laboratory. As the LDL-C concentration of samples in this study ranges from 100 – 180 mg/dL, our study demontrate that this quantification method has the linearity range from 100 to 180 mg/dL. For the further study, the sample with lower and higher concentration of the LDL-C will be studied to complete the linearity of this quantification method.

The quantification of small dense LDL particles has medical decision level at low level of LDL-C or 100 – 180 mg/dL. Patients who have same LDL-C level but different LDL particle size take different risk of developing atherosclerosis. The patients who have small dense LDL particles predominate or phenotype B take more risky than patients who have large bouyant LDL particles predominate or phenotype A. So the physicians should have strong emphasis on this risky group.

This quantification method will influent the medical decision and treatment. The miss detection of patients who have small dense LDL particles predominate let them develop the disease or continue the progression of the disease without the appropriate treatment. The quantification method can provide the early detection for this group of patients and protect them from wasting the time for receiving treatment.

For patients who have high level of LDL-C but there are large bouyant LDL particles predominate, they receive too early and unnecessary treatment. Patients may not only have side effects from drugs and treatments, but they also have to pay lots of money for the unnecessary expensive drugs and treatments.

The further study of the others parameters of method validation will be done to fulfill the simple method for quantification of serum small dense LDL particles.

CHAPTER VII
CONCLUSION

Present study determines and modifies the detection of serum small dense LDL particles and reported in term of particle number. The samples were collected from the Center of Medical Laboratory Services, Faculty of Medical Technology, Mahidol University during July to August 2008. Following the LDL-C determination, the LDL-C levels of each groups was 102.5 mg/dL, 143.7 mg/dL, and 181.6 mg/dL respectively.

The separation of serum small dense LDL particles was done by both ultracentrifugation and precipitation method. The result of ultracentrifugation showed that there were 3 major bands from all serum samples. The upper and middle bands were selected for polyacrylamide gel electrophoresis and apo B-100 analysis. The aliquot from precipitation method were also collected for further analysis.

The quantification of serum small dense LDL particles by precipitation method showed its process simplicity compared to the others separation methods such as analytical ultracentrifugation, gradient gel electrophoresis, and proton nuclear magnetic resonance.

The accuracy of this quantification method was determined by polyacrylamide gel elecltrophoresis. All bands of samples from ultracentrifugation and precipitation method were moved in the same line of bands from thyroglobulin dimer which was used as standard size marker of small dense LDL particle. This quantification method also showed good precision with a separated technique of 20 times. The %CV of 3 levels of samples were 3.91, 3.54, and 3.89 respectively.

The total error of the quantification of serum small dense LDL particles by precipitation method was 12% that was within the apo B-100 allowable total error from the American Association of Bioanalysts. This method can be used for routine clinical laboratory with linearity range from 100 – 180 mg/dL of LDL-C.

The method presented herein will influence the medical decision and treatment. Patients who have small dense LDL particles predominate or phenotype B will receive appropriate treantments and medicines, whereas patients who have large bouyant LDL particles predominate or phenotype A will not have to receive unnecessary treatments or medicines.

The results from apo B-100 analysis were converted into molecule number by dividing with 8.55×10^{-16}. Mean of the apo B-100 levels in term of molecule number of each sample groups was 2.51×10^{-16} molecule/dL, 2.90×10^{-16} molecule/dL, and 3.20×10^{-16} molecule/dL respectively.

The further study of the others parameters of method validation will be done to fulfill the simple method for quantification of serum small dense LDL particles.

REFERENCES

1. World Health Organization (WHO). Avoiding Heat Attacks and Strokes: Don't be a victim. Protect yourself. 2005.
2. Yach D, Hawkes C, Gould CL, Hofman KJ. The global burden of chronic diseases: overcoming impediments to prevention and control. JAMA 2004;291(21):2616-22.
3. Mitka M. Heart disease a global health threat. JAMA 2004;291 (21):2533.
4. Rodenburg J, Vissers M, Wiegman A, Trip M, Bakker H, Kastelein JJ. Familial hypercholesterolemia in children. Curr Opin Lipidol 2004;15 (4):405-11.
5. Enos WF, Holmes RH, Beyer J. Coronary disease among United States soldiers killed in action in Korea. J Am Med Assoc 1953; 152:1090-3.
6. Stary C, Chandler B, Glagov S, Guyton J, Insull W. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Atherosclerosis, American Heart Association. Circulation 1994;89(5):2462-78.
7. Adams G, Simoni D, Bordelon C, Vick G, Kimball K. Bilateral symmetry of human carotid artery atherosclerosis. Stroke 2002;33(11):2575-80.
8. McGill C Jr, McMahan A, Herderick E, Malcom T, Tracy E, Strong P. Origin of atherosclerosis in childhood and adolescence. Am J Clin Nutr 2000;72 (5 Suppl):1307S-15S.
9. Fuster V, Lewis A. Conner Memorial Lecture. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. Circulation 1994;90(4):2126-46.
10. Awareness of stroke warning signs – 17 states and the U.S. Virgin Islands, 2001. MMWR Morbid Mortal Wkly Rep 2004;53(17):359-62.
11. Glass K, Witztum L. Atherosclerosis: the road ahead. Cell 2001;104(4):503-16.

12. Mooteri N, Petersen F, Dagubati R, Pai G. Duration of residence in the United States as a new risk factor for coronary artery disease (the Konkani Heart Study). Am J Cardiol 2004;93(3):359-61.
13. Plutzky J. The vascular biology of atherosclerosis. Am J Med 2003;115(Suppl 8A):55S-61S.
14. Newby C. An overview of the vascular response to injury: a tribute to the late Russell Ross. Toxicol Lett 2000;112-3:519-29.
15. Behrendt D, Ganz P. Endothelial function. From vascular biology to clinical applications. Am J Cardiol 2002;90(10C):40L-48L.
16. Cannon O, 3rd. Role of nitric oxide in cardiovascular disease: focus on the endothelium. Clin Chem 1998;44(8 Pt2):1809-19.
17. Furchgott F, Zawadzki V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288(5789): 373-6.
18. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher M. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399(6736):601-5.
19. Libby P. Vascular biology of atherosclerosis: overview and state of the art. Am J Cardiol 2003;91(3A):3-6A.
20. O'Brien D, Chait A. The biology of the artery wall in atherogenesis. Med Clin North Am 1994 ;78(1):41-67.
21. Flavahan A. Atherosclerosis or lipoprotein-induced endothelial dysfunction. Potential mechanisms underlying reduction in EDRF/nitric oxide activity. Circulation 1992;85(5):1927-38.
22. Weiss N, Keller C, Hoffmann U, Loscalzo J. Endothelial dysfunction and atherothrombosis in mild hyperhomocysteinemia. Vasc Med 2002;7(3):227-39.
23. Dandona P, Aljada A, Chaudhuri A, Mohanty P. Endothelial dysfunction, inflammation and diabetes. Rev Endocr Metab Disord 2004;5(3):189-97.
24. Landmesser U, Hornig B, Drexler H. Endothelial function: a critical determination in atherosclerosis? Circulation 2004;109(21 Suppl 1):1127-33.

25. Felmeden D, Spencer C, Chung N, Belgore F, Blann A. Relation of thrombogenesis in systemic hypertension to angiogenesis and endothelial damage/dysfunction (a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial [ASCOT]). Am J Cardiol 2003;92(4):400-5.
26. Libby P, Ridker M, Maseri A. Inflammation and atherosclerosis. Circulation 2002;105(9):1135-43.
27. Noll G. Pathogenesis of atherosclerosis: a possible relation to infection. Atherosclerosis 1998;140(Suppl 1):S3-9.
28. Andel M, Tsevegjav A, Roubalova K, Hruba D, Dlouhy P, Kraml P. Infectious and inflammatory factors in the etiology and pathogenesis of atherosclerosis. Vnitr Lek 2003;49(12):960-6.
29. Viles-Gonzalez F, Anand X, Valdiviezo C. Update in atherothrombotic disease. Mt Sinai J Med 2004;71(3):197-208.
30. Callow D. Endothelial dysfunction in atherosclerosis. Vascul Pharmacol 2002;38(5):257-8.
31. Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. Atherosclerosis 2003;170(2):191-203.
32. Blann D, Nadar K, Lip Y. The adhesion molecule P-selectin and cardiovascular disease. Eur Heart J 2003;24(24):2166-79.
33. Huo Y, Ley K. Adhesion molecules and atherosclerosis. Acta Physiol Scand 2001;173(1):35-43.
34. Walpola L, Gotlieb I, Cybulsky I, Langille L. Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. Arterioscler Thromb Vasc Biol 1995;15(1):2-10.
35. Davies M, Gordon J, Gearing A, Pigott R, Woolf N. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. J Pathol 1993;171(3):223-9.
36. Johnson-Tidey R, McGregor J, Taylor P, Poston R. Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques. Co-expression with intercellular adhesion molecule-1. Am J Pathol 1994;144(5):952-61.

37. Johnson R, Chapman S, Dong Z, Ordovas J, Mayadas T, Herza J, et al. Absence of P-selectin delays fatty streak formation in mice. J Clin Invest 1997;99(5):1037-43.
38. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of P- and E-selectins in atherosclerosis. J Clin Invest 1998;102(1):145-52.
39. Schober A, Manka D, von Hundelshausen P, Huo Y, Hanrath P. Deposition of platelet RANTES triggering monocyte recruitment requires P-selectin and is involved in neointima formation after arterial injury. Circulation 2002;106(12):1523-9.
40. Brown MS, Herz J, Goldstein JL. LDL-receptor structure. Calcium cages, acid baths and recycling receptors. Nature 1997;388(6643):629-30.
41. Navab M, Berliner J, Watson A, Hama S, Shih D. The yin and yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arterioscler Thromb Vasc Biol 1996;16(7):831-42.
42. Heinecke JW. Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low-density lipoprotein hypothesis. Atherosclerosis 1998;141(1):1-15.
43. Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, Maeda N. Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. J Clin Invest 2000;105(4):451-8.
44. Harats D, Shaish A, George J, Mulkins M, Kurihara H. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol 2000;20(9):2100-5.
45. Cyrus T, Witztum J, Rader D, Tangirala R, Fazio S. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. J Clin Invest 1999;103(11):1597-604.

46. Detmers P, Hernandez M, Mudgett J, Hassing H, Burton C. Deficiency in inducible nitric oxide synthase results in reduced atherosclerosis in apolipoprotein E-deficient mice. J Immunol 2000;165(6):3430-5.
47. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med 2000;342(3):154-60.
48. Warmington KS, Boring L, Ruth JH. Effect of C-C chemokine receptor 2 (CCR2) knockout on type-2 (schistosomal antigen-elicited) pulmonary granuloma formation: analysis of cellular recruitment and cytokine responses. Am J Pathol 1999;154(5);1407-16.
49. Hogaboam CM, Steinhauser ML, Chensue SW, Kunkel SL. Novel roles for chemokines and fibroblasts in interstitial fibrosis. Kidney Int 1998;54(6):2152-9.
50. Hogaboam C, Bone-Larson CL, Lipinski S, Lukacs N, Chensue S. Differential monocyte chemoattractant protein-1 and chemokine receptor 2 expression by murine lung fibroblasts derived from Th1- and Th2-type pulmonary granuloma models. J Immunol 1999;163(4):2193-201.
51. Gosling J, Slaymaker S, Gu L, Tseng S, Zlot C. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. J Clin Invest 1999;103(6):773-8.
52. Libby P. Molecular bases of the acute coronary syndromes. Circulation 1995;91(11):2844-50.
53. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of P- and E-selectins in atherosclerosis. J clin Invest 1998;102(1):145-52.
54. Suzuki H, Kurihara Y, Takeya M. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. Nature 1997;386(6622):292-6.
55. Febbraio M, Podrez E, Smith J, Hajjar D, Hazen S. Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. J Clin Invest 2000;105(8):1049-56.

56. Tall AR, Jiang X, Luo Y, Silver D. 1999 George Lyman Duff memorial lecture: lipid transfer proteins, HDL metabolism, and atherogenesis. Arterioscler Thromb Vasc Biol 2000;20(5):1185-8.
57. Thyberg J. Differentiated properties and proliferation of arterial smooth muscle cells in culture. Int Rev Cytol 1996;169:183-265.
58. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362(6423):801-9.
59. Benditt EP, Benditt JM. Evidence for a monoclonal origin of human atherosclerotic plaques. Proc Natl Acad Sci USA 1973;70(6):1753-6.
60. Stoneman VE, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. Clin Sci (Lond) 2004.
61. Falk E. Plaque vulnerability and disruption. Rev Clin Esp 1996;196(4 Monografico):6-12.
62. Orekhov A, Andreeva E, Krushinsky A, Novikov I. Intimal cells and atherosclerosis. Relationship between the number of intimal cells and major manifestations of atherosclerosis in the human aorta. Am J Pathol 1986;125(2):402-15.
63. Swei A, Lacy F, DeLano FA, Schmid-Schonbein GW. Oxidative stress in the Dahl hypertensive rat. Hypertension 1997;30(6):1628-33.
64. Wesley RB, 2nd, Meng X, Godin D, Galis ZS. Extracellular matrix modulates macrophage functions characteristic to atheroma: collagen type I enhances acquisition of resident macrophage traits by human peripheral blood monocytes *in vitro*. Arterioscler Thromb Vasc Biol 1998;18(3):432-40.
65. Vanhoutte PM, Boulanger CM. Endothelium-dependent responses in hypertension. Hypertens Res 1995;18(2):87-98.
66. Mercurius KO, Morla AO. Inhibition of vascular smooth muscle cell growth by inhibition of fibronectin matrix assembly. Circ Res 1998;82(5):548-56.
67. Assoian RK, Marcantonio EE. The extracellular matrix as a cell cycle control element in atherosclerosis and restenosis. J Clin Invest 1996;98(11):2436-9.

68. Gordon T, Kannel WB, Castelli WP, Dawber TR. Lipoproteins, cardiovascular disease and death. The Framingham Study. Arch Intern Med 1981;141:1128-30.

69. Folsom AR. 'New' risk factors for atherosclerotic disease. Exp Gerontol 1999;34:483-90.

70. Fallon U, Ben-Shlomo Y, Elwood P, Ubbink J, Smith G. Homocysteine and coronary heart disease in the Caerphilly cohort: A 10 year follow up. Heart 2001;85:153-8.

71. Fallon U, Elwood P, Ben-Shlomo Y, Ubbink J, Smith G. Homocysteine and ischaemic stroke in men: the Caerphilly study. J Epidemiol Community Health 2001;55(2):91-6.

72. Gale C, Ashurst H, Phillips N, Stuart J. Renal function, plasma homocysteine and carotid atherosclerosis in elderly people. Atherosclerosis 2001;154(1):141-6.

73. Jacobsen DW. Homocysteine and vitamins in cardiovascular disease. Clin Chem 1998;44:1833-43.

74. Taylor BV, Oudit GY, Evans M. Homocysteine, vitamins, and coronary artery disease. Comprehensive review of the literature. Can Farm Physician 2000;46:2236-45.

75. Guerci B, Antebi H, Meyer L, Durlach V, Ziegler O. Increased ability of LDL from normolipidemic type 2 diabetic women to generate peroxides. Clin Chem 1999;45:1439-48.

76. Polidori M, Merocci P, Stahl W, Parente B, Cecchetti R, Cherubini A. Plasma levels of lipophilic antioxidants in very old patients with type 2 diabetes. Diabetes Metab Res Rev 2000;6:15-9.

77. Assmann G, Schulte H. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Prospective Cardiovascular Münster study. Am J Cardiol 1992;70:733-7.

78. Castelli WP, Anderson K, Wilson PWF, Levy D. Lipids and risk or coronary heart disease. The Framingham Study. Ann Epidermiol 1992;2:23-8.

79. Blankenhorn DH, Azen S, Kramsch D, Mack W. The MARS Research Group. Coronary angiographic changes with lovastatin therapy. The Monitored Atherosclerosis Regression Study (MARS). Ann Intern Med 1993;119:969-76.

80. The West of Scotland Coronary Prevention Study Group. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. N Engl J Med 1995;333:1301-7.

81. Downs J, Clearfield M, Weis S, Whitney E, Shapiro D. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels. Results of AFCAPS/TexCAPS. JAMA 1998;279:1615-22.

82. McGill HC Jr, McMahan CA, Kruski AW, Mott GE. Relationship of lipoprotein cholesterol concentrations to experimental atherosclerosis in baboons. Atherosclerosis 1981;1:3-12.

83. Keys A. Coronary heart disease in seven countries. Circulation 1970;40:I1-211.

84. Kannell WB, Castelli WP, Gordon T, McNamara PM. Serum cholesterol, lipoproteins, and risk of coronary heart disease. Ann Intern Med 1971;74:1-12.

85. Hoff HF, Karagas M, Heideman CL, Gaubatz JW, Gotto AM Jr. Correlation in human aorta of apo B fractions with tissue, cholesterol, and collagen content. Atherosclerosis 1979;32:259-68.

86. Hoff HF, Bradley WA, Heideman CL, Gaubatz JW, Karagas MD, Gotto AM Jr. Characterization of low-density lipoprotein-like particles in human aorta from grossly normal and atherosclerotic regions. BioChim Biophys Acta 1979;573:361-74.

87. Goldstein JL, Brown MS. Familial hypercholesterolemia. In:Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS, eds. The metabolic basis of inherited disease, 5th ed. New York: McGraw-Hill, 1973:672-713.

88. Vega GL, Grundy SM. In vivo evidence for reduced binding of low-density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. J Clin Invest 1986;78:1410-4.

89. Innerarity TL, Mahley RW, Weisgraber KH, Bersot TP, Krauss RM, Vega GL, et al. Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia. J Lipid Res 1990;31:1337-49.

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

91. Ross R. The pathogenesis of atherosclerosis—an update. N Engl J Med 1986;314:488-500.

92. Stender S, Zilversmit DB. Transfer of plasma lipoprotein components and of plasma proteins into aortas of cholesterol-fed rabbits: molecular size as a determinant of plasma lipoprotein influx. Arteriosclerosis 1981;1:28-49.

93. Small DM. Progression and regression of atherosclerotic lesions; insights from lipid physical biochemistry. Arteriosclerosis 1988;8:103-29.

94. Srinivasan S, Radhakrishnamurthy B, Pargainkar P, Berenson G, Dolan P. Lipoprotein-acid mucopolysaccharide complexes of human atherosclerotic lesions. BioChim Biophys Acta 1975;388:58-70.

95. Hollander W. Unified concept on the role of acid mucopolysaccharides and connective tissue proteins in the accumulation of lipids, lipoproteins, and calcium in the atherosclerotic plaque. Exp Mol Pathol 1976;25:106-20.

96. Sambandam T, Baker J, Christner J, Ekborg S. Specificity of low-density lipoprotein-glycosaminoglycan interaction. Arterioscler Thromb 1991;11:561-8.

97. Kramsch D, Franzblau C, Hollander W. Components of the protein-lipid complex of arterial elastin: their role in the retention of lipid in atherosclerotic lesion. Adv Exp Med Biol 1973;43:193-210.

98. Guyton J, Bocan T, Schifani T. Quantitative ultrastructural analysis of perifibrous lipid and its association with elastin in nonatherosclerotic human aorta. Arteriosclerosis 1985;5:644-52.

99. Winlove C, Parker K, Ewins A. Reversible and irreversible interactions between elastin and plasma lipoproteins. Biochim Biophys Acta 1985;838:374-80.

100. Bocan T, Guyton J. Human aortic fibrolipid lesions: progenitor lesions for fibrous plaques, exhibiting early formation of the cholesterol-rich core. Am J Pathol 1985;120:193-206.

101. Oorni K, Pentikainen M, Ala-Korpela M, Kovanen P. Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions. J Lipid Res 2000;41:1703-14.

102. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115-26.

103. Lusis J. Atherosclerosis. Nature 2000;407:233-41.

104. Navab M, Imes S, Hama S, Hough G, Ross L. Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. J Clin Invest 1991;88:2039-46.

105. Xiao D, Wang Z, She M. Minimally modified low-density lipoprotein induces monocyte chemotactic protein-1 expression in vivo and a novel method for monocyte adhesion to arterial intima. Clin Med J (Engl) 1999;112:438-42.

106. Frostegard J, Nilsson J, Haegerstrand A, Hamsten A, Wigzell H. Oxidized low density lipoprotein induces differentiation and adhesion of human monocytes and the monocytic cell line U937. Proc Natl Acad Sci USA 1990;87:904-8.

107. Kunjathoor V, Febbraio M, Podrez E, Moore K, Andersson L. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 2002;277:49982-8.

108. Tsukamoto K, Kinoshita M, Kojima K. Synergically increased expression of CD36, CLA-1 and CD68, but not of SR-A and LOX-1, with the progression to foam cells from macrophages. J Atheroscler Thromb 2002;9:57-64.

109. Haraldsen G, Kvale D, Lien B, Farstad I, Brandtzaeg P. Cytokine-regulated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human microvascular endothelial cells. J Immunol 1996;156:2558-65.

110. Uzui H, Harpf A, Liu M. Increased expression of membrane type 3-matrix metalloproteinase in human atherosclerotic plaque: role of activated macrophages and inflammatory cytokines. Circulation 2002;106:3024-30.

111. Yang P, Rui Y. Intercellular adhesion molecule-1 and vascular endothelial growth factor expression kinetics in macrophage-derived foam cells. Life Sci 2003;74:471-80.

112. Stamler J, Wentworth D, Neaton J, for the MRFIT Research Group. Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). JAMA 1986;256:2823-8.

113. Krauss R, Burke D. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. J Lipid Res 1981;23:236-44.

114. Swinkels D, Hak-Lemmers H, Demacker P. Single spin density gradient ultracentrifugation method for the detection and isolation of light and heavy low density lipoprotein subfractions. J Lipid Res 1987;28:1233-9.

115. Berneis K, Rizzo M. LDL size: does it matter? Swiss Med Wkly 2004;134(49-50):720-4.

116. Berneis K, Krauss R. Metabolic origins and clinical significance of LDL heterogeneity. J Lipid Res 2002;43:1363-79.

117. Krauss R, Blanche P. Detection and quantification of LDL subfractions. Curr Opin Lipidol 1992;3:377-83.

118. Packard C, Shepherd J. Lipoprotein heterogeneity and apolipoprotein B metabolism. Arterioscler Thromb Vasc Biol 1997;17:3542-56.

119. Austin M, King M, Vranizan K, Krauss R. Atherogenic lipoprotein phenotype. A proposed genetic marker for coronary heart disease risk. Circulation 1990;82:495-506.

120. Campos H, Blijlevens E, McNamara J, Ordovas J, Posner B, Wilson P, et al. LDL particle size distribution. Results from the Framingham Offspring Study. Arterioscler Thromb 1992;12:1410-9.

121. Selby J, Austin M, Newman B, Zhang D, Quesenberry C Jr, Mayer E, et al. LDL subclass phenotypes and the insulin resistances syndrome in women. Circulation 1993;88:381-7.

122. Austin M. Genetic epidemiology of low-density lipoprotein subclass phenotypes. Ann Med 1992;24:477-81.

123. Rizzo M, Barbagallo C, Severino M, Polizzi F, Onorato F, Noto D, et al. Low-density-lipoprotein peak particle size in a Mediterranean population. Eur J Clin Invest 2003;33:126-33.

124. Terry R, Wood P, Haskell W, Stefanick M, Krauss R. Regional adiposity patterns in relation to lipids, lipoprotein cholesterol, and lipoprotein subfraction mass in men. J Clin Endocrinol Metab 1989;68:191-9.

125. de Graaf, Swinkels D, Demacker P, de Haan A, Stalenhoef A. Differences in the low density lipoprotein subfraction profile between oral contraceptive users and controls. J Clin Endoclinol Metab 1993;76:197-202.

126. Dreon D, Fernstrom H, Williams P, Krauss R. LDL subclass patterns and lipoprotein response to a low-fat, high-carbohydrate diet in women. Arterioscler Thromb Vasc Biol 1997;17:707-14.

127. Austin M, Brunzell J, Fitch W, Krauss R. Inheritance of low density lipoprotein subclass patterns in familial combined hyperlipidemia. Arteriosclerosis 1990;10:520-30.

128. Teng B, Thompson G, Sniderman A, Forte T, Krauss R, Kwiterovich P Jr. Composition and distribution of low density lipoprotein fractions in hyperapobetalipoproteinemia, normolipidemia, and familial hypercholesterolemia. Proc Natl Acad Sci USA 1983;80:6662-6.

129. Genest J Jr, Bard J, Fruchart J, Ordovas J, Schaefer E. Familial hypoalphalipoproteinemia in premature coronary artery disease. Arterioscler Thromb 1993;13:1728-37.

130. Sacks F, Campos H. Low-density lipoprotein size and cardiovascular disease: a reappraisal. J Clin Endocr Metab 2003;88:4525-32.

131. Krauss R, Burke D. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. J Lipid Res 1982;23:97-104.

132. Griffin B, Caslake M, Yip B, Tait G, Packard C, Shepherd J. Rapid isolation of low density liporptoein (LDL) subfractions from plasma by density gradient ultracentrifugation. Atherosclerosis 1990;83:59-67.

133. Rizzo M, Taylor J, Barbagallo C, Berneis K, Blanche P, Krauss R. Effects on lipoprotein subclasses of combined expression of human hepatic lipase and human apoB in transgenic rabbits. Arterioscler Thromb Vasc Biol 2004;24:141-6.

134. Georgieva A, van Greevenbrock M, Krauss R, Brouwers M, Vermeulen V, Robertus-Teunissen M, et al. Subclasses of low-density lipoprotein and very low-density lipoprotein in familial combined hyperlipidemia: relationship to multiple lipoprotein phenotype. Arterioscler Thromb Vasc Biol 2004;24:1-7.

135. Bjornheden T, Babyi A, Bondjers G, Wiklund O. Accumulation of lipoprotein fractions and subfractions in the arterial wall, determined in an in vitro perfusion system. Atherosclerosis 1996;123:43-56.

136. Galeano N, Al-Haideri M, Keyserman F, Rumsey S, Deckelbaum R. Small dense low density lipoprotein has increased affinity for LDL receptor-independent cell surface binding sites: a potential mechanism for increased atherogenicity. J Lipid Res 1998;39:1263-73.

137. Tribble D, Rizzo M, Chait A, Lewis D, Blanche P, Krauss R. Enhanced oxidative susceptibility and reduced antioxidant content of metabolic precursors of small, dense low-density lipoproteins. Am J Med 2001;110:103-10.

138. O'Neal D, Lewicki J, Ansari M, Matthews P, Best J. Lipid levels and peripheral vascular disease in diabetes and non-diabetic subjects. Atherosclerosis 1998;136:1-8.

139. Knott T, Pease R, Powell L, Wallis S, Rall S Jr, Innerarity T, et al. Complete protein sequence and identification of structural domains of human apolipoprotein B. Nature 1986;323:734-8.

140. Cladaras C, Hadzopoulou-Cladaras M, Nolte R, Atkinson D, Zannis VI. The complete sequence and structural analysis of human apolipoprotein B-100: relationship between apo B-100 and apo B-48 forms. Embo J 1986;5:3495-507.

141. Chen S, Yang C, Chen P, Setzer D, Tanimura M, Li W, et al. The complete cDNA and amino acid sequence of human apolipoprotein B-100. J Biol Chem 1986;261:12918-21.

142. Goldstein J, Brown M. The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem 1977;46:897-930.

143. Schonfeld G, Krul E. Immunologic approaches to lipoprotein structure. J Lipid Res 1986;27:583-601.

144. Milne R, Theolis R Jr, Maurice R, Pease R, Weech P, Rassart E, et al. The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. J Biol Chem 1989;264:19754-60.

145. Pease R, Milne R, Jessup W, Law A, Provost P, Fruchart J, et al. Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B100. J Biol Chem 1990;265:553-68.

146. Innerarity T, Weisgraber K, Arnol K, Mahley R, Krauss R, Vega G, et al. Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding. Proc Natl Acad Sci USA 1987;84:6919-23.

147. Young S, Bertics S, Curtis L, Witztum J. Characterization of an abnormal species of apolipoprotein B, apolipoprotein B-37, associated with familial hypobetalipoproteinemia. J Clin Invest 1987;79:1831-41.

148. Teng B, Sniderman A, Krauss R, Kwiterovich P, Milne R. Modulation of apolipoprotein B antigenic determinants in human low density lipoprotein subclasses. J Biol Chem 1985;260:5067-72.

149. Schonfeld G, Patch W, Pfleger B, Witztum J, Weidman S. Lipolysis produces changes in the immunoreactivity and cell reactivity of very low density lipoproteins. J Clin Invest 1979;64:1288-97.

150. Mills G, Lane P, Weech P. A guidebook to lipoprotein technique. Amsterdam, Elsevier, 1984, pp 18-116.

151. Lindgren F, Jensen L, Hatch E. The isolation and quantitative analysis of serum lipoproteins. Blood lipids and lipoproteins: quantification, composition, and metabolism. New York, Wiley Interscience, 1972, pp 181-274.

152. Kahlon T, Glines L, Lindgren F. Analytic ultracentrifugation of plasma lipoprotein. Orlando, Academic Press, 1986, pp 26-45.

153. Mills G, Lane P, Weech P. A Guidebook to lipoprotein technique. Amsterdam, Elsevier, 1984, pp 308-83.

154. Lindgren F. Serum lipoproteins: isolation and analysis with the preparative and analytical ultracentrifuge. Missouri, BI-Sci. Publ. Div., 1974, pp 475-510.

155. Jeffrey P, Nichol L, Smith G. The determination of density and molecular weight distributions of lipoproteins by sedimentation equilibrium. J Biol Chem 1975;250:533-41.

156. Ma S, Schumaker V, Knobler C. Turbidimetric ultracentrifugation. Application to the study of human serum very low density liporptoein distributions. J Biol Chem 1977;250:1728-31.

157. Schumaker V, Puppione D. Sequential floatation ultracentrifugation. Orlando, Academic Press, 1986, pp 155-70.

158. Kunitake S, Kane J. Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. J Lipid Res 1982;23:936-40.

159. Kulkami K, Garber D, Jones M, Segrest J. Identification and cholesterol quantification of low density lipoprotein subclasses in young adults by VAP-II methodology. J Lipid Res 1995;36:2291-302.

160. Tornvall P, Karpe F, Carlson L, Hamsten A. Relationships of low density lipoprotein subfractions to angiographically defined coronary artery disease in young survivors of myocardial infarction. Atherosclerosis 1991;90:67-80.

161. Austin M, Breslow J, Hennekens C, Buring J, Willet W. Low-density lipoprotein subclass patterns and risk of myocardial infarction. JAMA 1988;260:1917-21.

162. McNamara J, Campos H, Ordovas J, Peterson J. Effect of gender, age, and lipid status on low density lipoprotein subfraction distribution. Results from the Framingham Pffspring Study. Arteriosclerosis 1987;7:483-90.

163. Otvos J, Jeyarajah E, Bennett D, Krauss R. Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement. Clin Chem 1992;38:1632-8.

164. Otvos J, Jeyarajah E, Bennett D. Quantification of plasma lipoproteins by proton nuclear magnetic resonance spectroscopy. Clin Chem 1991;37:377-86.

165. Langlois M, Blaton V. Historical milestones in measurement of HDL-cholesterol: Impact on clinical and laboratory practice. Clin Chim Acta 2006;369:168-78.

166. Naito H. Coronary artery disease and disorders of lipid metabolism. In Kaplan LA, Pesce AJ (eds.). Clinical Chemistry: Theory, Analysis and Correlation, 3rd ed. St. Louis, CV Mosby, 1996, pp 642-82.

167. Lehman C. Lipids and lipoproteins. In Lehman CA (ed.). Saunders Manual of Clinical Laboratory Science. Philadelphia, WB Saunders, 1998, pp 59-76.

168. Stein E, Myers G. Lipids, apolipoproteins and lipoproteins. In Burtis CA, Ashwood ER (eds.). Tietz Fundamentals of Clinical Chemistry, 4th ed. Philadelphia, WB Saunders, 1996, pp 375-401.

169. Flegg H. An investigation of the determination of serum cholesterol by an enzymatic method. Ann Clin Biochem 1973;10:79-83.

170. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adults Treatment Panel III). JAMA 2001;285(19):2486-97.

171. Benlian P, Cansier C, Hennache C, Khallouf O, Bayer P. Comparison of a new method for the direct and simultaneous assessment of LDL- and HDL-cholesterol with ultracentrifugation and established methods. Clin Chem 2000;46:493-505.

172. Nauck M, Winkelr K, März W. Quantitative determination of high-, low- and very-low-density lipoproteins and lipoprotein (a) by agarose gel electrophoresis and enzymatic cholesterol staining. Clin Chem 1995;41:1761-7.

173. Cobbaert C, Zwang L, Ceriotti F, Modenese A, Cremer P. Reference standardization and triglyceride interference of a new homogeneous HDL-cholesterol assay compared with a former chemical precipitation assay. Clin Chem 1998;44:779-89.

174. Nauck M, März W, Wieland H. New immunoseparation-based homogeneous assay for HDL-cholesterol compared with three homogeneous and two heterogeneous methods for HDL-cholesterol. Clin Chem 1998;44:1443-51.

175. Simó J, Castellano I, Ferré N, Jovon J, Camps J. Evaluation of a homogeneous assay for high-density lipoprotein cholesterol: limitations in patients with cardiovascular, renal, and hepatic disorders. Clin Chem 1998;44:1233-41.

176. Friedewald W, Levy R, Fredrickson D. Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.

177. Warnick R, Knopp R, Fitzpatrick V. Estimating low density lipoprotein cholesterol by Friedewald equation is adequate for classifying patients on the basis of nationally recommended cutpoints. Clin Chem 1990;36:15-9.

178. Vrga L, Contacos C, Li S, Sullivan R. Comparison of methods for measurement of apolipoprotein B and cholesterol in low-density lipoproteins. Clin Chem 1997;43:390-3.

179. Esteban-Salán M, Guimón-Bardesi A, de la Viuda-Unzueta J. Analytical and clinical evaluation of two homogeneous assays for LDL-cholesterol in hyperlipidemic patients. Clin Chem 2000;46:1121-31.

180. Jialal I, Hirany S, Devaraj S, Sherwood T. Comparison of an immunoprecipitation method for direct measurement of LDL-cholesterol with beta-quantification (ultracentrifugation). Am J Clin Pathol 1995;41:232-40.

181. Rifai N, Iannotti E, DeAngelis K, Law T, Nauck M. Analytical and clinical performance of a homogeneous enzymatic LDL-cholesterol assay compared with the ultracentrifugation-dextran sulfate-Mg^{2+} method. Clin Chem 1998;44:1242-50.

182. Stavropoulous W, Crouch R. A new colorimetric procedure for the determination of serum triglycerides. Clin Chem 1974;20:857.

183. Graziani M, Zanolla L, Righetti G, Marchetti C, Mocarelli P. Plasma apolipoproteins A-I and B in survivors of myocardial infarction and in a control group. Clin Chem 1998;44:134-40.

184. Tate J, Rifai N, Berg K, Couderc R, Dati F. International federation of clinical chemistry standardization project for the measurement of lipoprotein (a). Phase I. Evaluation of the analytical performance of lipoprotein (a) assay systems and commercial calibrators. Clin Chem 1998;44:1629-40.

185. Austin M, Hokanson J. Epidemiology of triglycerides, small dense low-density lipoprotein, and lipoprotein (a) as risk factors for coronary heart disease. Med Clin North Am 1994;78:99-115.

186. Danesh J, Collins R, Peto R. Lipoprotein (a) and coronary heart disease. Circulation 2000;102:1082-5.

187. Rhoads G, Dahlen G, Berg K, Morton N, Dannenberg A. Lp (a) lipoprotein as a risk factor for myocardial infarction. JAMA 1986;256:2540-4.

188. Nguyen T, Ellefson R, Hodge D. Predictive value of electrophoretically detected lipoprotein (a) for coronary heart disease and cerebrovascular disease in a community-based cohort of 9936 men and women. Circulation 1997;96:1390-7.

189. Kronenberg F, Kronenberg M, Kiechl S, Trenkwalder E, Santer P. Role of lipoprotein (a) and apolipoprotein (a) phenotype in atherosclerosis: Prospective results from the Bruneck study. Circulation 1999;100:1154-60.

190. Havel R, Eder H, Bragdon J. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34(9):1345-53.

191. Beckman Coulter, Inc. Applications Data: Ultracentrifuge Methods for Lipoprotein Research [Online]. [cited on 2008 Feb 20]; Available at http://www.beckmancoulter.com/literature/Bioresearch/0514B(DS).pdf.

192. Hirano T, Ito Y, Saugusa H, Yoshino G. A novel and simple method for quantification of small, dense LDL. J Lipid Res 2003;44:2193-201.

193. Grandjean P, Alhassan S. Essential laboratory methods for blood lipid and lipoprotein analysis. In Moffatt RJ, Stamford B (eds.). Lipid Metabolism and Health. Florida, CRC Press, 2006, pp 123-30.

APPENDIX

APPENDIX A

Documentary Proof of Ethical Clearance

No. COA. MU-IRB 2008/001.2503

Documentary Proof of Ethical Clearance
The Committee on Human Rights Related to
Human Experimentation
Mahidol University, Bangkok

Title of Project: Simple Method for Quantification of Serum Small Dense LDL Particles
(Thesis for Master Degree)

Principle Investigator: Mr. Nattapol Niyompong

Name of Institution: Faculty of Medical Technology

Approved by the Committee on Human Rights Related to Human Experimentation

Signature of Chairman: ..... Shusee Visalyaputra .....
(Professor Shusee Visalyaputra)

Signature of Head of the Institute: ..... P. Sakul .....
(Clinical Professor Piyasakol Sakolsatayadorn)

Date of Approval: 25 March 2008

Date of Expiration: 24 March 2009

BIOGRAPHY

NAME	Mr. Nattapol Niyompong
DATE OF BIRTH	15 February 1983
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2001-2004 Bachelor of Science (Medical Technology) Mahidol University, 2005-2008 Master of Science (Medical Technology)
FELLOWSHIPS	Thesis is partly supported by; 1. The Graduate Scholarship of the Faculty of Medical Technology, Mahidol University
HOME ADDRESS	7/350 Soi Vipavadee 36, Vipavadee Rd., Jatujak, Bangkok, Thailand 10900
E-MAIL ADDRESS	earth_and_som@hotmail.com