METHOD COMPARISON OF LIPID, LIPOPROTEIN AND BIOMARKER FOR CARDIOVASCULAR DISEASE IN THE LARGE POPULATION

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
(CLINICAL PATHOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2004

ISBN 974-04-5130-6

Thesis

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was submitted to the Faculty of Graduate Studies, Mahidol University

for the degree of Doctor of Philosophy (Clinical Pathology)

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ACKNOWLEDGEMENTS

This thesis is successful by the extensive support and assistance from my major advisor, Professor Porntip Lolekha, and my co advisors, Associate Professor Paibul Suriyawongpaisal, Associate Professor Piyamitr Sritara. I would like to gratefully thank them for their valuable suggestions, kindly guidance and comments. I also would like to thank Associate Professor William L. Roberts and Dr.Sayan Cheepudomwit who were an external examiner and a member of the thesis defense for their valuable advices and kindly comments. Finally, I wish to thank all members of the Division of Clinical Chemistry, Ramathibodi Hospital for their excellent cooperation.

These studies were supported in part by the Thailand Research Fund Royal Golden Jubilee Ph.D Program.

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METHOD COMPARISON OF LIPID, LIPOPROTEIN AND BIOMARKER FOR CARDIOVASCULAR DISEASE IN THE LARGE POPULATION

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ABSTRACT

Laboratory methods for HDL, LDL and hs-CRP measurements are commercially available. Although the new homogeneous HDL and LDL assays have been developed for use in a routine laboratory, a homogeneous method for LDL measurement offers little advantage over the calculated LDL using the Friedewald formula. Therefore, the estimation of LDL is still the most common analytic approach in the clinical laboratory. Several studies have been published in the performance and clinical utility of these assays. However, their comparability and risk assessment using various combinations of methods of these analytes in a large population has not been well studied.

This study compared performance of two HDL direct assays, hs-CRP methods and calculated LDL obtained from two homogeneous HDL methods using serum samples from the International Collaborative Study on Atherosclerosis and Stroke in Asia (InterASIA) participants. The overall risk of coronary heart disease was assessed using each method and combinations of calculated LDL and two hs-CRP methods based on these analytes' cut-points.

The method comparison results demonstrated that a pair of homogeneous HDL, estimated LDL results when using two homogeneous HDL assays and hs-CRP methods are highly correlated. According to Deming regression analysis, the slopes of the regression line for all assays were close to 1 with a small intercept. The mean differences were close to zero. Although there was little difference in paired results, the percentages of overall consistency among the subjects for all assays were very high. The subjects were assigned to nine risk groups according to the LDL cut-points and the tertiles of hs-CRP. The highest percentage of subjects were classified into the low risk groups for both LDL and hs-CRP. Using combinations of calculated LDL and two hs-CRP methods shows more variability as a result of hs-CRP method differences than that resulting from method differences in calculated LDL values.

We conclude that each pair of homogeneous HDL, hs-CRP and estimated LDL results correlate and agree well. Thus, either method of these analytes are suitable for screening a large population. For method combination the hs-CRP method had more effect on risk assessment than that of the calculated LDL method. Variations in population risk assessment due to differences in HDL methods appear to be less than those due to differences in hs-CRP methods.

KEY WORDS: HOMOGENEOUS HDL/ FRIEDEWALD FORMULA/ HS-CRP/ LARGE POPULATION/ INTERASIA

106 pp ISBN 974-04-5130-6

การเปรียบเทียบวิธีวิเคราะห์และความมีประสิทธิภาพทางคลินิกของไลปิด ไลโปโปรตีน และสารชีวโมเลกุลที่เป็นปัจจัยเสี่ยงต่อการเกิดโรคหลอดเลือดและหัวใจในกลุ่มประชากรขนาดใหญ่ (METHOD COMPARISON OF LIPID, LIPOPROTEIN AND BIOMARKER FOR CARDIOVASCULAR DISEASE IN THE LARGE POPULATION)

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บทคัดย่อ

วิธีการตรวจวิเคราะห์ทางห้องปฏิบัติการสำหรับ HDL, LDL และ hs-CRP มีจำหน่ายสำเร็จรูปอย่างแพร่หลาย ถึงแม้วิธีการตรวจ HDL และ LDL โดยไม่ต้องตกตะกอนได้ถูกพัฒนาขึ้นเพื่อนำมาใช้ในงานประจำวัน วิธีดังกล่าวสำหรับการตรวจวิเคราะห์ LDL มีประโยชน์เหนือกว่าค่า LDL ที่ได้จากการคำนวณโดยใช้สูตร Friedewald เพียงเล็กน้อยเท่านั้น ดังนั้นการประมาณค่า LDL โดยใช้สูตรดังกล่าวจึงคงยังเป็นวิธีที่ใช้กันมากที่สุดในห้องปฏิบัติการทางคลินิก หลายการศึกษาได้เคยตีพิมพ์ในเรื่องของสมรรถภาพและคุณประโยชน์ทางคลินิกของวิธีการเหล่านี้ อย่างไรก็ดีการเปรียบเทียบวิธีการและการประเมินความเสี่ยงโดยใช้การตรวจวิเคราะห์สารต่าง ๆ เหล่านี้ร่วมกันในกลุ่มประชากรขนาดใหญ่ยังไม่ได้มีการศึกษามากนัก

การศึกษานี้เปรียบเทียบสมรรถภาพของวิธีการตรวจวิเคราะห์ HDL โดยวิธีไม่ตกตะกอนจำนวน 2 วิธี, ค่า LDL ซึ่งได้จากการคำนวณโดยใช้ค่า HDL ที่ได้จากทั้งสองวิธี และ hs-CRP จำนวน 2 วิธี โดยใช้ตัวอย่างซีรัมจากผู้เข้าร่วมโครงการการศึกษาวิจัย InterASIA ทั้งนี้ความเสี่ยงโดยรวมของการเป็นโรคหลอดเลือดหัวใจนั้นถูกประเมินโดยอิงค่าจุดตัดของแต่ละวิธีและมีการใช้ค่าคำนวณของ LDL ประเมินร่วมกับค่าของ hs-CRP

ผลการเปรียบเทียบวิธีการตรวจวิเคราะห์แสดงให้เห็นว่า วิธีการตรวจวิเคราะห์แต่ละคู่ของ HDL, ค่าคำนวณ LDL ที่ได้จากการตรวจวิเคราะห์ HDL ทั้งสองวิธี และ hs-CRP มีความสัมพันธ์กันอย่างสูง จากการวิเคราะห์สมการเส้นตรง Deming พบว่า ความชันของเส้นตรงที่แสดงความสัมพันธ์สำหรับทุกวิธีการมีค่าเข้าใกล้ 1 และมีจุดตัดแกน y แคบ ค่าความแตกต่างของค่าเฉลี่ยของแต่ละวิธีการมีค่าใกล้ศูนย์ ถึงแม้ผลที่ได้ของแต่ละคู่จะมีความแตกต่างกันบ้าง อย่างไรก็ดีแต่ละวิธีสามารถจำแนกตัวอย่างตามกลุ่มเสี่ยงต่าง ๆ ได้สอดคล้องกันในเปอร์เซ็นต์ที่สูง เมื่อใช้ค่าจุดตัดของ LDL ร่วมกับ tertile ของ hs-CRP ตัวอย่างจะถูกจัดให้อยู่ตามกลุ่มเสี่ยงต่าง ๆ จำนวน 9 กลุ่ม ทั้งนี้ตัวอย่างในกลุ่มที่มีความเสี่ยงต่ำจะมีจำนวนเปอร์เซ็นต์สูงที่สุดทั้ง LDL และ hs-CRP จากการใช้ค่าคำนวณ LDL ร่วมกับการตรวจวิเคราะห์ hs-CRP แสดงให้เห็นว่า ความแตกต่างของวิธีการตรวจวิเคราะห์ hs-CRP ทำให้ผลการตรวจวิเคราะห์มีความแปรผันมากกว่าความแตกต่างของวิธีในการคำนวณค่า LDL

การศึกษาครั้งนี้สรุปว่าผลการวิเคราะห์ที่ได้จากวิธีการตรวจวิเคราะห์ HDL ด้วยวิธีตรวจวัดโดยตรง, วิธีการตรวจวิเคราะห์ hs-CRP และ ค่าคำนวณของ LDL แต่ละคู่วิธี มีความสัมพันธ์และสอดคล้องกันดี สามารถนำมาใช้ในการตรวจกรองกลุ่มประชากรขนาดใหญ่ได้ เมื่อตรวจวิเคราะห์ hs-CRP ร่วมกับค่าคำนวณ LDL พบว่า ความผันผวนในการประเมินความเสี่ยงของกลุ่มตัวอย่างอันเนื่องมาจากวิธีการตรวจวิเคราะห์ HDL ที่แตกต่างกันนั้นมีน้อยกว่าความผันผวนอันเนื่องมาจากวิธีการตรวจวิเคราะห์ hs-CRP ที่แตกต่างกัน

106 หน้า ISBN 974-04-5130-6

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LIST OF ABBREVIATIONS

%	: percent
<	: less than
≤	: less than or equal
>	: more than
≥	: more than or equal
α	: alpha
β	: beta
ACAT	: acylcholesterol acyltransferase
AHA	: American Heart Association
Apo	: apolipoprotein
ATP	: Adult Treatment Panel
CDC	: Centers for Disease Control and Prevention
CE	: cholesterol esterase
CETP	: cholesterol ester transfer protein
CHD	: Coronary Heart Disease
CHOD	: cholesterol oxidase
CI	: confidence interval
COD	: cholesterol oxidase
CRM	: certified reference material
CRMLN	: Cholesterol Reference Method Laboratory Network
CRP	: C-reactive protein
CV	: coefficient of variation, defined as SD/mean
Da	: dalton
DM	: definitive method
EDTA	: sodium ethylenediamine tetraacetate
FDA	: food and drugs administration
FFAs	: free fatty acids

LIST OF ABBREVIATIONS (CONT.)

g/mL	: gram per mililitter
GDH	: glycerol dehydrogenase
H_2O_2	: hydrogen peroxide
HDL	: high density lipoprotein
HMG-CoA	: β-hydroxy-β-methylglutaryl CoA reductase
HPLC	: high performance liquid chromatography
hs-CRP	: highly sensitivity assays for CRP
HTGL	: hepatic triglycerides lipase
ICAM	: intercellular adhesion molecule
IDL	: intermediate density lipoprotein
IL	: interleukin
L	: liter
LCAT	: lecithin cholesterol acyltransferase
LDL	: low density lipoprotein
LOA	: limits of agreement
LPL	: lipoprotein lipase
Lp(a)	: lipoprotein (a)
MCP-1	: monocyte chemotactic protein-1
MRFIT	: Multiple Risk Factor Intervention Trial
NCEP	: National Cholesterol Education Program
NHLBI	: National Heart, Lung, and Blood Institute
NIH	: National Institutes of Health
MCP	: monocyte chemotactic protein
mg	: miligram
mg/dL	: miligram per deciliter
mg/L	: miligram per liter
rnL	: mililiter
mmol/L	: milimole per liter

LIST OF ABBREVIATIONS (CONT.)

nm	: nanometer
PDGF	: Platelet Derived Growth Factor
r	: correlation coefficient
RF	: risk factor
RM	: reference method
SD	: standard deviation
$S_{y/x}$	: standard error of the estimation
TC	: total cholesterol
TF	: tissue fator
TG	: triglycerides
TGF	: transforming growth factor
TFN	: interferon
TNF	: tumor necrosis factor
VCAM	: vascular adhesion molecule
VLDL	: very low density lipoprotein
WHO	: World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Background of the problems

Decreased high density lipoprotein cholesterol (HDL) and elevated low density lipoprotein cholesterol (LDL) concentrations are associated with risk of coronary heart disease (CHD) (1-3). In 2001, the Adult Treatment Panel (ATP) III of the National Cholesterol Education Program (NCEP) provides guidelines for assessment, diagnosis and treatment of CHD based on lipoprotein concentrations. The new guidelines recommended that all adults 20 years or older should evaluated a complete fasting lipoprotein profile which is total cholesterol (TC), LDL, HDL and triglycerides (TG) measurements as the preferred initial test every 5 years (4). The NCEP ATP III established HDL cut points of $\geq$1.55 mmol/L (60 mg/dL), 1.03-1.54 mmol/L (40-59 mg/dL) and <1.03 mmol/L (40 mg/dL) that classified asymptomatic patients into low, normal and high risk groups, respectively and the goal of LDL treatment targets are <2.58 mmol/L (100 mg/dL), <3.36 mmol/L (130 mg/dL) and <4.13 mmol/L (160 mg/dL) for persons with CHD or CHD risk equivalents, persons with multiple (2+) risk factors and persons having 0-1 risk factor, respectively (4). Accordingly, the lipid and lipoprotein determinations are included in most national coronary heart disease prevention programs to predict an individual's risk and to guide treatment. Thus, rapid and accurate methods have been designed for use in the clinical laboratory.

The NCEP Working Group on Lipoprotein Measurement requires the performance goals of total error for HDL and LDL should be within 13% and 12% of the true value when compared to the reference method that used by the Centers for Disease Control and Prevention (CDC), respectively (5). Various techniques for HDL quantification including ultracentrifugation, electrophoresis, high performance liquid chromatograph, and precipitation based methods have been described (6-8). Recently, new homogeneous methods have been developed and are becoming widely used (9-13).

The β-quantification method is an accepted reference method to determine LDL. However, this method is tedious and requires ultracentrifugation. The NCEP Working Group on Lipoprotein Measurement recommended the Friedewald calculation, which estimates LDL from measurements of TC, TG, and HDL, for use in the routine laboratory (14, 15). However, this calculated LDL is invalid when TG concentrations are >4.52 mmol/L or when chylomicrons or dysbetalipoproteinemia is present. This calculation requires the determination of three different measurements and may not always meet the NCEP performance criteria. Homogeneous LDL methods have been introduced to eliminate the limitations and improve the analytical performance goals.

In recent years clinical studies indicate that atherosclerosis, in addition to being a disease of lipid accumulation, also represents a chronic inflammatory process and its complication lead to CHD (16). Thus, researchers have hypothesized that inflammatory markers such as cell adhesion molecules, cytokines, pro-atherogenic enzymes and C-reactive protein (CRP) may provide an adjunctive method for global assessment of cardiovascular risk (17-19). Several prospective studies (20-23) have shown that CRP can predict the risk of future coronary events in apparently healthy men and women. In comparisons with traditional and novel biomarkers of CHD risk, CRP has been shown to be the single strongest risk marker of future coronary events (19, 24, 25). The algorithms for risk assessment of future coronary risk combining both CRP and lipids have been purposed (26, 27). Regular CRP assays, designed to detect acute inflammation, lack the sensitivity necessary to assess cardiovascular risk in healthy populations (28). In response to this purpose, the more sensitive immunoassays so called high sensitivity CRP (hs-CRP) have been developed (29-33). Various methods including the use of fluorescent, luminescent, or radioactive adducts to antibodies have been applied. However, the most common approach is particle enhanced nephelometry or turbidimetry, in which monoclonal antibody is adsorbed onto latex particles to increase signal at low concentration of the analyte. Hs-CRP was mentioned in the NCEP ATP III guidelines as an emerging risk factor. Three cutpoint values for low risk (<1.0 mg/L), average risk (1.0 - 3.0 mg/L) and high risk (>3.0 mg/L) of CHD were recommended by a Centers for Disease Control and Prevention (CDC) and American Heart Association (AHA) consensus conference (34).

1.2 Problems

Laboratory methods for HDL, LDL and hs-CRP measurement are commercially available. In routine clinical chemistry laboratories, HDL is frequently measured by a chemical precipitation method with various kinds of the precipitation reagents (8, 35-40). A major problem with precipitation method is the interference from increased triglycerides (41). In addition, these methods do not meet the imprecision goal for usefulness of a medical test (42). Moreover, precipitation methods are time-consuming, require a relatively large sample volume, and are not fully automated. The new homogeneous assays can be fully automated, eliminating the tedious manual steps of precipitation and meet the NCEP analytical performance goals. There have been several studies between a homogeneous assay and a conventional assay or a reference method (12, 43-45). However, no data have been reported on the diagnostic performance of homogeneous assays using a large population study.

The most accurate method for determination of LDL is the β-quantification but this method is not applicable to routine analysis. Thus, the estimation of LDL using the Friedewald formula has been recommended as the routine method despite well established limitations (14). Recently, fully automated homogeneous HDL methods have been adopted and may decrease the inaccuracy of the LDL calculations. Although new homogeneous LDL methods have been developed and can be useful for specimens with TG >4.52 mmol/L (400 mg/dL), they offer little advantage over the Friedewald calculation at low TG concentrations (46, 47). The estimation of LDL by the Friedewald formula is still the most common analytic approach in the clinical laboratory. The elevated concentrations of both LDL and hs-CRP are associated with CHD. Several studies have been published in the performance and clinical utility of hs-CRP (29-33). However, their comparability and risk assessment using various combinations of methods for these analytes in a large population has not been well studied.

1.3 Objectives

The over all objectives of this present study were

1.3.1 Comparison of two homogeneous HDL cholesterol methods in a large population study

We compared the HDL direct assays on the Dade Behring Dimension RxL using Automated High Density Lipoprotein Flex® reagent cartridge (the Dade method) and the Roche COBAS INTEGRA using High Density Lipoprotein Direct method cassettes (the Roche method) for screening a large population. Assessment of their abilities to classify the subjects into the low, normal, and high risk groups recommended by the NCEP ATP III is tabulated.

1.3.2 Comparative study of two automated high sensitivity C-reactive protein methods in a large population

We compared the Tina-quant CRP immunoturbidimetric method to the N High Sensitivity latex enhanced immunonephelometric assay in 4,118 sera. Agreement between the two methods for tertile risk assessment was also assessed.

1.3.3 Comparison of risk stratification using combinations of two HDL-cholesterol methods and two high sensitivity C-reactive proteins assays in a large population study

We compared the calculated LDL obtained from the two different homogeneous HDL assays in 3,728 sera, assessed agreement between LDL calculated from the two HDL methods using the thresholds recommended by the NCEP ATP III guidelines and compared overall risk of CHD using a combination of calculated LDL and hs-CRP for each combination of HDL and hs-CRP methods.

CHAPTER 2
LITERATURE REVIEW

2.1 Physiology of Lipids

Lipids are organic compounds that are actually or potentially esters of fatty acids, utilized by living organisms, and soluble in organic solvents but insoluble in water. The lipids play an important role in human health by acting as critical structural components of biological membranes, providing readily available energy reserves, serving as essential vitamins and hormones and aiding in solubilization of dietary lipids. Lipids can be subdivided into five groups based on their chemical structure as summarized in the following (48):

Classification of Clinically Important Lipids

Sterol derivatives
- Cholesterol and cholesteryl esters
- Steroid hormones
- Bile acids
- Vitamin D

Fatty Acids
- Short chain (2 to 4 carbon atoms)
- Medium chain (6 to 10 carbon atoms)
- Long chain (12 to 26 carbon atoms)
- Prostaglandins

Glycerol Esters
- Triglycerides, diglycerides, and monoglycerides (acylglycerols)
- Phosphoglycerides

Sphingosine Derivation
- Sphingomyelin
- Glycosphingolipids

Terpenes (Isoprene Polymers)
- Vitamin A
- Vitamin E
- Vitamin K

The main composition of animal lipid is triglycerides, about 98% to 99%, of which 92% to 95% is fatty acid and the remainder is glycerol. The remaining lipids include cholesterol, phospholipids, diglycerides, monoglycerides, fat-soluble vitamins, steroids, terpenes, and other fats. The small amount of nonhydrolyzable matter in food fats consists of sterols, fatty alcohols, hydrocarbons, pigments, g1ycerol esters, and various other compounds. Cholesterol is founded in all animal fats. Most sterols are cholesterol, but depending on the diet. Cholesterol from plant source is called phytosterol. Despite their close similarity to cholesterol, phytosterols are poorly absorbed in the intestinal mucosa. The presence of phytosterols will compete with animal cholesterol for uptake by the mucosal cells. Therefore, they significantly inhibit the absorption of cholesterol when they are administered in amount of 5 to 15 g/day.

2.1.1 Digestion, absorption and metabolism of lipids

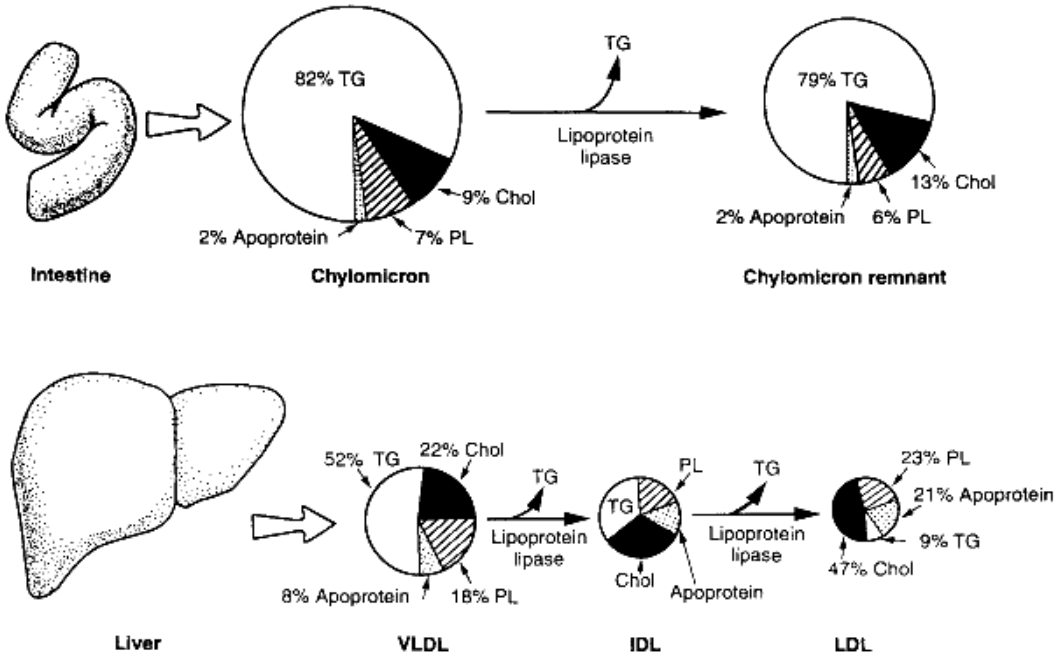
Fat absorption occurs in three phases: intraluminal phase or digestive phase, absorptive phase or cellular phase, and transport phase. The intraluminal phase occurs inside the lumen of the intestines which the dietary fats are modified both physically and chemically in a process known as emulsification before absorption. Most digestion of food fat is carried on in the intestine through the action of bile acids and intestinal and pancreatic enzymes (lipase). The bile acids break up lipids globules into smaller units, called micelles, that can be readily acted on by digestive enzymes produced by the pancreas and secreted into the duodenum. Lipase acts on dietary triglycerides to release free fatty acids forming monoglycerides, diglycerides and glycerol. Only a small percentage of the fat is completely hydrolyzed to free fatty acids (FFAs) and glycerol. Cholesterol esters are hydrolyzed to free cholesterol and free fatty acid; the reaction is catalyzed by the enzyme cholesterol esterase. Phospholipase A hydrolyzes the exogenous phospholipids.

During the absorption phase, the digested lipids diffuse into the intestinal mucosal cells. Cholesterol and other lipids are also absorbed. In the mucosal cells, FFAs will bind to coenzyme A (acyl or acetyl CoA) by the action of enzyme fatty acid CoA ligase. Thus long-chain fatty acids appear in thoracic duct lymph transported as triglycerides in the chylomicrons, which are mixtures of triglycerides (82%), some

proteins (2% as apoproteins), small amounts of cholesterol (9%, mainly as ester), and phospholipids (7%) (49).

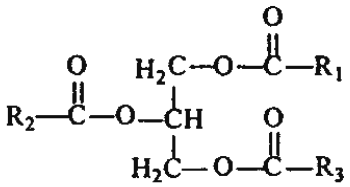
In the transport phase the chylomicrons are moved to the mucosal cell membranes and released by a reverse pinocytosis mechanism. Short- and medium-chian FFAs are also released bound to albumin and transported in the portal circulation while chylomicrons are relased into the throacic duct of lymphatic system eventually to enter the circulatory system (48, 49). The bloodstream transports chylomicrons to all tissues in the body, including adipose tissue, which is their principal site of uptake. The large chylomicrons are removed rather rapidly. Chylomicrons are normally present in only trace amounts in blood samples taken from individuals after an overnight fast.

In normal condition, chylomicron catabolism proceeds in two phases. In the first catabolic phase, apolipoprotein (Apo) C-II in the chylomicrons will activate lipoprotein lipase, which will hydrolyze the fatty acids from the triglycerides within the chylomicrons (48). The process results in a relatively triglyceride-poor, cholesterol-rich remnant particle, called chylomicron remnant, and the chylomicron remnants will be released back into circulation. In the second phase, the chylomicron remnants, which has been implicated as a highly atherogenic lipoprotein is removed by the liver. Remaining chylomicron remnants are cleared by the liver. The liver can synthesize lipoprotein particles from recently absorbed dietary constituents. Newly synthesized hepatic triglycerides are coupled with phospholipid, cholesterol, and protein to form very low density lipoprotein (VLDL). Then VLDL are release into bloodstream and transported to adipose tissue. Further catabolism of the VLDL occurs at an extracellular site and results in the formation of LDL, a cholesterol-rich particle. Within tissue cells, the FFAs derived from triglycerides in chylomicrons or VLDL will be used by the cells for energy or end up stored for later use in adipose cells. The origin and catabolic pathway of chylomicron and VLDL are shown in below figure (49):



2.1.2 Biological functions of triglycerides

Triglycerides are lipids that combine fatty acids to 3 carbon atoms of the glycerol back bone. Their major function is to provide energy for the cell. One gram of fatty acids liberates about 9 kcal. The human body stores large amounts of fatty acids in ester linkages with glycerol in the adipose tissue. This form of reserve energy storage is highly efficient because of the magnitude of the energy released when fatty acids undergo catabolism. The structure of triglycerides is shown below (48):



Most of the fatty acids come from the diets, can be synthesized endogenously, and are called nonessential fatty acids. There are three fatty acids (linoleic, linolenic, and arachidonic acids) that cannot be synthesized in the human body. These fatty acids are called essential fatty acids and are important for proper growth and development of cells, cell membrane integrity, prostaglandin synthesis, and myelinization of the central nervous system. Insufficient intake of the essential fatty acids will lead to an essential fatty acid deficiency.

2.1.3 Triglycerides metabolism

Because of property in water insolubility, triglycerides are transported in the plasma in combination with other more polar lipids (phospholipids) and proteins, as well as with cholesterol and cholesteryl esters, in the complex lipoprotein macromolecules. The center of lipoprotein molecule is largely of nonpolar triglycerides and cholesteryl esters, whereas the more polar protein and phospholipid components are at the surface, with their polar groups directed outward to stabilize the whole structure in the aqueous plasma environment.

2.1.3.1 Triglycerides synthesis

Triglycerides concentration in the plasma is a balance between the rate of entry into the plasma and the rate of removal. A change in concentration may result in a change in either or both of these factors. Plasma triglycerides are derived from intestinal and liver. Intestinal triglycerides are synthesized from dietary fat. The source of the fatty acids present in the triglycerides entering the blood from the liver depends greatly on the individual's nutritional state. Thus in the fasting state, fatty acids derived from adipose cell triglycerides are taken up by the liver and a portion is reexcreted as VLDL. The liver takes up and converts dietary carbohydrates to triglycerides, which are secreted as lipoproteins. Thus, the liver is the main contributor of triglyceride to the plasma, except during the absorption of dietary fat.

2.1.3.2 Triglycerides catabolism

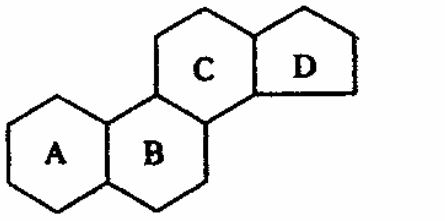
The actions of clearing-factor lipase at the endothelial cell surface are the removal of triglyceride fatty acid from the blood and also determining where it is used. Adipose tissue will take up triglyceride fatty acid when its concentration in the blood stream is excess. Most fatty acids are reconverted to intracellular triglyceride and stored. In contrast, unesterified fatty acids, which are mobilized from adipose tissue, will be oxidized and served as the primary energy for the body tissues in a state of caloric deficit (as during fasting). Triglyceride is still present in the blood in VLDL under these conditions, but it is directed away from this tissue and toward muscle to supplement the supply of energy from the mobilized fatty acids instead of being taken up by adipose tissue. This switch in triglyceride fatty acid uptake is achieved through changes in the activity of intracellular lipase in the tissues concerned. Thus fasting results in a decrease in the activity of the enzyme in adipose tissue and an increase in

its activity in muscle.

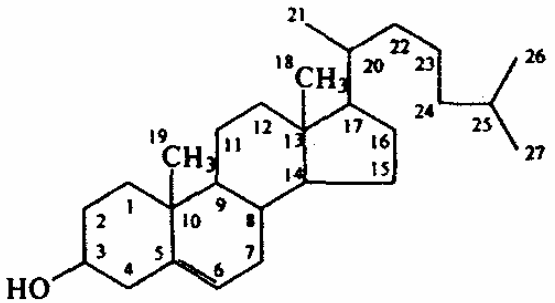
The intracellular adipose triglyceride enzyme is distinct from the plasma enzyme because it is converted from an inactive to an active form by epinephrine, norepinephrine, adrenocorticotropin, thyroid-stimulating hormone, and glucagon. Moreover, its activity is promoted by growth hormone and is inhibited by insulin. Therefore, it is called hormone-sensitive lipase. This enzyme of other tissue, not of adipose tissue, exhibits increased activity during fasting, possibly from falling of insulin levels. Thus, it is believed that hormone-sensitive lipase plays an important role in fat mobilization from adipose tissue.

2.1.4 Biological functions of cholesterol

Cholesterol is a member of a large class of biological compounds called steroids that have a cyclopentanoperhydrophenanthrene ring (sterane) skeleton in its molecule as shown below (48):



Perhydrocyclopentanophenanthrene (sterane) skeleton

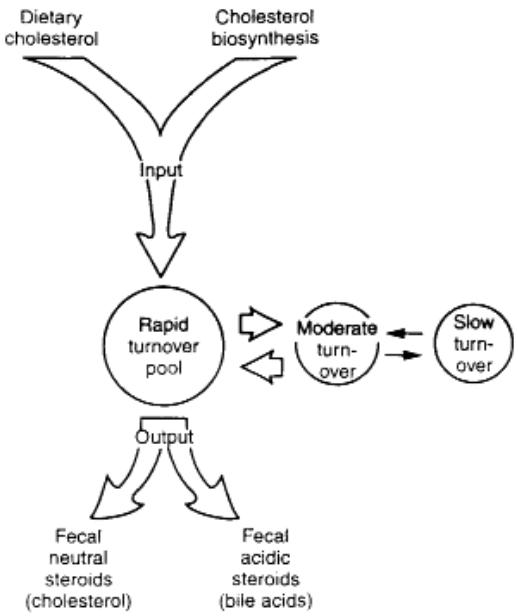


Cholesterol

It is found almost in animals, in which virtually all cells and body fluids contain some cholesterol. Cholesterol and its esters are essential structural component of the membranes of all animal cells and subcellular particles, obligatory precursor of bile acids and precursor of all steroid hormones, including sex and adrenal hormones.

2.1.5 Cholesterol metabolism

The turnover rate and the amount of tissue cholesterol that is exchangeable with the plasma cholesterol will vary from one tissue to another. About 2% of body's cholesterol is renewed each day. The concentration of a given cholesterol pool is under the influence of cholesterol, input, output, and turnover rates. The scheme of dynamics of cholesterol metabolism is shown follow (49):



Because of the continuous cycling of cholesterol into and out of the bloodstream, the plasma cholesterol concentration is not a simple additive function of dietary cholesterol intake and endogenous cholesterol synthesis. Rather, it reflects the rates of synthesis of the cholesterol-carrying lipoproteins and the efficiency of the receptor mechanisms that determine their catabolism.

2.1.5.1 Cholesterol absorption

Cholesterol enters the intestine from the diet, bile and intestinal secretions, and cells. The bulk of dietary cholesterol is provided by animal products, especially meat, egg yolk, seafood, and whole fat dietary products. The average daily intake of dietary and intestinal cholesterol is 400-500 mg/day. However, cholesterol absorption varies considerably according to an individual's dietary intake, amount of cholesterol in bile and absorption efficiency of cholesterol. Although dietary cholesterol may reach up to 3 g/day, the greatest amount that can be absorbed is about 1 g/day (48). All cholesterol in the intestine is present in the nonesterified (free) form. Esterified cholesterol in the diet is hydrolyzed rapidly in the intestine to unesterified cholesterol and free fatty acids by cholesterol esterase secreted from the pancreas and small intestine.

First, unesterified cholesterol is solubilized in order to be absorbed by the luminal cells. Solubilization process occurs through the formation of mixed micelles that contain unesterified cholesterol, fatty acids, monoglycerides (derived

from triglycerides), phospholipids (lysolecithin), and conjugated bile acids (48). Formation of mixed micelle not only assists cholesterol absorption by solubilizing the cholesterol but also facilitates its transport to the surface of the luminal cells. The bile acids are the most important factor in micelle formation by acting as detergents. Digestion and absorption of both cholesterol and triglycerides are impaired severely if bile acids are absent.

Most cholesterol is absorbed in the small intestine (jejunum and proximal ileum). Once it enters the intestinal mucosal cells, cholesterol is incorporated with triglycerides, phospholipids, and several specific apolipoproteins into a large lipoprotein called the chylomicron (50-53). Apo B-48 is important to the formation of chylomicrons. Chylomicrons formation, consequently cholesterol and fat absorption are severely impaired in people with a rare deficiency of apo B-48 synthesis. Chylomicrons enter the thoracic duct and then the systemic venous circulation at the junction of the left subclavian and left internal jugular vein.

2.1.5.2 Cholesterol synthesis

Cholesterol can be synthesized endogenously by the liver and other tissues from smaller molecules, particularly acetate, except its absorption from the intestine (48). The liver and intestinal wall probably supplies over 90% of endogenous cholesterol. The rate of cholesterol synthesis is determined by the capacity of β-hydroxy-β-methylglutaryl CoA (HMG-CoA) reductase, which catalyze a rate-limiting step in the biosynthetic sequence from acetyl CoA (coenzyme A) to cholesterol. The other sites of suppression are likely in the biosynthetic cholesterol pathway. Hepatic HMG-CoA is inhibited by several hormones, dietary factors, and drugs. Feedback control of hepatic cholesterogenesis is also mediated by dietary cholesterol and directly or indirectly by bile acids.

The first stage of cholesterol biosynthesis is formation of the six-carbon thioester HMG-CoA from acetyl CoA. In the second stage, HMG-CoA is reduced to mevalonate, then carboxylated to isoprene units. These units are condensed to form geranyl pyrophosphate and then farnesyl pyrophosphate. Final product of this stage is squalene, which is combination of two farnesyl pyrophosphate molecules. This stage includes the step involving the enzyme HMG-CoA reductase, which is the rate-limiting enzyme in cholesterol biosynthesis. The final stage of synthesis is in the

endoplasmic reticulum, where the intermediate products being bound to a specific carrier protein. Squalene forms the 4-ring, 30-carbon intermediate, lanosterol. Then a number of side chains are removed from the pentanophenanthrene structure in a sequence of oxidation-decarboxylation reactions to form 27 carbon molecule of cholesterol.

2.1.5.3 Cholesterol esterification

Cholesterol is transported in the plasma predominantly as cholesteryl esters associated with lipoproteins. Esterification of cholesterol enhances the capacity of the lipoprotein in lipid-carrying and prevents intracellular toxicity by unesterified cholesterol. The reaction can occurs both in cytoplasm and in plasma. The plasma reaction requires the enzyme lecithin cholesterol acyltransferase (LCAT), and the intracellular reaction utilizes acylcholesterol acyltransferase (ACAT) (54). Esterified cholesterols are formed when long chain fatty acids, transferred from triglycerides, bind to the hydroxyl group located at C3. The major reaction in the liver, intestine, adrenal cortex and probably in the arterial wall is an intracellular ACAT pathway. About 70% of total cholesterol in humans is found in the form of esterified cholesterol. LCAT serves for the formation of virtually all this cholesterol ester.

2.1.5.4 Cholesterol catabolism

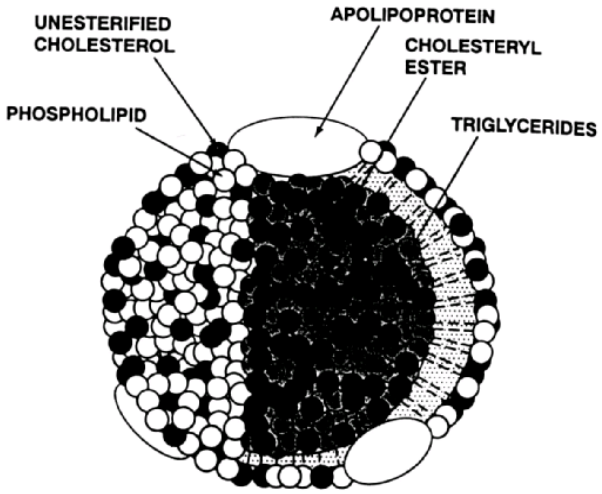
Once lipoprotein cholesterol enters the cells, the cholesteryl esters are hydrolyzed by lisosomal acid lipase. The lack or malfunction of this enzyme results in intracellular accumulation of cholesterol esters and produces a clinical disorder called cholesteryl storage disease (48).

Cholesterol reaching the liver is either secreted unchanged into blie or metabolized to bile acids. These mechanisms for excretion of cholesterol depend on the receptor-mediated activity in the hepatocytes, which have receptor sites specific for Apo B and E. The major function of the liver in lipoprotein clearance is to remove lipoproteins containing Apo E such as chylomicron remnants and VLDL remnants and Apo B such as LDL remnants from the blood circulation. Because the Apo E containing lipoproteins are cleared with much greater efficiency than the Apo B containing lipoproteins, chylomicron remnants and VLDL remnants (intermediate density lipoprotein, IDL) are not normally measurable in healthy individuals.

The uptake of LDL by the peripheral tissues is also receptor-site dependent. The binding of LDL to the receptor site followed by internalization and hydrolysis of the LDL serves to deliver free cholesterol to the cell. The intracellular free cholesterol then functions (55) as a regulator for the rate of receptor synthesis, a regulator for cholesterol synthesis by the end-product negative-feedback mechanism, or a regulator for ACAT activity, which determines how much cholesterol is stored in the cell as a cholesterol ester. The availability of HDL is one of the determining factors for the efflux of cholesterol from the cell into the blood. By this process, the cholesteryl ester in the cell is hydrolyzed to free cholesterol and fatty acid. The liver, gastrointestinal tract and other organs, such as the adrenal glands and gonadal tissues, take up the HDL and catabolize it to its protein and lipid constituents including cholesterol.

2.2 Physiology of Lipoprotein

Lipoproteins are spherical particles with nonpolar lipids, triglycerides and cholesterol esters, in their core and more polar lipids, phospholipids and free cholesterol, oriented near the surface. Specific proteins, called Apo, are also located on their surface. Lipoprotein enables the lipids to remain partially soluble in plasma and facilitates their transport and utilization. Scheme of lipoprotein complex is shown in the following (48):



Lipoprotein complexes contain different proportion of lipids and proteins. The main systems to isolate, separate, and characterize lipoproteins are based on their

hydrated density, which is determined mainly by the triglycerides content, or on their electrical charge. Ultracentrifugation separates lipoproteins in plasma into chylomicrons, VLDL, IDL, LDL and HDL (56-58). Lipoproteins also can be separated from each other by electrophoresis on agarose, cellulose, acetate and paper, as well as polycrylamide gels (59). The bands are identified by comparison to the serum protein bands. HDL is an alphalipoprotein, LDL is a betalipoprotein, VLDL is a pre-beta lipoprotein, and IDL is found in the float beta band located between beta and pre-beta, which in some instances blend together to form one broad beta band. Chylomicrons are found near or at the origin. A new high-definition agarose support media can identify Lipoprotein (a) [Lp(a)] as a fast migrating pre-beta lipoprotein that is structurally related to LDL. The physical and chemical properties of human lipoproteins are summarized in the following (49):

Feature	**Chylomicrons**	**VLDL**	**IDL**	**LDL**	**HDL**	**Lp(a)**
Density (g/mL)	<0.95	<1.006	1.006 to 1.019	1.019 to 1.063	1.063 to 1.21	1.040 to 1.130
Electrophoretic mobility	Origin	Pre-beta	Beta	Beta	Alpha	Pre-beta
Molecular weight (Daltons)	0.4 to 30 x 10^9	5 to 10 x 10^6	3.9 to 4.8 x 10^6	2.75 x 10^6	1.8 to 3.6 x 10^5	2.9 to 3.7 x 10^6
Diameter (nm)	>70	25 to 70	22 to 24	19 to 23	4 to 10	25 to 30
Lipids-protein ratio	99:1	90:10	85:15	80:20	50:50	75:25 to 64:36
Major lipids	Exogenous triglycerides	Endogenous triglycerides	Endogenous triglycerides, Cholesteryl esters	Cholesteryl esters	Phospholipids	Cholesteryl esters Phospholipids
Major proteins	A-I, A-II B-48 C-I C-II, C-III	B-100 C-I, C-II, C-III E	B-100 E	B-100	A-I, A-II	(a) B-100

Chylomicrons contain mainly triglyceride combined with cholesterol, small amounts of phospholipid, and specific apoproteins (Apo B-48, A-I, A-II, C-I, C-II, C-III**,** with small amounts of Apo B and E-II, E-III, E-IV). Most models for chylomicron structure have been made under the assumption that the neutral lipids (triglycerides and cholesteryl ester) are partially surrounded by an outer shell of phospholipid, free cholesterol, and protein. Because of a high triglycerides and low protein content, they also have the lowest density. This low density permits high concentrations of chylomicrons to float on top of the serum or plasma, which is appear turbid or milky.

Under fasting conditions (more than 10 to 12 hours after a meal), no chylomicrons are generally found in the blood of healthy persons.

VLDL is synthesized in the liver from chylomicron remnants. This lipoprotein consists of 52% triglyceride, 18% phospholipid, 22% cholesterol, and about 8% protein. A ratio of cholesterol and cholesteryl esters in VLDL is about 1:1 by weight. Sphingomyelin and phosphatidylcholine are the major phospholipids. The larger the size of a VLDL particle, the greater the proportion of triglycerides and Apo C and the smaller the proportion of phospholipid, Apo B, and other apoproteins. Apo B occurs in a constant absolute quantity in all VLDL fractions. Apo B-100 accounts for approximately 30% to 35%, with Apo C-I, C-II, and C-III making up over 50% of the apoprotein content in VLDL. Apo E-II, E-III, and E-IV and varying quantities of other apoproteins (A-I, A-II, B-48) may also be present. The relative quantity of each protein varies with the individual and with the degree of hyperlipidemia. Partially degraded VLDL, commonly called remnant lipoprotein is a triglyceride-poor lipoprotein, known to be highly atherogenic.

IDL is a lipoprotein derived from VLDL catabolism, which is persists for short periods of time and contains approximately equal amounts of cholesterol and triglycerides and Apo B-100 and E. The liver takes up IDL after it has interacted with the LDL receptor to form a complex, which is endocytosed by the cell. For LDL receptors in the liver to recognize IDL requires the presence of both Apo B-100 and Apo E (the LDL receptor is also called the Apo B-100/Apo E receptor) for the liver uptake and further degradation of IDL to LDL by the action of hepatic lipase. A deficiency of Apo E results in elevation of both chylomicron remnants and IDL.

LDL is formed as the IDL is degraded. LDL contains, by weight, 80% lipid and 20% protein. Consistent with this increased protein content, the size of LDL is smaller and denser than the VLDL and chylomicrons. About 50% of LDL lipid is cholesterol. LDL constitutes 40% to 50% of the plasma lipoprotein mass in humans. LDL is the major carrier of cholesterol and is considered an atherogenic lipoprotein. Apo B-100 is the major apoprotein of normal LDL, and LDL Apo B represents 90% to 95% of the total plasma Apo B-100. LDL is frequently separated into two classes base on the basis of flotation density, LDL_1 (or IDL) and LDL_2. The lower-density fraction, IDL (1.006 to 1.109 g/mL), is more lipid-rich than LDL_2 (1.019 to 1.063 g/mL) and

probably represents an intermediate in VLDL catabolism. Thus a comparison of IDL with LDL_2 demonstrates the gradual disappearance triglyceride and of apoprotiens characteristic of VLDL: (Apo C and E) and an enrichment with Apo B-100 cholesterol ester. There is a significant size heterogeneity in all the major lipoprotein classes, including that of LDL. It has been shown that small, dense LDL is metabolically more active and is more atherogenic than the conventional-size LDL. The ATP III report has acknowledged this as a CHD risk factor.

HDL is produced in both the liver and intestinal walls and contains proportionally more protein and less cholesterol than any of other lipoproteins, approximately 50% protein and 50% lipid. The HDL is the smallest of the lipoproteins and floats at the highest density of any of the lipoprotein molecules. The most important HDL lipid is phospholipid, though HDL cholesterol is of particular interest. The major phospholipid species is phosphatidylcholine, also known as lecithin, which accounts for 70% to 80% of the total phospholipid. Phosphatidylcholine plays an important functional role as a reactant in plasma cholesterol esterification, which is catalyzed by the enzyme LCAT. HDL may be further subfractionated by differential ultracentrifugation into HDL_2 (with a density of 1.063 to 1.110 g/mL) and HDL_3 (1.110 to 1.21 g/mL). Person with lower HDL_2 levels are apparently more susceptible to premature CHD.

Lp(a), or sinking pre-beta-lipoprotein is similar in lipid composition, concentration, and density (1.05 to 1.10 g/mL) to LDL. Apo B-100 makes up 65% of Lp(a) protein, but another 15% is albumin, and the remainder is an apoprotein unique to Lp(a), called Apo Lp(a). Lp(a) structurally resembles LDL and the Apo B protein is connected to the Apo Lp(a) by disulfide bridges. It is polymorphic in size and has several isomers. Despite its high frequency in the population, the functional significance of this lipoprotein is still not entirely clear. However, it is known that Lp(a) competes with plasma plasminogen for the latter's binding sites, resulting in decreased synthesis of plasmin and inhibition of fibrinolysis. Thus Lp(a) may have a role in thrombogenesis. It also plays a role in atherogenesis by causing cholesterol deposition in the arterial wall, inducing monocyte-chemotactic activity in the arterial wall subendothelial space, enhancing foam cell formation, and promoting smooth muscle cell proliferation. Most perspective studies have demonstrated that Lp(a) is a primary, and independent CHD risk factor that aggravates the coronary risk exerted by

elevated LDL cholesterol, low HDL cholesterol, hypertension, or the combined effects of multiple risk factors (e.g., smoking, hypertriglyceridemias, diabetes, angina pectoris, and a family history for premature myocardial infarction)

2.2.1 Lipoproteins metabolism

The pathways of lipoprotein metabolism are complex and consist of the exogenous pathway, endogenous pathway, intracellular LDL receptor, and HDL reverse-cholesterol pathway (60-62). The exogenous pathway involves transport of dietary lipid from the intestine to the liver. The endogenous pathway involves transport of lipids synthesized in the hepatocytes to peripheral tissues. The two pathways overlap at the stage of hydrolysis by lipoprotein lipase (LPL) in the periphery, and by hepatic triglycerides lipase (HTGL).

2.2.1.1 Exogenous pathway

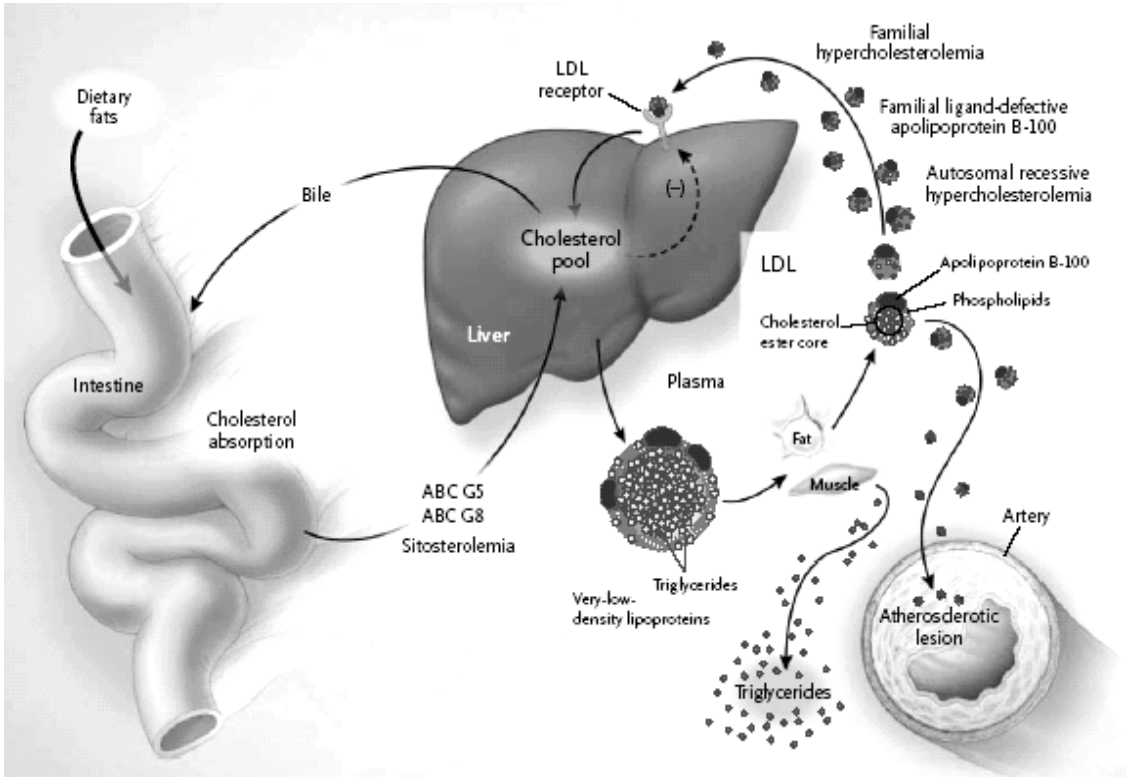
Lipoproteins in this pathway are the dietary origin. Nascent chylomicrons are assembled from dietary triglycerides and cholesterol in secretory vesicles in the Golgi apparatus and introduced into circulation through the intestinal villi. These particles require Apo C and Apo E from circulating HDL after shortly enter the circulation. The presence of Apo C-II on the surface of chylomicrons activates the LPL, attached to the luminal surface of endothelial cells, which hydrolyzes the triglycerides to FFAs. The FFAs then bound to albumin for either being taken up by muscle cells as an energy source or into adipose cells for storage. Some of the phospholipids and the A apolipoproteins are simultaneously transferred from the chylomicron particle onto HDL. This process results in chylomicron remnant, contains 80% to 90% of the triglycerides content of the original chylomicron, primarily cholesterol, and also Apo E and Apo B-48 on its surface. The chylomicron remnant can be recognized by Apo B-48 and Apo E specific hepatic remnant receptors and internalized by endocytosis to be hydrolyzed in the lysosome. The cholesterol released can form bile acids, be incorporated into newly synthesized lipoprotein, or be stored as cholesteryl ester. Furthermore, the cholesterol from these remnants can down-regulate HMG-CoA reductase, which is the main of the rate-limiting enzyme of cholesterol biosynthesis.

2.2.1.2 Endogenous pathway

In contrast of the endogenous pathway, the lipoproteins in this pathway are the hepatic origin. The hepatocytes synthesize triglycerides from carbohydrates and fatty acids. They also synthesize their own cholesterol by increasing the enzyme HMG-CoA reductase when dietary cholesterol is insufficient. In the exocytosis process, the endogenous triglycerides and cholesterol are assembled in secretory vescicle in the Golgi apparatus for transporting into the extracellular space and introduced into bloodstream in the form of nascent VLDL. This particle contains Apo B-100, Apo E, and small amount of Apo C on its surface. More C apolipoproteins are transferred after secretion from circulating HDL. In chylomicron metabolism, triglycerides in VLDL particle are hydrolyzed to FFAs by endothelial LPL, which is influenced by Apo C-II on VLDL surface. The C apolipoproteins are transferred back to HDL particles during the hydrolysis of VLDL triglycerides. VDLD particles are transformed to VLDL remnants. Some of these remnants are taken up by the liver and the remainders are converted to IDL. The IDL particles are removed from circulation by binding of Apo E on their surface to the hepatic remnant receptors. About 50% of IDL is removed by the hepatocytes.

Some phospholipids, free cholesterol, and apolipoproteins on IDL surface, are transferred to HDL, or form de novo HDL in the circulation. HDL transfers cholesteryl esters to LDL. The coupled lypolysis and the cholesteryl esters exchange reaction results in the replacement of much of the triglycerides core of the original VLDL with the cholesteryl esters. The further hydrolysis of IDL undergoes for removing the remaining triglycerides. All apolipoproteins except Apo B-100 are delivered to other lipoproteins. The ultimate formation of LDL is the end of this process.

The overview of exogenous and endogenous pathways is shown in the following (63):

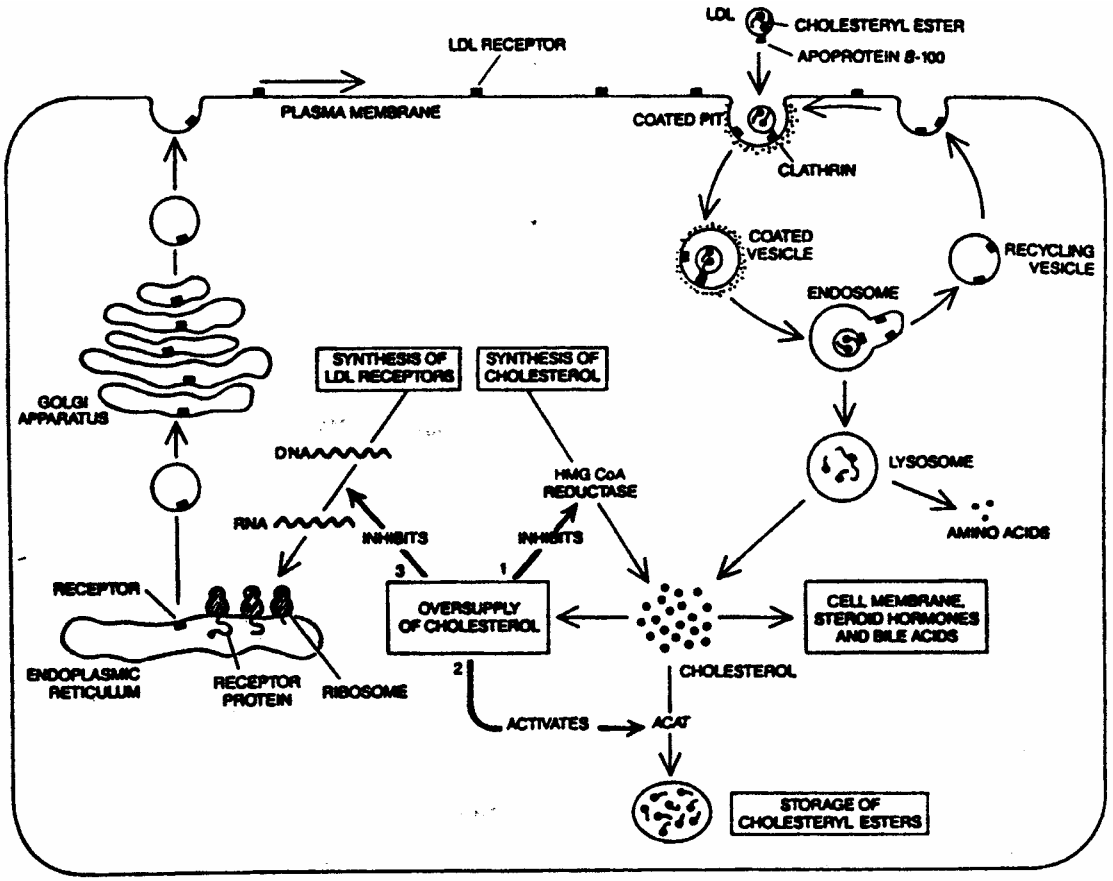


2.2.1.3 LDL pathway

As discussed previously, LDL formation occurs primarily from the catabolism of VLDL. When Apo B on LDL surface binds to high-affinity receptors presented on the plasma membrane called coated pits, the LDL particles are internalized in coated vesicles and fuse to form an endosome. Fusion of the vesicle membrane with the lysosomal membrane exposes the LDL to a host of hydrolytic enzymes, which degrade the Apo B to small peptide and amino acids. After the LDL disassociates, the receptors return to the cell surface for reuse. The cholesteryl esters are hydrolyzed by an acid lipase, and liberated free cholesterol leaves the lysosomes for the synthesis of cell membranes, steroid hormones in the tissues, and bile acids in hepatocytes. Cells have the abilities to regulate their cholesterol levels. Excess of free cholesterol leads to decrease the rate of endogenous cholesterol synthesis by inhibition of the rate-limiting enzyme HMG-CoA reductase, increasing formation of cholesterol esters, and inhibition of the synthesis of new LDL receptors by suppression of the transcription of the receptor gene.

In addition to its normal degradation mechanism in the high-affinity LDL receptor pathway, plasma LDL can be take up by scavenger cells (macrophages)

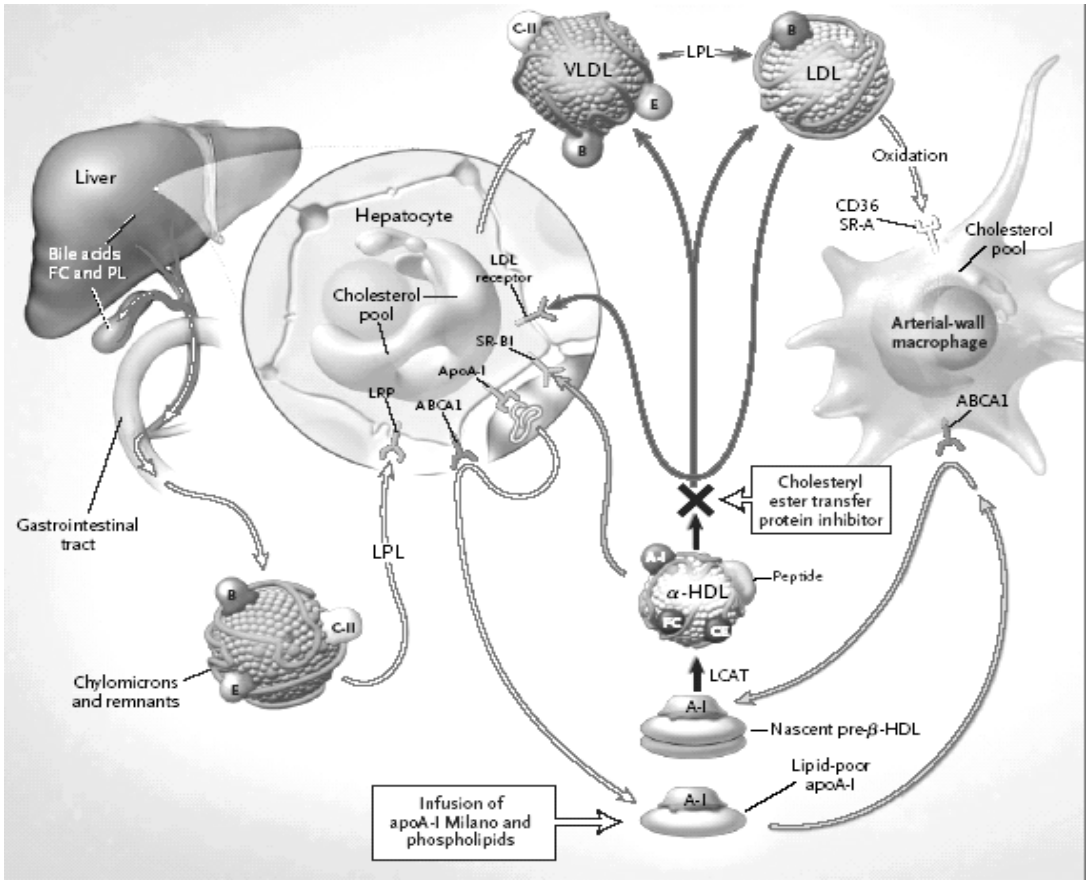
of the reticuloendothelial system. When the plasma level of LDL rises, these scavenger cells degrade increasing amounts of LDL. When macrophages are overloaded with cholesteryl esters, they are transformed to foam cells, which are considered as a components of atherosclerotic plaques. In human, the range of proportion of plasma LDL degraded by the LDL receptor system is 33% to 66%. The remainder is degraded by the scavenger cell system. Scheme of the LDL pathway and regulation of cholesterol as shown below (49):



2.2.1.4 HDL reverse cholesterol transport pathway

Nascent HDL particles, containing of Apo A-I, lecithin and free cholesterol, are initially released from the liver or intestine as a disk-shaped. LCAT, activated by the presence of Apo A-I, catalyzes the esterification of cholesterol, forming the functional spherical form that actively participates in reactions with the lipoproteins. HDL cholesteryl esters are provided to the liver from three mechanisms. First, the hepatic receptors take up cholesteryl esters from HDL, and HDL particles are returned to circulation for further transport. Second, the cholesterol ester transfer

protein (CETP) influences HDL to transfer cholesteryl esters to Apo B-100 containing lipoprotein. Third, Hepatic remnant receptors recognize HDL Apo E. According to these process, cellular and lipoprotein cholesterol are returned to the liver for reuse or disposal. This important role of HDL, along with LCAT, CETP and Apo A-I, in reverse cholesterol transport may be the basis for the protection afforded by HDL against CVD (64-66). Schematic model of reverse cholesterol transport mediated by HDL is shown in the following (67):



2.3 Clinical Implication of Hyperlipidemia

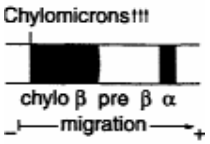
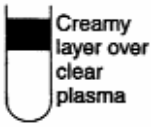
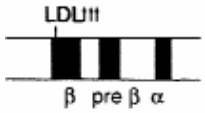
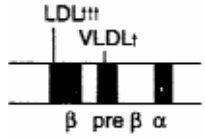
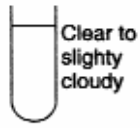
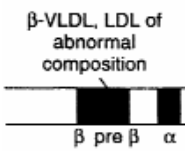
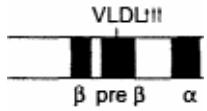
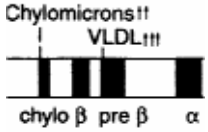
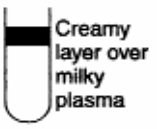
Hyperlipidemia is an elevated concentration of lipids in the blood. The major plasma lipids of interest are total cholesterol and the triglycerides. Over 90% of persons with hyperlipidemia have hyperlipoprotienemia, which is an elevation of serum lipoprotein concentrations. However, in clinical chemistry the use of term lipids generally refers to lipoprotein metabolism and atherosclerosis, a major cause of CHD.

The terms of hyperlipidemia and hyperlipoproteinemia are used interchangely to describe disorders that are result in elevated lipids. According to the identification of elevated concentration of blood lipid and type of abnormal lipoprotein pattern, hyperlipoproteinemia are classified into six types. The causes of lipid and lipoprotein disorders may be primary causes such as a mutation in gene encoding Apo A-I or secondary causes, known conditions associated with hyperlipoproteinemia, such as diabetes millitus. The secondary causes and types of hyperlipoproteinemia are shown in below (49):

Secondary causes of hyperlipoproteinemia

Pattern	**Secondary causes**
Hyperchylomicronemia	Insulinopenic diabetes mellitus, Dysglobulinemia, Lupus erythematosus, Pancreatitis
Hyperbeta-lipoproteinemia	Nephrotic syndrome, Hypothyroidism Obstructive liver disease, Porphyria Multiple myeloma, Portal cirrhosis Acute phase of viral hepatitis, Myxedema Stress, Anorexia nervosa Idiopathic hypercalcemia
Dysbeta-lipoproteinemia	Hypothyroidism, Dysgammaglobulinemia Myxedema, Primary biliary cirrhosis Diabetic acidosis
Hyperpre-beta-lipoproteinemia	Diabetes mellitus, Nephrotic syndrome Pregnancy, Hormone use (oral contraceptives) Glycogen-storage disease, Alcoholism Gaucher's disease, Niemann-Pick disease Pancreatitis, Hypothyroidism Dysglobulinemia
Mixed type of lipoproteinemia	Insulinopenic diabetes mellitus, Nephrotic syndrome, Alcoholism, Myeloma Idiopathic hypercalcemia, Pancreatitis Macroglobulinemia, Diabetes mellitus (insulinindependent)

Types of hyperlipoproteinemia

Pattern	Electrophoretic pattern	24hr standing plasma (4 °C)	Cholesterol	Triglycerides
Hyperchylo micronemia (very rare)				
Hyperbetalipoprotein-emia (common)				
Combined hyperlipoproteinemia (common)				
Dysbeta-lipoprotein-emia (very rare)				
Hyperpre-beta-lipo-proteinemia (very common)				
Mixed hyperlipo-proteinemia (rare)				

2.3.1 Association of hyperlipidemia and CHD

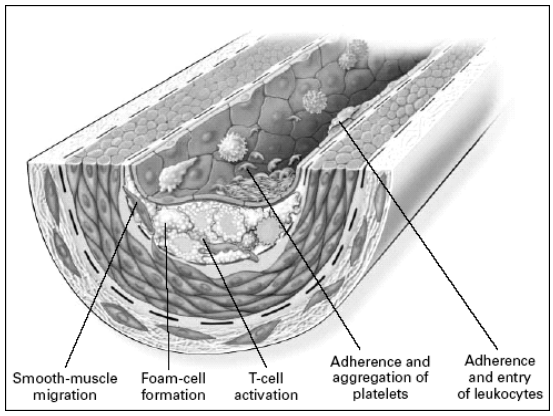
High blood cholesterol and LDL, and low concentration of HDL, are major risk factor for CHD. In large-scale randomized trial of lipid reduction represent that lowering cholesterol can reduce CHD risk (68). The significance of an increased TG is still doubted in it contributes to the risk factor. According to the Multiple Risk Factor Intervention Trial (MRFIT), baseline total cholesterol is a strong predictor of CHD mortality after 6 years especially for cholesterol levels above 5.2 mmol/L (69). A similar result is obtained from in the well known Framingham Heart Study. This study is instrumental in establishing elevated blood cholesterol as a major risk factor for all clinical manifestations of CHD and demonstrate every 1% increase in TC there is a 2% increase in risk factor for CHD (70). Furthermore, in individuals under 50 years of age, total cholesterol levels are directly related to 30-year overall and CHD mortality rates. Total overall mortality increase 5% and CHD mortality increase 9% for each increment of 0.259 mmol/L (10 mg/dL) of cholesterol (71). Additional analyses of Framingham data help to define the role of different cholesterol-rich lipoproteins in the pathogenesis of atherosclerosis. LDL levels are found to be positively related to CHD, even more strongly than TC levels (70-71). HDL levels are on the other hand inversely related to the development of CHD (72). Other risk factors of CHD are listed in below table and identified as primary or secondary factors (49):

Primary Risk Factors Associated with CHD
Primary
Genetic predisposition for CHD
Family history of premature CHD in first-degree relatives
(<45 years for males, <55 years for females)
Hypertension
Cigarette smoking
Elevated total cholesterol (LDL cholesterol)
Decreased HDL cholesterol
Elevated triglycerides (VLDL cholesterol, remnant lipoproteins)
Increasing age
Male gender

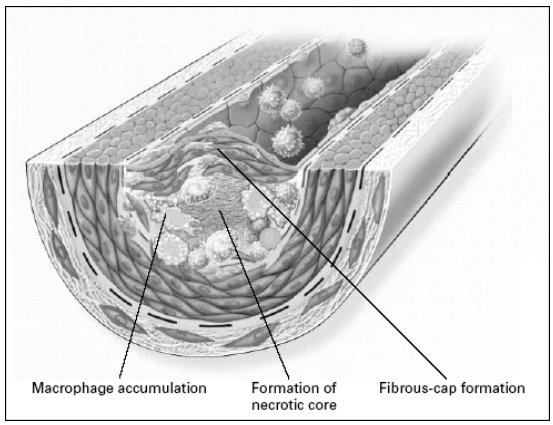
Secondary Risk Factors Associated with CHD
Lack of exercise
Obesity
Stress
Diabetes mellitus
Elevated lipoprotein (a)
Elevated homocysteine
Elevated intermediate-density lipoproteins
Renal failure patients receiving hemodialysis
Postmenopausal state
Certain thrombogenic disorders

2.3.2 Pathophysiology of atherosclerosis

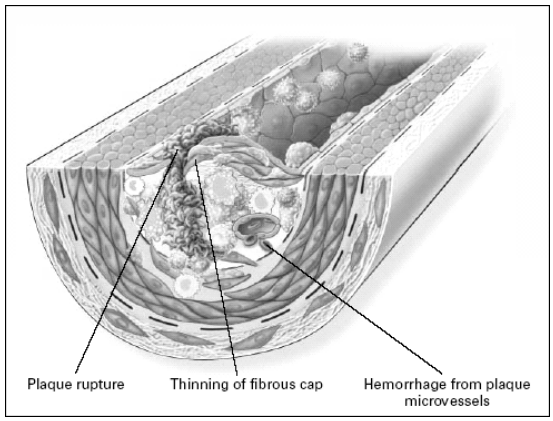
Atherosclerosis, hardening of the artery, is a major cause of CHD. This condition results from the accumulation of lipids in the coronary artery walls. The major pathophysiology of atherosclerosis is associated with plaque formation, that leads to reduce blood flow and initiate formation of a thrombus. Atherosclerotic plaque formation occurs in three progressive stages. The initiate stage is fatty streak, which gradually develops into raised lesions, called fatty plaques. Then the fibrous plaque, which has a proliferation of smooth muscle cells and a collagen-rich fibrous cap that covers a lipid core which is lined by foam cells and surrounds an amorphous extracellular accumulation of cholesteryl esters. The last stage is the complicated lesion, which can manifest calcification, hemorrhage, ulceration (rupture), and thrombosis. This complicated lesion frequently underlies the acute clinical event of arterial occlusion that leads to myocardial injury (MI). The diagram of atherogenesis is shown in the following (73):



Fatty-Streak Formation in Atherosclerosis



Formation of an Advanced, Complicated Lesion of Atherosclerosis



Unstable Fibrous Plaques in Atherosclerosis

Pathogenesis of atherosclerotic lesions are believed in two mechanisms include the response to injury (74) and elevated lipids (48). The endothelium may become damaged by various factors as shown in the previous table. The loss of endothelium along with increased adhesion of platelets to subendothelium results in aggregation of platelets and the chemotaxis of monocytes and T-lymphocytes to the

site of endothelial injury. Platelets and monocytes release growth factors that induce smooth muscle cells to migrate into the site of injury. Migration of smooth muscle cells result in plaque, also called the fatty plaque, features cells loaded with lipids. Oxidative stress has been established as a significant causative mechanism of chronic endothelial cells injury that then triggers a variety of responses, especially oxidation of lipids, which manifests as atherosclerosis. According to the Steinberg et al (75), oxidized LDL may play an important role in atherogenesis by acting as a chemoattractant for the blood-borne monocytes to enter the subendothelial space, causing the transformation of the monocytes to macrophages, causing the trapping of macrophages in the endothelial spaces by inhibiting their motility and acting as the toxic substance to the endothelial cells. The lipid-laden macrophage forms the foam cells that contribute to the development of the fatty streaks. Also, these activated macrophages can form at least four different growth factors such as interleukin-1 (IL-1) and platelet derived growth factor (PDGF), which may be responsible for the migration of smooth muscle cells and fibroblasts into the intima and for their subsequent proliferation. The platelets, in this hypothesis, are involved in atherogenesis by aggregating and forming mural thrombi at particular anatomical sites where the blood flow properties produce shearing effects and cause some sort of injury to the endothelium.

2.3.3 The Guideline for evaluation ,detection, treatment and prevention of CHD

On the basis finding of the previous study (68, 69), hypercholesterolemia in adults has been redefined in terms of CHD risk. Since 1988, the National Cholesterol Education Program (NCEP) has issued guidelines identifying LDL as the primary target of cholesterol therapy. This guideline had a significant effect on reducing the CHD morbidity and mortality in the United States, where CHD is still a major disease. The panel eliminated the age and sex stratification to simplify the classification system and to make this guideline more convenient to remember the cutoff levels (76, 77). The goals of the NCEP ATP I, II and III programs were to establish criteria that defined the high-risk person for medical intervention and to provide clear guidelines on how to detect, set goals for, treat, and monitor these patients over time. ATP I (77)

outlined a strategy for primary prevention of CHD in persons with high levels of LDL [≥4.14 mmol/L (160 mg/dL)] or those with borderline-high LDL [3.36-4.12 mmol/L (130-159 mg/dL)] and multiple (2+) risk factors. ATP II (78) restated the importance of this approach as well as recommending the intensive management of LDL in persons with established CHD. For such patients, ATP II set a new, lower LDL goal of 100 mg/dL. According to the recent clinical trial evidence, ATP III emphasized the need for more intensive LDL-lowering therapy in certain groups of people but its core is based on ATP I and ATP II. The new features of recent guideline are as follows (79):

New Features of ATP III

Focus on Multiple Risk Factors

- Raises persons with diabetes without CHD, most of whom display multiple risk factors, to the risk level of CHD risk equivalent.
- Uses Framingham projections of 10-year absolute CHD risk (i.e., the percent probability of having a CHD event in 10 years) to identify certain patients with multiple (2+) risk factors for more intensive treatment.
- Identifies persons with multiple metabolic risk factors (metabolic syndrome) as candidates for intensified therapeutic lifestyle changes.

Modifications of Lipid and Lipoprotein Classification

- Identifies LDL cholesterol <2.58 mmol/L (100 mg/dL) as optimal.
- Raises categorical low HDL cholesterol from <0.90 mmol/L (35 mg/dL) to <1.03 mmol/L (40 mg/dL) because the latter is a better measure of a depressed HDL.
- Lowers the triglyceride classification cutpoints to give more attention to moderate elevations.

Support for Implementation

- Recommends a complete lipoprotein profile (TC, LDL, HDL, and TG) as the preferred initial test, rather than screening for total cholesterol and HDL alone.
- Encourages use of plant stanols/sterols and viscous (soluble) fiber as therapeutic dietary options to enhance lowering of LDL.
- Presents strategies for promoting adherence to therapeutic lifestyle changes and drug therapies.
- Recommends treatment beyond LDL lowering for persons with TG ≥ 2.26 mmol/L (200 mg/dL).

For screening purpose, the NCEP guideline recommended that all adults 20 years of age or older should have a fasting TC, TG and lipoprotein profile done every 5 years. In nonfasting individuals, only the values for TC and HDL should be used. If the TC [≥ 5.17 mmol/L (200 mg/dL)] or HDL [< 1.03 mmol/L (40 mg/dL)] value is abnormal, a follow-up fasting lipoprotein profile is required for the development of therapeutic goals. More aggressive of lipids values have been classified as shown in the following (79):

NCEP ATP III Risk Classification for TC, LDL, HDL and TG

TC mmol/L	***(mg/dL)***	***Classification of Risk***
<5.2	(200)	Desirable
5.2 - 6.2	(200-240)	Borderline high
>6.2	(240)	High
LDL mmol/L	***(mg/dL)***	
<2.58	(100)	Optimal
2.58 – 3.33	(100-129)	Near optimal/above optimal
3.36 – 4.11	(130-159)	Borderline high
4.13 – 4.88	(160-189)	High
≥4.91	(190)	Very high
HDL mmol/L	***(mg/dL)***	
≥1.55	(60)	Low
<1.03	(40)	High
TG mmol/L	***(mg/dL)***	
<1.70	(150)	Normal
1.70 - 2.25	(150-199)	Borderline high
2.26 - 5.64	(200-499)	High
≥5.65	(500)	Very high

In this guideline, LDL is served as the primary target of CHD risk assessment instead of TC and HDL. In addition to the patient's lipid profile, accompanying CHD risk factors are considered in overall risk assessment. The patient is considered to have a high risk status when there are the following events; CHD-related events, the presence of two or more other CHD risk factors and a lipid or lipoprotein abnormality with the presence of one other CHD risk factor. Also considered at risk are patients who may not have a CHD event, but who are still at risk

for myocardial injury (MI) or cardiac death in the next 10 years according to the Framingham projections of 10-year absolute CHD risk.

A significant change noted in ATP III is recognition of the seriousness of risk for CHD in patients with diabetes. Because such patients frequently display multiple risk factors, even in the absence of CHD, and have the same risk for CHD as nondiabetics with CHD, ATP III defines diabetes as equivalent to existing CHD in terms of projecting coronary risk. Therefore, persons with diabetes who have an MI have an unusually high cardiovascular death rate, either in the short-term or long-term, and a more intensive herapeutic strategy is warranted. Risks for such patients are also considered to be equivalent to those of patients with known CHD. Certain patients who have multiple (two or more) risk factors and a Framingham risk score of >20% over the next 10 years are classified as having a CHD risk equivalent because of the number and severity of their risk factors. Patients with multiple metabolic risk factors, also known as the metabolic syndrome, are candidates for more intensive lifestyle therapy.

For the established therapeutic goals, ATP III sets LDL goals based on the degree of patient risk as summarized in the following (79):

ATP III: LDL Cut-points for Treatment According to Risk Category

Risk category (10-yr CHD risk)	LDL Level mmol/L (mg/dL)		
	Goal	**Initiate TLC**	**Consider drug therapy**
CHD or CHD risk equivalent (>20%)	<2.58 (100)	≥2.58 (100)	≥3.36 (130) 2.58-3.34 (100-129) drug optional
2+ risk factors (≤20%)	<3.36 (130)	≥3.36 (130)	10-year risk: 10-20% ≥3.36 (130) 10-year risk: <10% ≥4.13 (160)
0-1 risk factor (<10%)	<4.13 (160)	≥4.13 (160)	≥4.91 (190) 4.13-4.90 (160-189) drug optional

TLC = Therapeutic lifestyle changes.

For example, in patients with CHD or CHD risk equivalents, such as diabetes and therosclerotic disease, the risk for major coronary events within the next 10 years

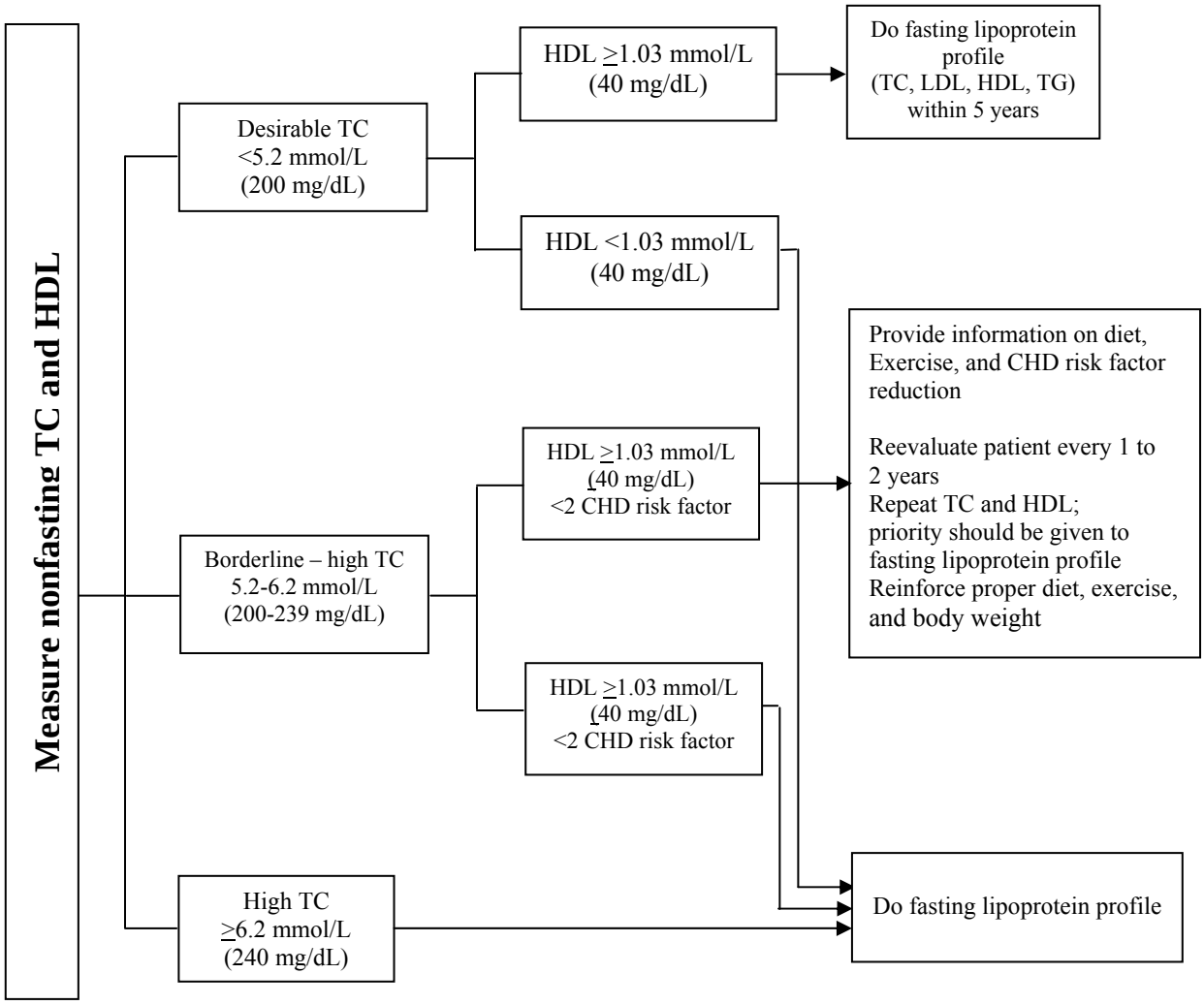
is greater than 20%. Treatment for such patients is intended to be aggressive, with the goal of decreasing LDL to <2.58 mmol/L (100 mg/dL). Patients with two or more of the risk factors are considered to be at intermediate risk, with the LDL goal being dependent on the individual's degree of risk. For those whose 10-year risk for CHD is 10% to 20%, the LDL goal is <3.36 mmol/L (130 mg/dL), while those whose 10-year CHD risk is less than 10% have an LDL-C goal of 4.13 mmol/L (160 mg/dL). Patients with 1 or 0 risk factors generally have a 10-year risk of less than 10% and have a more lenient LDL goal of <4.13 mmol/L (160 mg/dL).

The selection of therapeutic LDL cholesterol intervention strategies requires two additional major therapeutic modalities. The first involves therapeutic lifestyle changes, including a low saturated fat and cholesterol intake diet and/or weight reduction and increased physical activity if the patient has a metabolic syndrome or life-habit risk factors such as abdominal obesity, atherogenic dyslipidemia (elevated TG, small LDL particles, low HDL), high blood pressure, and insulin resistance (with or without glucose intolerance). The second major intervention strategy is drug therapy, including statins, bile acid sequestrants, and nicotinic acid.

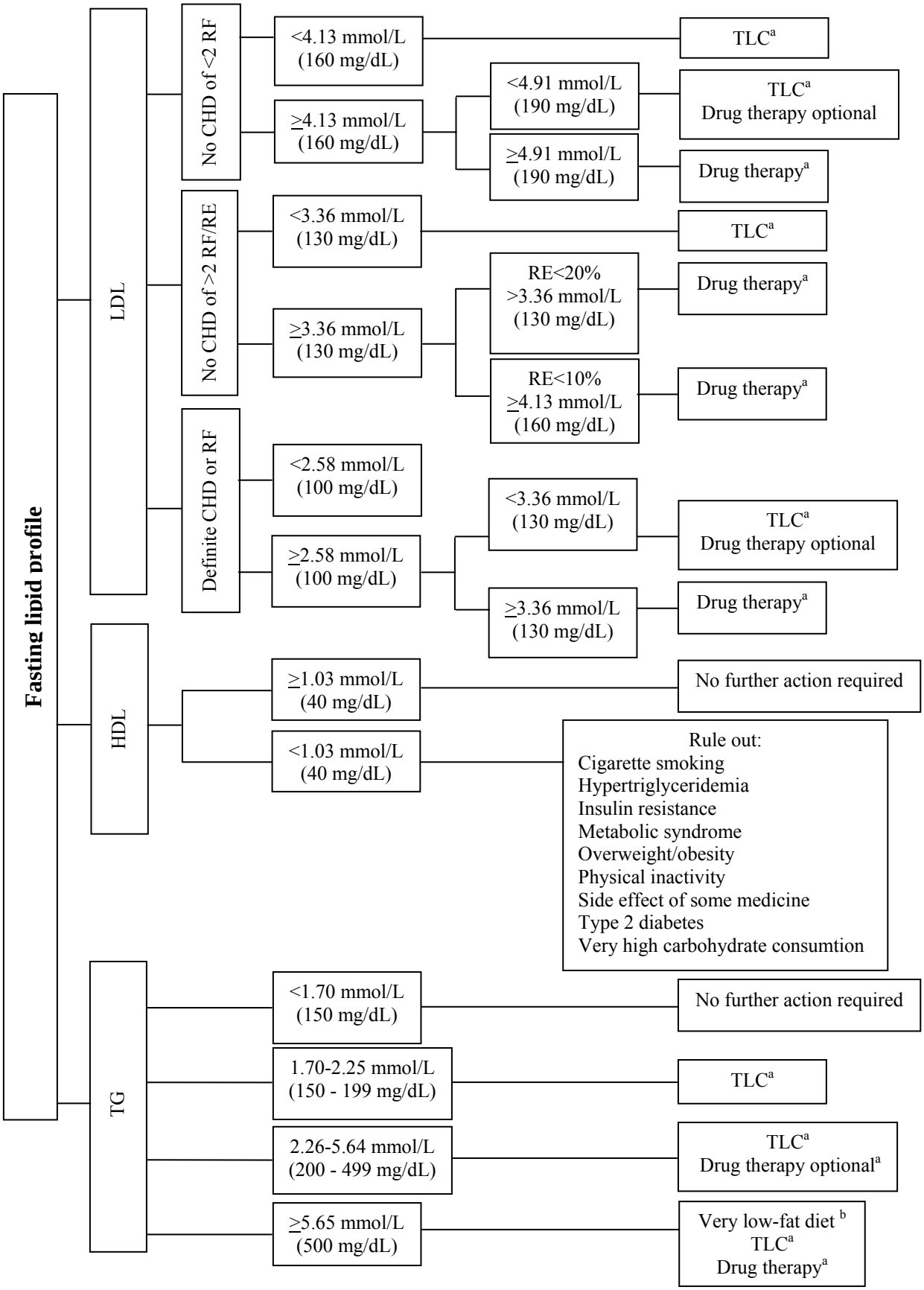
The ATP III report also emphasizes primary prevention of CHD along with LDL-lowering therapy. Therapeutic lifestyle changes are the foundation of clinical primary prevention. Nonetheless, some persons at highest risk for CHD, because of high LDL concentrations or multiple risk factors, are candidates for LDL-lowering drugs. Secondary prevention with LDL-lowering therapy is also beneficial, and the goal of therapy should be aggressive (i.e., LDL cholesterol <2.58 mmol/L (100 mg/dL). Clinical trials have demonstrated that the LDL-lowering therapy reduces total mortality, coronary mortality, major coronary events, coronary artery procedures, and stroke in persons with established CHD. It should be stressed that any person with elevated LDL or other form of hyperlipidemia should undergo clinical or laboratory assessment to rule out secondary dyslipidemia before initiation of lipid-lowering therapy.

Treatment strategies still focus on lowering the high blood level of LDL in order to provide primary prevention of CHD. The algorithm of CHD risk assessment, treatment, and monitoring using the NCEP ATP III guidelines for primary prevention in adults with and without evidence of CHD as in the following diagram based on

nonfasting results of both TC and HDL concentrations (49):



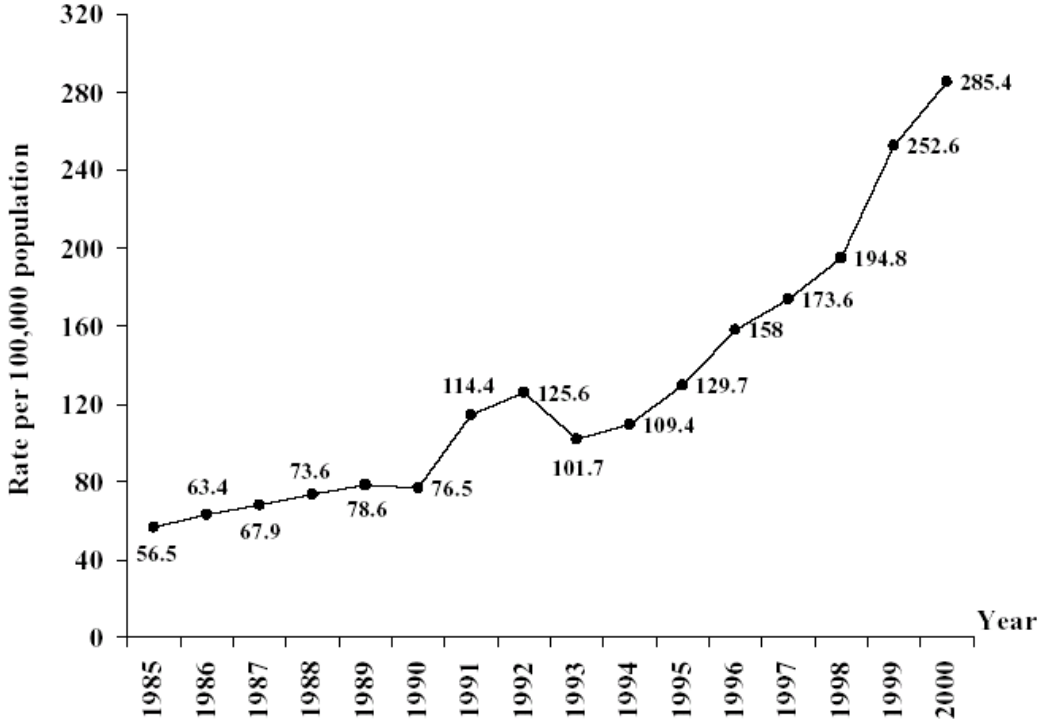
For secondary prevention of disease in adults with evidence of CHD or other clinical atherosclerotic disease, lipoprotein analyses are required, and the LDL cholesterol concentration is the key index for classification of CHD risk and therapy. Algorithm of CHD risk assessment, treatment, and monitoring for primary and secondary prevention of CHD in adults with and without evidence of CHD as shown in the following diagram (49). Classification of risk is based on fasting results on LDL, HDL, TG concentrations, and other risk factors (RF).


Fasting lipid profile
LDL
No CHD of <2 RF
<4.13 mmol/L (160 mg/dL)
TLC[a]
≥4.13 mmol/L (160 mg/dL)
<4.91 mmol/L (190 mg/dL)
TLC[a] Drug therapy optional
≥4.91 mmol/L (190 mg/dL)
Drug therapy[a]
No CHD of >2 RF/RE
<3.36 mmol/L (130 mg/dL)
TLC[a]
≥3.36 mmol/L (130 mg/dL)
RE<20% >3.36 mmol/L (130 mg/dL)
Drug therapy[a]
RE<10% ≥4.13 mmol/L (160 mg/dL)
Drug therapy[a]
Definite CHD or RF
<2.58 mmol/L (100 mg/dL)
≥2.58 mmol/L (100 mg/dL)
<3.36 mmol/L (130 mg/dL)
TLC[a] Drug therapy optional
≥3.36 mmol/L (130 mg/dL)
Drug therapy[a]
HDL
≥1.03 mmol/L (40 mg/dL)
No further action required
<1.03 mmol/L (40 mg/dL)
Rule out:
Cigarette smoking
Hypertriglyceridemia
Insulin resistance
Metabolic syndrome
Overweight/obesity
Physical inactivity
Side effect of some medicine
Type 2 diabetes
Very high carbohydrate consumtion
TG
<1.70 mmol/L (150 mg/dL)
No further action required
1.70-2.25 mmol/L (150 - 199 mg/dL)
TLC[a]
2.26-5.64 mmol/L (200 - 499 mg/dL)
TLC[a] Drug therapy optional[a]
≥5.65 mmol/L (500 mg/dL)
Very low-fat diet [b] TLC[a] Drug therapy[a]

2.3.4 Prevalence of CHD in Global and Thailand

CHD is the leading cause of death world wide (80). According to World Health Organization (WHO) estimates, 17 million people around the globe die from CHD each year. In 1999, CHD contributed to nearly one-third of global deaths. Low and middle income countries contributed to 78% of CHD deaths. By 2010 CHD is estimated to be the leading cause of death in developing country. By 2020 the WHO estimates nearly 25 million CHD deaths worldwide (80). In Thailand, CHD has been the leading cause of death of all ages since 1989, and mortality rates have increased each year since then (81). The prevalence rate has risen from 56.5 in 1985 to 109.4 in 1994 and up to 285.4 per 100,000 populations in 2000. Data from the Ministry of Public Health has shown that heart disease have become the leading causes of morbidity and mortality among Thai population and become public health problems with a rising trend. Such increasing trend results from unhealthy consumption behaviours and physical inactivity, as evidently demonstrated by the following hospital admission rate as shown in the following (81):

Admission Rate of Heart Disease in 1985 - 2000



2.4 Measurement of Lipid and Lipoprotein in Clinical Laboratory

Because of their predictively association with CHD, measurements of lipids and lipoproteins in the clinical laboratory have become increasingly important. The NCEP has developed national consensus guidelines for diagnosis and treatment of CHD which provide risk cut-points and define use of the lipids and lipoproteins analytes in case finding and therapy (77-79). Lipid and lipoprotein require a different approach when normal expected values are being defined because theses analytes are risk factors, not diagnostic factors, decision cut-points cannot be easily established by an individual laboratory or manufacturer as is done for other diagnostic analytes. Decision points are set by expert panels, based on population distributions and CHD risk relationships established in large epidemiological studies. The Centers for Disease Control and Prevention (CDC) have established the reference methods for TC, TG and also HDL. A reference method (RM) for LDL is currently being developed. These reference methods establish the accuracy base for the measurement of these analytes.

The NCEP sponsored expert laboratory panel has developed guidelines for measurements with requisite analytical performance targets for total error and corresponding precision and bias based on clinical needs for reliable patient classification (82-85). The total error goal, calculated from %bias plus 2 x coefficient of variation (CV), requires a level of imprecision and bias such that 95% of individual measurements fall within plus or minus the percent different from the reference method. The imprecision is calculated by replicate analyzing of a single specimen, and described as standard deviation (SD) and CV (CV = SD/mean * 100%). Using the mean of replicate analyses can be used to calculate bias, which represents the systemic error or overall inaccuracy. The percentage difference between the mean of the test method compared to the RM is the relative bias. Bias and CV targets represent analytical performance, which meets the ultimate goal of the total error target. The NCEP analytical performance goals for lipid and lipoprotein measurement are summarized in the following (82-85):

	Bias	Imprecision	Total error
Cholesterol	≤3% RM	CV ≤3%	≤9%
TG	≤5% RM	CV ≤5%	≤15%
HDL	≤5% RM	CV ≤4% at ≥1.9 mmol/L (42 mg/dL) SD ≤1.7% at < 1.9 mmol/L (42 mg/dL)	≤13%
LDL	≤4% RM		≤12%

The standardization programs for research laboratories and a Cholesterol Reference Method Laboratory Network for diagnostic manufacturers and clinical laboratories provide reliable access and documentation of traceability to accepted reference methods. Because of a definitive method (DM), involving isotope dilution mass spectrometry, is expensive, complicated for frequent use and time consuming, the CDC developed RM and applied to use in a network of standardization program (83, 86). This method is an accepted gold standard and calibrated by an approved primary reference standard to the DM. The CDC sponsored Cholesterol Reference Method Laboratory Network (CRMLN) is established to extend standardization to diagnostic firm and clinical laboratories (83). This network is also established to provide direct accuracy comparisons using fresh native serum specimen.

2.4.1 Cholesterol measurement

The CDC reference method for cholesterol is based on a chemical method devised by Abell, Levy and Brodie. In this method, alcoholic KOH serum is used to hydrolyze the cholesterol esters. Then the total cholesterol is extracted with hexane and dried. The dry residue is treated with a mixture of acetic acid, acetic anhydride, and sulfuric acid, called Liebermann-Burchard reagent, to develop color. Absorbance is read at 620 nm. This method is calibrated by the pure cholesterol, and exhibits 1.6% positive bias, compared with DM.

Because RM is time-consuming and hazardous, requisite manual organic phase extractions and using strong acids, considerable skill, and technologist time, the new enzymatic method has been developed and used in routine laboratory (87). This method is carried out the analysis in three enzymatic steps as shown in the following schematic (48):

$$\text{Cholesteryl ester} + H_2O \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesteryl esterase}} \text{Cholestenone} + H_2O_2$$

$$H_2O_2 + \text{dye} \xrightarrow{\text{Peroxidase}} \text{color}$$

This coupled enzyme reaction is fast, easily automated, accurate, precise and has relative few interference with endogenous reducing substance such as uric acid, bilirubin, ascorbic acid, and glutathione.

2.4.2 Triglycerides measurement

In the CDC reference method for TG, the triglycerides are first extracted with chloroform to remove water soluble interfering substances such as glucose and glycerol from the serum. Silicic acid then is used to treat the extract to remove phospholipids. The alkaline hydrolyze TG in the extract to produce unesterified fatty acids and glycerol. The produced glycerol is oxidized to produce formaldehyde, which is reacted with chromotropic acid for color development. Absorbance of the chromogen in the reaction mixture is measured at 570 nm. The schematic of TG reaction is shown in following (49):

$$\text{Triglycerides} + \text{KOH} \longrightarrow \text{Fatty acids} + \text{Glycerol}$$

$$\text{Glycerol} + \text{Periodate} \longrightarrow \text{Formic acid} + \text{Formaldehyde}$$

$$\text{Formaldehyde} + \text{Chromotropic acid} \longrightarrow \text{Chromogen}$$

In routine laboratory, triglycerides are measured enzymatically directly in plasma or serum. The first step of this assay is the lipase catalyzed hydrolysis of triglycerides to glycerol and fatty acids. Then glycerol phosphorylated in an ATP requiring reaction catalyzed by glycerokinase. In a glycerophosphate oxidase catalyzed reaction, glycerolphosphate is oxidized to dihydroxy acetone and H_2O_2. Finally, H_2O_2 is measured as described in the following reaction (49):

$$\text{Triglyceride} + 3\ H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + 3\ \text{Fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerophosphate} + \text{ADP}$$

$$\text{Glycerophosphate} + O_2 \xrightarrow{\text{Glycerophosphate oxidase}} \text{dihydroxyacetone} + H_2O_2$$

Alternatively, glycerophosphate can be measured in an NADH producing reaction and the absorbance is measured at 340 nm or in diaphorase catalyzed reaction to form a reaction product whose absorbance can be measured at 500 nm. The schematic of this alternative reaction is shown below (49):

$$\text{Glycerophosphate} + \text{NAD} \xrightarrow{\text{Glycerophosphate dehydrogenase}} \text{Dihydroxyacetone phosphate} + \text{NADH} + H^+$$

$$\text{NADH} + \text{Tetrazolium dye} \xrightarrow{\text{Diaphorase}} \text{Formazan} + NAD^+$$

Other methods measure production of ADP in reaction. The loss of NADH is measured photometrically at 340 nm. The equations are shown below (49):

$$\text{ADP} + \text{Phosphoenol pyruvate} \xrightarrow{\text{Pyruvatekinase}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}$$

Glycerol is present generally in serum so the measuring glycerol in serum is overestimation if the endogenous is not corrected. Normally, the endogenous glycerol presents the equivalent of less than 0.11 mmol/L triglycerides. This over concentration

is not clinical significant. However, in the conditions such as diabetes mellitus, emotion stress, intravenous administration of drugs or nutrient containing glycerol, endogenous glycerol containing are significantly higher and can impart greater error.

2.4.3 HDL measurement

Measurement of HDL as an inverse risk factor is also important in characterizing CHD risk. Therefore, accuracy in the measurement is especially important. Furthermore, errors in HDL measurement also contribute directly to inverse errors in the calculation of LDL.

The physical and chemical characteristics of lipoprotein have been used to achieve their separation. The presence of lighter lipids in varying proportions with the heavier protein facilitates separations by density, leading to use of ultracentrifugation method. The CDC reference method uses a combination of ultracentrifugation to remove chylomicrons and VLDL and precipitation with heparin-$MnCL_2$ to remove IDL, LDL and Lp(a), leaving HDL in the supernatant. Then cholesterol in this fraction is measured by use of the CDC reference method for cholesterol. This method is tedious and time-consuming. Moreover, the labile lipoproteins can be substantially altered by the high salt concentrations and centrifugal force used. Furthermore, this method uses the different type of equipment, making conditions extremely difficult to reproduce from one laboratory to another, and also needs the skills of the technicians. In addition, the fractions are heterogeneous and contain other functional lipoprotein, which may be cross-contaminate. Although ultracentrifugation is used as the comparison method for validation of other methods, and is more useful in research laboratory. However, this method is not considered to use in routine laboratory.

Electrophoretic methods separate the lipoproteins in a single operation using a variety of electrophoretic media such as paper, agarose gel, cellulose acetate membrane or polyacrylamide gel and result in bands. The lipoprotein bands have been named by comparison of the plasma protein as described previously. Although this method is considered to use for quantitative analysis but the discrepant results in samples with atypical lipoproteins may occur. Like the ultracentrifugation, these methods have disadvantages to use in clinical laboratory, especially when the

workload is high. Therefore, more practical chemical precipitation methods are considered to use in routine clinical laboratory.

Various high performance liquid chromatography (HPLC) methods have been introduced, but have been hindered by poor stability of the column. The improved HPLC method separates lipoproteins on the basis of size and quantifies of cholesterol with enzymatic reagent detection. Another method using nuclear magnetic resonance may be suitable for use in high workload laboratory. However, these methods are not suitable for routine work.

First generation of precipitation methods are introduced by Burstein and co-workers (88, 89). This chemical precipitation method selectively aggregate and render insoluble the lower density lipoprotein, leaving HDL in the solution by using polyanions, sometimes combined with divalent cations. Then insoluble lipoproteins are sedimented by low-speed centrifugation. The supernatant solution can be recovered by pipetting or decanting for cholesterol analysis as a measure of HDL. The earliest common precipitation method uses the polyanion heparin in combination with manganese and the CDC uses this method in combination with ultracentrifugation to assign target values to reference materials (83). However, manganese is interfered with EDTA and with enzymatically assays and heparin inconsistent in properties. Sodium phosphotungstate with magnesium become common in clinical laboratories. Because of sensitivity to reaction conditions and greater variability, it is replaced by dextran sulfate with magnesium and the 500 kDa dextran sulfate is replaced by 50kDa material for more specificity. Many commercial precipitate agents using various chemical sources and formulations are available, result in different results.

A significant interference with the HDL precipitation methods is elevated TG, which can prevent sedimentation of precipitate, leading to overestimated of HDL. Prior dilution and high speed centrifugation of specimens can reduce this error. Turbid specimen can be also cleared by ultracentrifugation. Because these methods need the manual pipetting step, their capabilities in routine laboratory do not reach the NCEP performance goal. In response to reduce error from the tedious manual step, improve in automated analyzers and progressively decreased specimen volume requirement, second generation methods, partially automated, were developed. In high volume laboratories manual pipetting was often replaced by automatic pipetting stations and

centrifugation by rack. Another method which gained rapid popularity linked dextran sulfate to magnetic beads, allowing the aggregated non-HDL particles to be pulled down with a magnet, sometimes placed right in the analyzer tray (90).

In this method, the precipitant has been complexed with magnetic particles. Once the lipoprotein-precipitant-magnetic particle complex has been formed, it can be removed rapidly without centrifugation by placing the reaction vessel on a magnetic disk that is supplied with the kit. The HDL-containing supernate is then removed and HDL cholesterol is measured in the usual way. The method can be also adapted for use in an automated clinical chemistry analyzer such that the supernate is analyzed without first removing it from the sedimented complex. The method was reported to have a bias of about 4 to 7% in the concentration range 0.78 to 1.30 mmol/L (30 to 50 mg/dL) when compared with ultracentrifugation combined with polyanion precipitation using a conventional dextran sulfate-Mg^{2+} method and a CV under 4%, similar to the usual dextran sulfate-Mg^{2+} method.

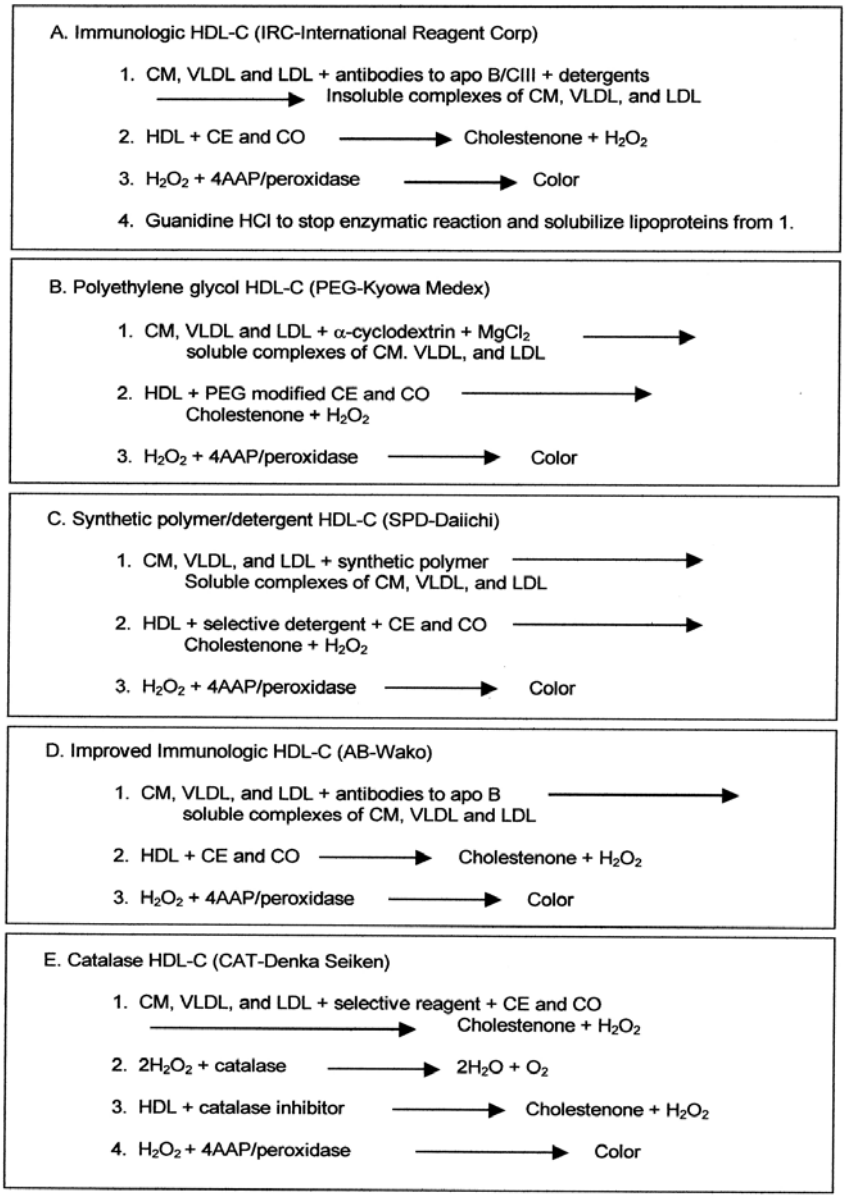
The most significant breakthrough in full automation, a “homogeneous” reagent, became widely known in 1994 through a poster presentation at the Annual Meeting of the American Association for Clinical Chemistry (9). This first method was evaluated favorably (91) but required four successive reagent additions making it impractical. In subsequent years four more homogeneous methods with only two reagents have been reported. Specimen is placed directly in the analyzer tray without pretreatment, followed first by addition of a reagent to block non-HDL, sulfated alpha-cyclodextrins (92), phosphotungstate with magnesium (93), or antibody (12, 94), and second by an enzymic reagent to measure HDL. One method (92) covalently links polyethylene glycol to cholesterol esterase and oxidase enzymes, blocking reaction with LDL. The latest homogeneous method (13) adds the cholesterol enzymes with catalase and a chemical which reacts specifically with the non-HDL while the catalase eliminates the hydrogen peroxide product. Then color reactants are added with a catalase inhibitor, developing color proportional to HDL.

The homogeneous reagents are more expensive than the pretreatment reagents but the labor savings in the developed countries more than compensate, resulting in an approximate 20% overall savings (93, 95). Proficiency survey reports from the US College of American Pathologists indicate that homogeneous reagents

first appeared as a separate peer group in 1997 and by 1998 accounted for over 25% of reporting laboratories (96). Recent evaluations of homogeneous reagents suggest consistent improvements in precision with CVs generally decreasing to about half those in simultaneously performed pretreatment methods (45-47, 97). Reports have suggested methods are accurate, but many lack credibility, because the comparison methods were not validated as traceable to an accepted RM. The most reliable assessment of method accuracy is a formal comparison study with one the CDC CRMLN laboratories; only one homogeneous method using the current reagent generation has completed such an evaluation (95). Another consideration is that at least three homogeneous methods have undergone modification during 1998, changing from partially lyophilized to fully liquid reagents or undergoing other major changes in formulation (95), so reports prior to 1999 may not be relevant to the current formulations. Assuming that current generations are at least equivalent to, or better than the previous generations for precision of the evaluations (91, 93-100), many study suggest that the homogeneous assays are capable of meeting NCEP analytical performance targets. This means they are calibrated accurately. The reports, including comparisons with pretreatment methods, indicate that the older methods used in clinical laboratories are unlikely to meet the NCEP precision targets, supporting the NCEP laboratory panel's prediction (50).

A concern for research laboratories and for specialty laboratories supporting lipid clinics and long-term clinical trials are reports that some homogeneous assays give discrepant, sometimes very different, results on unusual specimens; those from patients with dysbetalipoproteinemias, elderly patients, or those with kidney, liver, or cardiovascular diseases (101, 102). The homogeneous assays have been reported to be relatively free of the common interferences. Generally triglycerides below 20.7 to 25.8 mmol/L (800 to 1000 mg/dL) do not interfere but above 51.7 mmol/L (2000 mg/dL) they do. Normal to moderate bilirubin and hemoglobin concentrations generally do not interfere, but high levels are likely to result in inaccurate results. There are definitely trade-offs in the decision to replace a pretreatment method with a homogeneous method. Many clinical laboratories will appropriately use a homogeneous method to cut costs. At the other end of the spectrum research and specialty laboratories with a high proportion of unusual specimens will likely wait for additional validation before

adopting these methods. The schematic reaction mechanisms for each of five homogeneous methods as shown in following (103):



The accepted reference method for HDL is a three step procedure developed at CDC, involving ultracentrifugation to remove VLDL, heparin-manganese precipitation to remove LDL, and analysis of supernatant cholesterol by the Abell-Kendall assay (48). Because this method is tedious, expensive and time-consuming, the CDC and CRMLN laboratories validated an equivalent reference method, the so

called Designated Comparison Method, using a modified dextran sulfate (50kDa) precipitation on serum with Abell-Kendall cholesterol analysis (104).

2.4.4 LDL measurement

LDL, as the most validated lipoprotein risk factor, is the primary basis for treatment decisions in the NCEP clinical guidelines (4). Measurements of LDL are challenging because of the heterogeneity of LDL; the LDL fraction consists of a range of particles varying in composition, size and density. The common research method for accurate LDL quantitation and the basis for the reference method is designated beta-quantification, "beta" deriving from the electrophoretic term for LDL (105). Ultracentrifugation of serum at density of 1.006 g/mL floats VLDL and any chylomicrons, leaving LDL and HDL in the bottom fraction. Ultracentrifugation is a robust method that can give reliable results. However, this method is a tedious technique and impractical for the clinical laboratory. In a separate step, HDL is recovered in a supernate fraction by chemical precipitation and centrifugation of serum as described above. After cholesterol analysis of serum and fractions, the concentration of LDL is calculated as the difference between cholesterol measured in the infranate and in the HDL fraction. VLDL is calculated by subtracting bottom fraction cholesterol from that in serum. Cholesterol analysis in the top fraction is sometimes done as a quality control check on recovery of the fractions.

A simpler technique for LDL quantitation, common in both clinical and research laboratories, are the calculation of LDL by using the Friedewald formula , which VLDL is estimated as TG/5 (mg/dL) or TG/2.2 (mmol/L) (15). HDL is determined by one of the methods described above, and cholesterol and TG are measured in the fasting serum with LDL calculated by difference. The calculation gives reasonably reliable results in most normolipemic specimens because VLDL is usually relatively low and has little effect on the calculated LDL. Elevated TG, >10.3 mmol/L (400 mg/dL), chylomicrons and β-VLDL, characteristic of the rare type III hyperlipoproteinemia preclude estimation. The reliability of the Friedewald calculation has been controversial up to the present (106). In the specimens meeting the criteria listed above, calculation is reliable for patient classification provided the underlying rneasurements are made with appropriate accuracy and precision (107,

108). The NCEP expert laboratory panel, after reviewing quality control (QC) and proficiency testing performance in clinical laboratories, concluded that the Friedewald calculation would not meet NCEP analytical targets because of less than adequate precision in the underlying measurements. The panel recommended alternative methods be developed for use in clinical classification, preferably with direct separation of LDL (5).

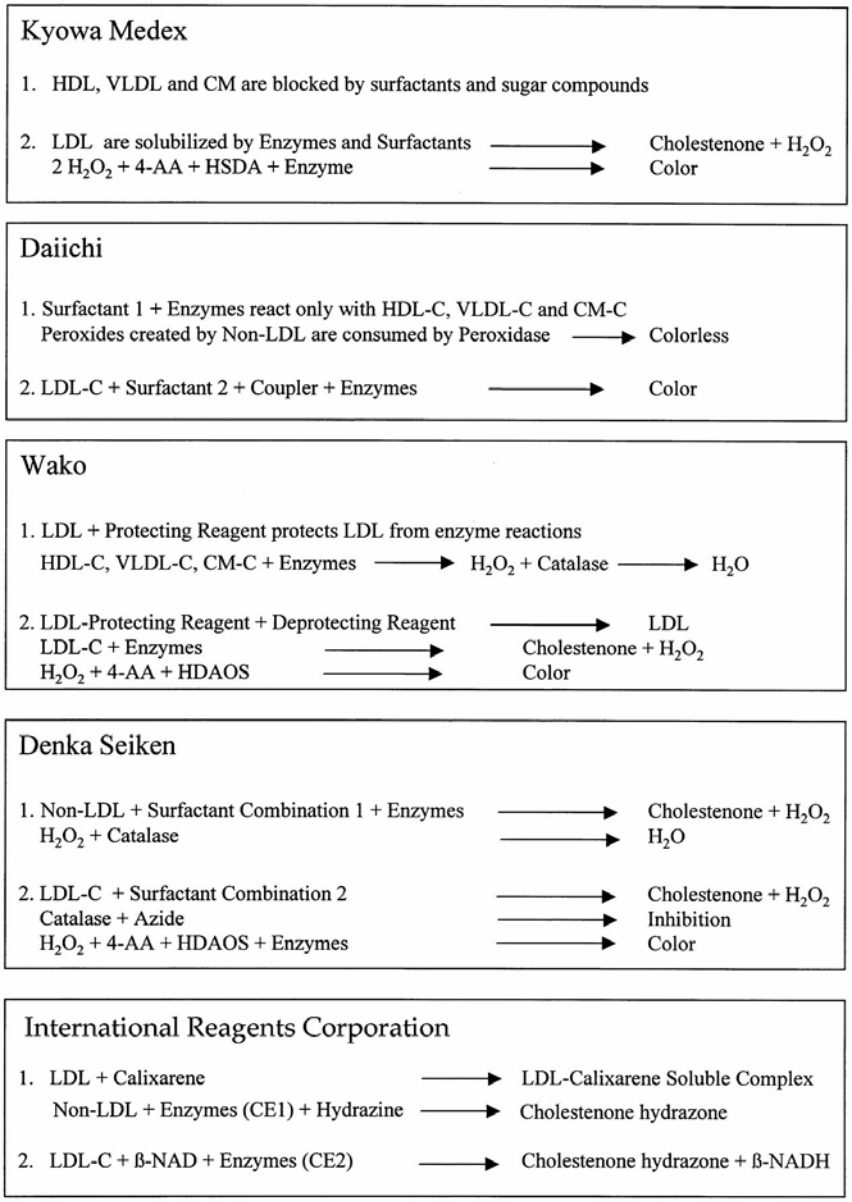
Subsequently, direct LDL methods were developed or refined for general use (109). The most common commercial method (110) used specific antibodies immobilized on latex beads to bind and remove non-LDL particles in a pretreatment step, with cholesterol analysis in the filtrate using a chemistry analyzer. The method was found to correlate reasonably well with β-quantification on both fasting and non-fasting specimens (111-113). This is a major advantage of direct methods that over the Friedewald calculation.

Following development of homogenous HDL methods a similar approach was used to develop homogenous methods for LDL, five to date (114). The first method, reported in the US in 1998, employed a non-ionic surfactant with sulfated α-cyclodextrin to selectively expose LDL to enzymic reagents (50) and reportedly correlated well with β-quantification. Subsequent reports (115-116) reached similar conclusions with different chemistries. As additional studies are reported on the new class of LDL methods, more reliable conclusions can be made about their performance. Of particular concern to specialty laboratories will be reliability of separations on unusual specimens.

Electrophoretic methods (117-118) for using in lipoprotein analysis, are more for qualitative than quantitative purposes. Electrophoresis allows separation and quantitation of major lipoprotein classes, while providing a visual display useful in detecting unusual or variant patterns. Agarose has been the most common medium for separation of whole lipoproteins, providing a clear background and convenient use (110). An automated commercial system has been reported to provide reasonably accurate and precise quantitation (120), but it is likely considered relatively less convenient than the homogeneous methods.

With homogeneous methods for LDL, the need for an RM and system for standardization of LDL becomes more urgent, since the Friedewald calculation was

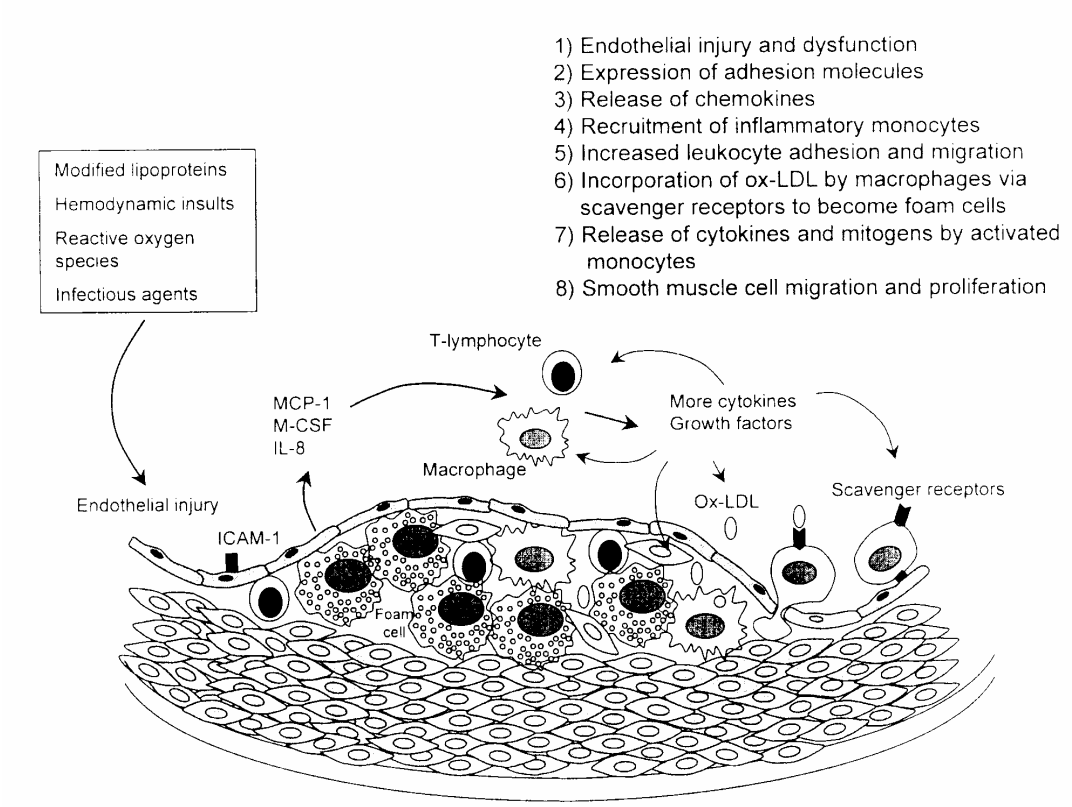
controlled by standardization of the underlying analytes. In response, CDC and the CRMLN laboratories are developing an RM and will be implementing a program for standardization (83). Homogeneous methods will probably replace the direct LDL methods, but it is yet to be resolved whether, and in what circumstances, homogeneous LDL methods should replace the Friedewald calculation, assuming that at east some of the commercial methods will prove to be sufficiently precise, accurate and robust. Nevertheless, homogeneous methods add additional cost, even considering the offset as TG measurements decline. Considerations of relative analytical performance, capability of measurement on non-fasting specimens, relative cost and reimbursement issues will impact the decision. The schematic reaction mechanisms for each of five homogeneous methods as shown in following (121):



2.5 Inflammation and CHD

2.5.1 Inflammation and atherosclerosis

Atherogenesis is believed to evolve in response to a succession of events triggered by vascular injury (122). The injured vascular endothelium and the associated inflammatory response are generally recognized as essential components of atherogenesis. Endothelial dysfunction appears to play a fundamental role in plaque formation (123). This process is marked by an up-regulation of adhesion molecules such as vascular adhesion molecule 1 (VCAM-l), and intercellular adhesion molecule 1 (ICAM-1), that mediate the increased adhesion of mononuclear leukocytes and T cells to the endothelium with subsequent migration into the subendothelial space (124). The attachment of monocytes and T-lymphocytes to the injured endothelium with subsequent migration into the intima is one of the first and most crucial steps in lesion development (125). Endothelial dysfunction also leads to the production of several vascular proinflammatory and prothrombotic molecules that will impact on vascular homeostasis, including interleukin-l (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), and tissue factor (TF), a membrane-bound glycoprotein with powerful pro-coagulant activities (126). Mononuclear cells within this inflammatory infiltrate are responsible for the local production of a variety of cytokines, including several interleukins, tumor necrosis factors α and β (TNF-α and TNF-β), interferon gamma (IFN-γ), and transforming growth factor beta (TGF-β) (127). These cytokines enhance the process of monocyte recruitment and stimulate endothelium and smooth muscle cells to release additional growth factors. At the same time, modified LDL begins to accumulate subendothelially. Macrophages take up this cholesterol-rich lipoprotein via scavenger and nonreceptor-mediated mechanisms becoming “foam cells” (128, 129), which together with the T-lymphocytes and smooth muscle cells form the “fatty streak,” the early lesion of atherosclerosis. As the lesion progresses, an increasing number of macrophages continue to scavenge more lipid. Ultimately, the proliferation of smooth muscle cells and the deposition of collagen lead to the evolution of the advanced atherosclerotic plaque. Formation of the advanced atherosclerotic plaque as shown follow (130):



Initially, the artery is compensated by dilation (remodeling) until it reaches the point at which it can no longer do so; then the lesion encroaches into the lumen altering blood flow (131). Various types of injury have been shown to initiate and sustain this inflammatory process, including the subendothelial accumulation of modified LDL, hemodynamic forces, hyperglycemia, and infection (125). Thus, inflammation appears to play a causal role in atherosclerosis and its complications. Local inflammation is accompanied by significant changes in the plasma concentration of the acute-phase proteins. The cytokines that are produced during the inflammatory process are the main stimulators of their production. Recent studies have demonstrated that baseline levels of acute-phase proteins in apparently healthy persons or patients with stable CHD constitute an independent risk factor for cardiovascular events, whereas the rise of these proteins after acute coronary events correlates with adverse outcome. Thus, the measurement of serological markers of inflammation may provide a novel method for identifying individuals with subclinical CHD and also for detecting those at increased cardiovascular risk.

2.5.2 C-reactive protein

CRP is the most sensitive of acute-phase proteins in humans, with serum levels rising as much as 1000-fold following injury, inflammation, or infection. CRP, a 206 amino acid polypeptide, was discovered in 1930 in the plasma of patients during the acute phase of pneumococcal pneumonia (133). It was named CRP after it was identified as a factor that binds to the C-polysaccharide of pneumococcus (134, 135). It is primarily, but not exclusively, produced by hepatocytes. Under certain conditions, neurons and other cells can also be a source of CRP. The gene for CRP is located on band q 2.1 of chromosome 1 in a region rich in host protective genes (136). IL-6 appears to play a major role in regulating the synthesis of CRP. Other proinflammatory cytokines may have important roles as well. The monocytes and macrophages at inflammatory sites are commonly thought to be the major source of circulating IL-6. The additional contributions sources are fibroblasts, endothelial cells, and adipose tissue.

CRP is present in relatively low levels in the serum of healthy subjects, with a median value of around 1 mg/L. The association of CRP concentration above 10 mg/L with active coronary disease has been established for many years. However, it was not until the development of highly sensitivity assays for CRP (hs-CRP) that the association of what was previously considered “normal levels” of CRP with future risk of cardiovascular disease was recognized. These highly sensitive assays allow the reliable measurement of hs-CRP concentrations as low as 0.1 mg/L.

Hs-CRP as a marker for underlying systemic inflammation has been consistently found to be increased in patients with various manifestations of atherosclerosis. Berk et al. were among the first to report that hs-CRP is increased in patients with acute ischemia (137). Not surprisingly, hs-CRP increases following an MI and peaks at 36 to 96 h after onset of symptoms (138-141). The magnitude of hs-CRP rise correlates weakly with infarction size as assessed by myocardial enzymes (139-142). The hs-CRP rise is attenuated with successful reperfusion with thrombolytic therapy. Moreover, hs-CRP response after myocardial infarction (MI) demonstrates modest association with future CHD morbidity and mortality, independently of infarct size (143).

2.5.3 hs-CRP as a predictor of future coronary events in apparently healthy men

A recent series of large prospective studies highlighted the strong association between baseline hs-CRP and the risk for future cardiovascular events in apparently healthy individuals. Mild elevations in hs-CRP were associated with increased cardiovascular events in asymptomatic but high-risk subjects. The MRFIT, a study of high-risk men followed for up to 17 years, found that baseline hs-CRP was predictive of MI and CHD death (144). However, this association was only detected in smokers (RR = 2.8; CI = 1.4-5.4). Several prospective studies also established a link between hs-CRP levels and cardiovascular events in apparently healthy, lower-risk men. In the PHS, hs-CRP was measured in 543 men who had developed coronary events and in 543 age- matched subjects who remained free from these events during the 14 years of the trial. Baseline hs-CRP was a strong, independent predictor of MI and ischemic stroke in both smokers and nonsmokers. Compared with those in the lowest quartile ($\leq$ 0.55 mg/L), subjects with hs-CRP levels in the highest quartile ($\geq$ 2.11 mg/L) had a twofold increase in the risk for stroke, a nearly threefold increase in the risk of MI, and a fourfold increase in the risk of symptomatic peripheral vascular disease (145). Hs-CRP was shown to be equally predictive of future coronary events, whether they occurred within a short period of time from baseline or many years later. Furthermore, the increased risk was independent of all other measured cardiovascular disease risk factors, including smoking and lipid levels. Moreover, the highest predictive values were achieved when both hs-CRP and the TC to HDL ratio were considered together (26).

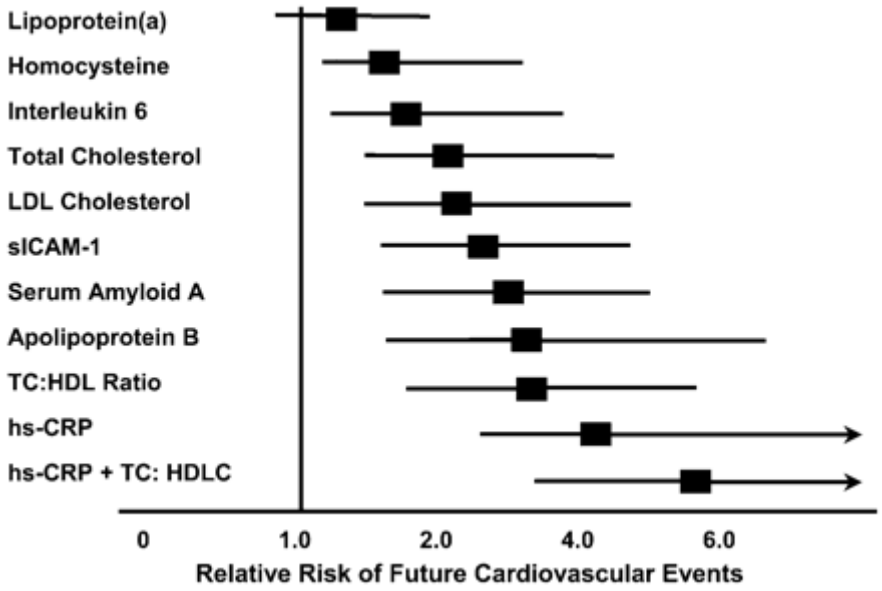
In a similar population of 936 healthy, middle-aged men, the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study also confirmed the value of hs-CRP in predicting CHD events (146). A one standard-deviation increase in the log-transformed value of baseline hs-CRP was associated with a 50% increase in CHD risk after 8 years of follow-up. In the Helsinki Heart Study, hyperlipidemic otherwise healthy middle-aged men who later developed an MI had mean hs-CRP levels of 4.4 compared with 2.0 mg/L seen in those who had no coronary event (p = 0.001) (147). Although some studies failed to demonstrate an independent relationship with CHD (148-149), most prospective case-control studies

suggest that hs-CRP is a strong, independent predictor of cardiovascular disease in men. Danesh et al. published a meta-analysis encompassing 14 prospective trials and 2,557 patients that demonstrate a risk ratio of 1.9 (95% CI = 1.5 to 2.3) for the highest third of hs-CRP (>2.4 mg/L) compared with the lowest third (< 1.0 mg/L). When the 11 trials of apparently healthy men were considered separately, the risk ratio was 2.0 (95% CI = 1.6 to 2.5) (150).

2.5.4 hs-CRP as a predictor of future coronary events in apparently healthy women

Although most studies have enrolled only men, the association between hs-CRP and cardiovascular morbidity has also been confirmed in women. In the Cardiovascular Health Studies, a high-risk but healthy group of 5,201 elderly men and women were followed for an average of 2.4 years (149). In women but not in men, hs-CRP was associated with increased coronary events, especially in those with evidence of subclinical atherosclerosis. Similar findings have also been reported from the Rural Health Promotion Project study (149).

A nested case-control study was conducted among the 28,263 apparently healthy participants of the WHS (19). Baseline hs-CRP and 11 other markers were measured, and subjects were followed for a median of 3 years. Compared with the lowest quartile of hs-CRP, the highest quartile was associated with a RR of 4.4 (95% CI 2.2 to 8.9, $p < 0.001$) for cardiovascular events (death from CHD, nonfatal MI or stroke, or coronary revascularization). In this direct comparison, hs-CRP was the single strongest predictor of future coronary event in women. In addition, the ability of hs-CRP to predict future coronary risk in women increased when considered with lipid values, a similar finding to that seen in the PHS. The schematic of relative risk for future cardiovascular events in the WHS study is shown in follow (130):



In stratified analysis, hs-CRP continued to be a strong predictor of future cardiovascular events among low-risk subgroups of women (absence of diabetes, hypertension, hyperlipidemia, smoking, or family history of CHD). In women with LDL below 3.37 mmol/L, a level considered low risk by the NCEP guidelines, the adjusted relative risk increased approximately 39% with each increasing quartile of hs-CRP. In fact, the mean LDL concentration in that group of women was 2.7 mmol/L. Therefore, hs-CRP could identify a subset of patients at high CHD risk who would have been missed if only lipid screening was used.

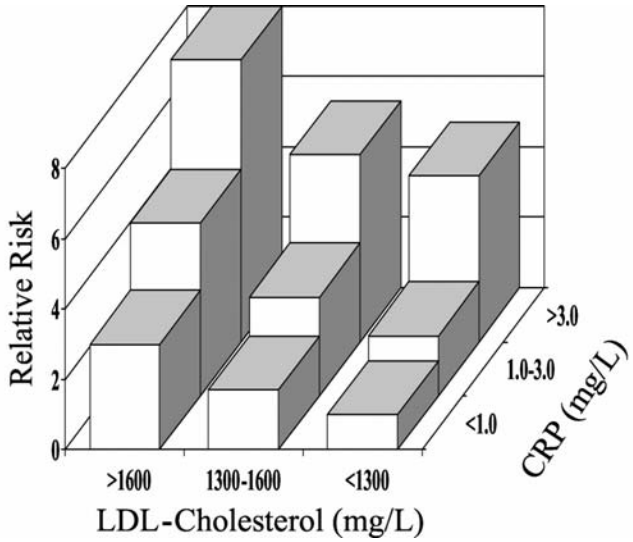
2.5.5 Clinical application of hs-CRP

Available data suggest that hs-CRP is a strong independent predictor of CHD risk in apparently healthy individuals. Risk prediction associated with hs-CRP either alone or in combination with lipid screening appears to be substantially greater than that associated with traditional and nontraditional risk factors such as levels of total and LDL, homocysteine, and Lp(a). Data from the PHS and WHS suggest that the addition of hs-CRP to standard lipid screening can significantly improve the ability to detect coronary risk (26). Furthermore, findings from the WHS have demonstrated that increased hs-CRP concentrations are associated with increased vascular risk even among those with normal lipid levels. Therefore, hs-CRP provides a tool to identify

those individuals at increased risk of future coronary events who otherwise would be missed if we only relied on the traditional lipid screening.

In clinical practice, hs-CRP may have an important role in risk assessment of CHD in primary prevention. It is believed that hs-CRP should not be reported to the clinician in mass concentration but in quintiles of risk established by prospective clinical studies. Recently, it has been shown that quintiles and tertiles of CRP are similarly associated with future risk of coronary events (151). The CDC and the American Heart Association (AHA) issued guidelines and specific recommendations for the utility of this marker (34). Three cut-point values for clinician interpretation were defined as low risk (<1.0 mg/L), average risk (1.0-3.0 mg/L) and high risk (>3.0 mg/L) of CHD. In addition, hs-CRP levels should be interpreted in combination with lipid levels. Ridker et al. showed that prediction models incorporating the TC: HDL ratio was far better that those based on hs-CRP alone. Based on these data, an algorithm for cardiovascular risk assessment using both hs-CRP and lipids was proposed recently (27). A simplified clinical approach to this issue based on the ATP III cut-points for LDL (4) and the CDC/AHA tertiles for CRP is shown in follow (152):

LDL-Cholesterol (mg/L)	CRP (mg/L)		
	<1	1-3	>3
<1300	1.0	1.7	4.7
1300-1600	1.7	2.8	5.3
>1600	3.0	4.9	8.0



Hs-CRP concentrations are relatively stable throughout the day, and their determinations may be performed without concern for diurnal variation (153). However, considerable within-subject variability does exist, and a single test will have

a wide confidence interval. Ockene et al. reported that the use of two sequential hs-CRP measurements is adequate for clinical use. Ideally, the two hs-CRP determinations should be done after 1 month apart and the lowest value used for risk prediction. In that study, it was shown that almost 90% of subjects were classified in the exact quartile or varied only by one quartile using two independent measurements for either hs-CRP or total cholesterol. It has been suggested that values >5 mg/L should be repeated to avoid possible false positives. Values above 10 mg/L most likely reflect subclinical infection or inflammation, and therefore measurements should be repeated after 3 to 4 weeks.

In addition to its ability to identify subjects at increased coronary risk, hs-CRP may also have a role in targeting specific preventive therapies. Current data suggest that the increased risk associated with systemic or vascular inflammation may be ameliorated with aspirin and statin therapies. Thus, it is possible that hs-CRP may help identify those patients who will benefit most from these pharmacological interventions. Likewise, hs-CRP measurement may have an important role in determining the risk/benefit ratio related to the initiation of hormone replacement therapy (HRT) in postmenopausal women. The use of HRT to prevent CHD is a difficult health decision for postmenopausal women. The recent findings from the Heart and Estrogen/Progestin Study (HERS) have challenged previous observational data regarding the role of hormones in preventing subsequent cardiovascular events. HERS demonstrated an early increase in cardiovascular events in association with randomized assignment to hormone replacement therapy in postmenopausal women with confirmed coronary disease (RR during 1^{st} year =1.5, 95% CI 1.01 to 2.29, p = 0.009). Recently, similar findings were reported from the Women's Health Initiative in participants with no prior history of CHD. Interestingly, HRT has been shown to significantly increase hs-CRP in observational and cross-sectional studies (154, 155). Data from the WHS showed that healthy women on HRT had median hs-CRP levels twice as high as women not taking HRT (2.7 vs. 1.4 mg/L; p = 0.001) (156). The Postmenopausal Estrogen/Progestin Intervention (PEPI) study, a 3-year randomized trial, showed an early and sustained increase in hs-CRP associated with assignment to HRT compared with placebo (157). Increased levels of hs-CRP were seen as well in the short-term study of postmenopausal women assigned to micronized estradiol when

compared with placebo. The clinical significance of the increased hs-CRP with HRT remains to be clarified, but the finding may have implications for women initiating hormone therapy, specifically because increased levels of hs-CRP are associated with increased cardiovascular risk among otherwise healthy women. The measurement of baseline levels of hs-CRP prior to the initiation of HRT may help to tailor the appropriate therapeutic regimen for women at high risk for cardiovascular complications.

2.5.6 Analytic considerations in hs-CRP measurement

Historically, CRP was measured in clinical laboratories to detect active inflammation and infection using immunoturbidimetric and immunonephelometric techniques. The detection range of these assays spans from 3 to well over 200 mg/L. Therefore, because these assays lack the appropriate sensitivity needed for the assessment of cardiovascular risk in apparently healthy individuals, high-sensitivity methods have had to be developed. Several approaches have been used by both manufacturers and investigators to achieve the desired limit of quantification, including the labeling of anti-CRP-antibodies with either an enzyme (ELISA) or a fluorescent compound, or attaching the antibodies to polystyrene beads. However, the most common approach is particle enhanced nephelometry or turbidimetry, which monoclonal or polyclonal antibody is adsorbed onto latex particles to increase signal at low concentrations of the analytes. Presently, six manufacturers hs-CRP assays (Dade Behring, Diagnosis Products Corporation, Hemagen Diagnostics, Kamiya, Olympus and Roche Diagnostics) have been cleared by the U.S. Food and Drugs Administration (FDA) for clinical use and only the Dade Behring assay has been approved by the FDA for use in assessing the risk of CHD. The commercial hs-CRP methods are listed in the following table (158).

Source	Methodology	Reference material	Lower limit of detection (mg/L)[1]	Assay range mg/L
Dade Behring	IN[3]	CRM470	0.02	0.175-11[2] (initial dilution)
Daiichi	IT[4]	CRM470	0.04	0.2-60
Denka Seiken	IT	CRM470	0.03	0.05-10
Diagnosis Products Corporation (DPC)	IL[5]	CRM470	0.02	0.1-250
Hemagen Diagnostics	EIA[6]	WHO 85/506	0.10	1-10 or 1-50
Iatron	IT	CRM470	0.005	0.05-4
Kamiya	IT	CRM470	0.03	0.1-20
Olympus	IT	CRM470	0.08	0.5-20
Roche Diagnostics	IT	CRM470	0.02	0.1-20
Wako	IT	CRM470	0.06	0.05-10

[1] Manufacturers claim; [2] Based on default dilution; [3] Immunonephelometry; [4] Immunoturbidimetry; [5] Immunoluminometry; [6] Enzyme immunoassay

It is important to note that not all hs-CRP assays possess similar sensitivity or lower limit of quantification (30). A recent evaluation of nine different assay systems showed that all these “second generation” hs-CRP methods were capable of reliably measuring concentrations as low as 0.3 mg/L (31). More importantly, five of these methods were in excellent agreement in classifying subjects into quartiles of risk. In fact, 92 to 95% of subjects were classified by these methods into the exact quartile and the remaining 5 to 8% fell almost equally in the adjacent two quartiles. Using the other four methods, however, only 65 to 75% of subjects were classified into the exact quartile, and the remaining 25 to 35% all fell in the adjacent upper quartile indicating a problem with standardization. The others studies also showed differences in values obtained on the same samples (33, 34). This is an interesting observation considering that manufacturers of these methods have indicated that their calibrators are traceable to the Certified Reference Material 470 (CRM 470). Unfortunately, this is not an unusual occurrence. Although manufacturers attempt to standardize their assays using the appropriate calibrators, they often fail to follow the

appropriate value transfer protocol from the reference materials to their own calibrators (132). Invariably, this results in a suboptimal standardization. Because the hs-CRP value of an individual patient is interpreted in the context of cut-points established by prospective clinical studies, standardization of hs-CRP assays is crucial. Poor agreement among methods will result in the misclassification and mismanagement of patients. To address this matter, the CDC (Atlanta, GA) originally initiated a standardization program in March 2001 which manufacturers of all hs-CRP assays worldwide were invited to participate (159). Several steps have been taken, including plan for an hs-CRP reference system that will be used with low-grade inflammation serum values that are associated with high cardiovascular risk. The CDC has also offered a long-term plan for developing a definitive reference method and is working on an immediate plan to develop a candidate hs-CRP serum that will be used as the reference material in all diagnostic products. Phase I of this project aims to identify a suitable reference materials (160) that will be used in phase II, which will seek to harmonize various hs-CRP assays. Race and ethnicity are also the sources of variability in CRP measurement. Based on the distribution of CRP concentrations studies, recent data from several American and European studies have clearly demonstrated the comparable distribution of CRP concentrations among women not receiving hormone replacement therapy and men (161-163). The 50^{th} percentile of CRP measured in the various populations was ~1.5 mg/L for both genders. Furthermore, data from the National Health and Nutrition Examination Survey III showed no significant difference in the distribution of CRP concentration among white, African-American, and Mexican-American men (161). Moreover, a comparable CRP distribution was seen in Japanese men (164). Japanese women, however, seem to have slightly lower CRP concentrations. Furthermore, the geometric mean for CRP in Indian Asians was reported to be 17% higher than in European whites (165), a difference that was no longer significant after the adjustment for central obesity and insulin resistance (166). The clinical implication of these findings is that no gender- or ethnic specific cut-points for CRP are indicated. Others sources of variations in CRP measurement are reviewed recently (167).

CHAPTER 3
MATERIALS AND METHODS

3.1 Equipment

We used three analyzers in our study. The Dimension RxL analyzer (Dade Behring Inc., Newark, USA) was used to perform triglycerides, cholesterol and the Dade Behring homogeneous HDL. The Behring Nephelometer 100 (BN 100) analyzer (Dade Behring Inc., Liederbach, Germany) was used to perform the N High Sensitivity CRP. The COBAS INTEGRA 400 analyzer (Roche Diagnostics, 4070 Basel, Switzerland) was used to perform the Roche homogeneous HDL and Tina-quant CRP.

3.2 Reagents

3.2.1 Triglycerides

Triglycerides was determined enzymatically with the Triglycerides Flex^{TM} reagent cartridge (Dade Behring Inc., Newark, USA). According to the manufacturer's specification, the sample is pre-incubated with lipase enzyme reagent which converts triglycerides into free glycerol and fatty acids. The liberated glycerol is determined enzymatically using glycrol dehydrogenase (GDH) and NAD. The change in absorbance at 340 nm due to the formation of NADH is directly proportional to the total amount of glycerol and its precursors in the sample and is measured using a bichromatic (340, 383 nm) rate technique.

3.2.2 Cholesterol

The cholesterol method used on the Dimension clinical chemistry system is based on the enzymatic method. Cholesterol was determined with the CHOL Flex^{TM} reagent cartridge (Dade Behring Inc., Newark, USA). Cholesterol esterase (CE) catalyzes the hydrolysis of cholesterol esters to produce free cholesterol which, along

with preexisting free cholesterol, is oxidized in a reaction catalyzed by cholesterol oxidase (CHOD) to form cholest-4-ene-3-one and hydrogen peroxide (H_2O_2). In the presence of horseradish peroxidase (HPO), the H_2O_2 thus formed is used to oxidize N,N-diethylaniline-HCl/4-aminoantipyrine (DEA-HCl/AAP) to produce a chromophore that absorbs at 540 nm. The absorbance due to oxidized DEA-HCl/AAP is directly proportional to the total cholesterol concentration and is measured using a polychromatic (540,452, 700 nm) endpoint technique.

3.2.3 Dade Behring homogeneous HDL (the Dade method)

HDL was determined using the Automated HDL Cholesterol Flex^{TM} reagent cartridge (Dade Behring Inc., Newark, USA). The assay is a homogeneous method for directly measuring HDL concentrations without the need for pretreatment or centrifugation steps. The method is in a two reagent format and depends on the properties of a unique detergent, which solubilizes only the HDL lipoprotein particles, thus releasing HDL cholesterol to react with CE and CHOD. The H_2O_2 is measured in a peroxidase (POD) catalyzed reaction that forms a dye. In addition to selectively disrupting the HDL lipoprotein particles, this detergent also inhibits the reaction of the cholesterol enzyme with LDL, VLDL and chylomicron lipoproteins by adsorbing to their surfaces. A polyanion is contained in the first reagent to assist with complexing LDL, VLDL and chylomicron lipoproteins, further enhancing the selectivity of the detergent and enzymes for HDL-C. For the Dade method, our laboratory was standardized through the National Heart, Lung and Blood Institute (NHLBI) – Centers for Disease Control and Prevention Lipid Standardization Program. Therefore, we used the Dade method as the comparative method.

3.2.4 Roche homogeneous HDL (the Roche method)

The HDL method used on the COBAS INTEGRA 400 analyzer is a homogeneous assay. HDL was determined with the direct HDL cassette reagent (Roche Diagnostics, 4070 Basel, Switzerland). The method is based on the adsorption of synthetic polyanions to the surface of lipoproteins. LDL, VLDL, and chylomicrons are thereby transform into a detergent-resistant form. HDL is not combined with the polyanions. The detergent solubilizes cholesterol from HDL, but not from LDL,

VLDL, and chylomicrons. Solubilized cholesterol is oxidized by the sequential enzymatic action of CE and CHOD. The H_2O_2 that is formed reacts with N,N-bis (4-sulfobutyl)-m-toluidine (DSBmT) and 4-aminoantipyrine (4-AAP) in the presence of POD and forms a red quinoneimine dye.

3.2.5 N High Sensitivity CRP (N High Sensitivity CRP method; hs-CRP_{DB})

N High Sensitivity CRP (Dade Behring Inc., Newark, USA) is an in vitro diagnostic assay for the quantitative determination of CRP in human serum and heparin- and EDTA plasma by means of particle enhanced immunonephelometry using BN™ system. Polystyrene particles coated with monoclonal antibodies to CRP are agglutinated when mixed with samples containing CRP. The intensity of the scattered light in the nephelometer depends on the CRP content of the sample and therefore the CRP concentration can be determined versus dilutions of a standard of a known concentration. This assay is designed to measure CRP concentrations within an overall range of approximately 0.175 to 1100 mg/L. The range applicable for different sample dilutions are 1:20, 1:100, 1:400 and 1:2000. The measuring range for each dilution is 0.175 to 11 mg/L, 0.875 to 55 mg/L, 3.5 to 220 mg/L and 17.5 to 1100 mg/L, respectively. The patient samples are automatically diluted of 1:20 with N Diluent for the high sensitivity application. If the hs-CRP is higher than the upper limit of the analytic measurement range, the samples have to be further diluted of the higher dilutions. In our study, we used the N High Sensitivity CRP assay as the comparison method because it was the only method approved by the FDA for cardiovascular and peripheral vascular risk assessment.

3.2.6 Tina-quant CRP (Tina-quant method; hs-CRP_R)

The Tina-quant CRP assay (Roche Diagnostics, 4070 Basel, Switzerland) is an in vitro diagnostic system intended for use to determine human CRP in serum and plasma. The principle of the assay is based on the particle enhanced immunoturbidimetry. Human CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies. The precipitate is determined turbidimetrically at 552 nm.

3.3 Samples

Venous blood samples were obtained from the participants of the International Collaborative Study on Atherosclerosis and Stroke In Asia (InterASIA) after an 8 hours overnight fast. The sample design has been described in detail previously (168). For each individual who agreed to participate, health professional staff performed a brief physical examination and administered a structured questionnaire on general information, medical history, cardiovascular disease risk factors and life style to assess their health status. Samples were stored immediately in ice, and centrifuged and separated on the day of collection. Sera were subsequently frozen and transferred on dry ice to our laboratory for biochemical analysis.

3.4 Reference samples

3.4.1 Calibrators

To calibrate triglycerides, the Dade Behring homogeneous HDL and the N High Sensitivity CRP methods, we used the following commercial calibrators for each method from the Dade Behring, CHEM II calibrator (Dade Behring Inc., Newark, USA), AHDL calibrator (Dade Behring Inc., Newark, USA) and the N Rheumatology Standard SL for Nephelometry, respectively. We used Cholesterol Quality Control Materials obtained from the CDC (Solomon Park Research Laboratories Atlanta, Georgia, USA) to calibrate cholesterol method.

To calibrate the Roche homogeneous HDL and the Tina-quant CRP methods, we used the Calibrator HDL Direct and CRP T Standard, respectively.

3.4.2 Quality control samples

To control the inaccuracy of each method, we used Dade® Moni-trol® Level 1 (Dade International Inc. Miami, FL 33152-0672, USA) and 2 (Dade International Inc. Miami, FL 33152-0672, USA) Chemistry Controls for TC and TG methods, Dade Liquid Moni-Trol® TOTAL (ASSAYED) Level 1 (AT0109-1) and 2 (AT0109-2) for the Dade Behring homogeneous HDL assay, Precinorm L (Cat. No. 10781827) and Precipath HDL/LDL-C (Cat. No. 11778552) for the Roche homogeneous HDL-C

assay, ApoB for the N High Sensitivity CRP and CRP T control for the Tina-qaunt CRP method.

3.5 Procedures

The 5,350 serum samples were separated into the 3 specific assay cups for each analyzer. TC and TG were determined using the Dimension RxL analyzer. HDL determination was performed using both homogeneous HDL assays on the Dade Dimension RxL analyzer and the Roche COBAS INTEGRA 400 analyzer. The CRP concentrations in all samples was determined using a nephelometric method on the BN 100 analyzer and a particle enhanced immunoturbidimetric method performed using a COBAS INTEGRA 400 analyzer. LDL was calculated by the Friedewald formula using the HDL value obtained from the Dade Behring method (LDL_{DB}) and from the Roche method (LDL_R):

$$\text{LDL (mmol/L)} = (\text{TC}) - (\text{HDL}) - (\text{TG}/2.2)$$

$$[\text{LDL (mg/dL)} = (\text{TC}) - (\text{HDL}) - (\text{TG}/5)]$$

Results of participants with TG concentration greater than 4.52 mmol/L (400 mg/dL) and hs-CRP concentration greater than 10 mg/L were excluded from the comparative study of two estimated LDL and two hs-CRP assays, respectively. The processing of our study was summarized as show in the following chart.

3.6 Statistical analysis

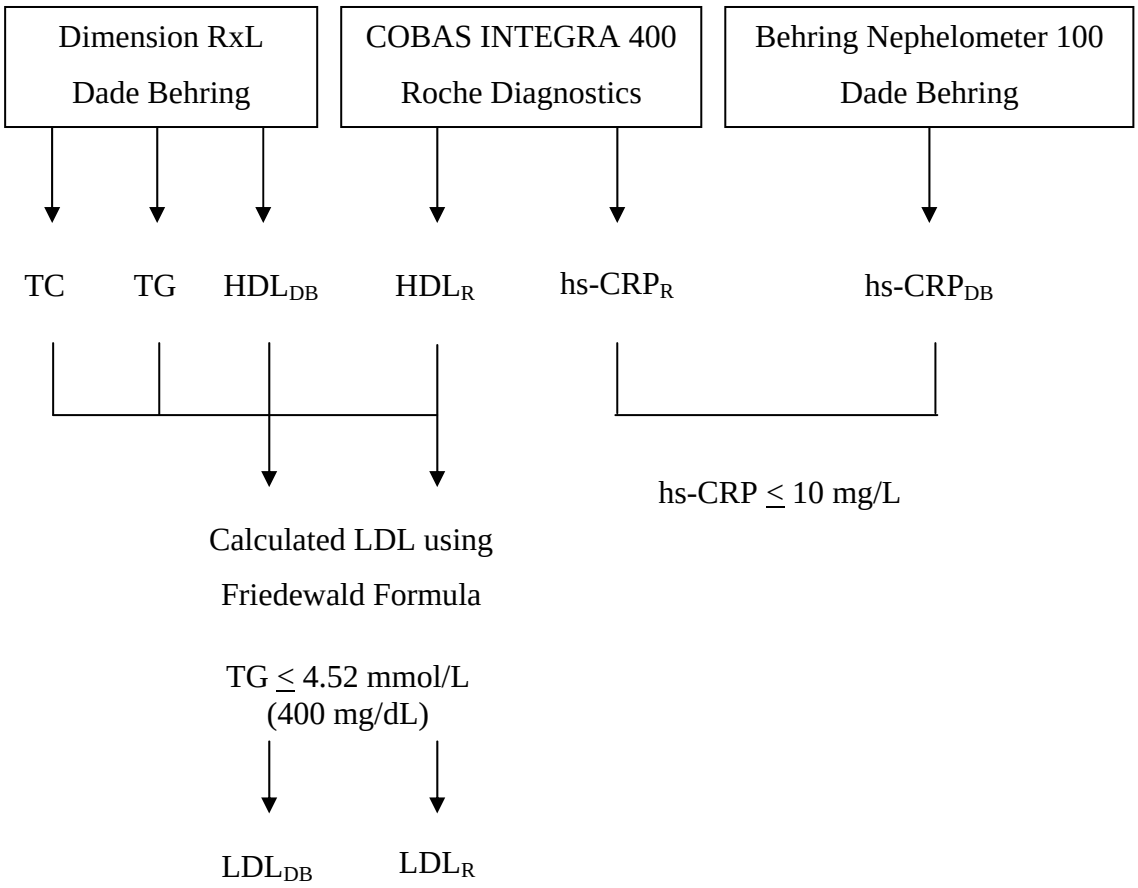
The HDL and estimated LDL values were expressed as means $\pm$ SD. Means were compared by using Student paired *t*-test. To determine the clinical concordance, the percentages of low, normal, and high risk groups of HDL results and the low, average, and high risk groups based on the LDL treatment targets recommended by the NCEP ATP III guidelines were calculated.

Because hs-CRP distributions were skewed rightward, their values were expressed as medians. Median comparisons were assessed by the Wilcoxon signed rank test. Each method was used to classify the subjects into the low, average, and high risk groups as recommended by the CDC/AHA consensus guidelines.

Slope, intercept, $S_{y/x}$ and r were estimated using Deming regression analysis. Significance levels were set at 0.05. Analyses were performed with EP Evaluator-

CLIA software (David G. Rhoads Associates, Kennett Square, PA). Bland-Altman analysis was performed to assess agreement between paired of HDL, estimated LDL and hs-CRP results. The 95% limits of agreement (LOA) were also determined calculating from bias ± 1.96 SD (169). We also compared the percentage of subjects in each LDL risk groups against the risk groups for hs-CRP using the two hs-CRP methods. Finally, the percentage of subjects for all LDL and hs-CRP method combinations assigned to each relative risk group were also determined.

5,350 InterASIA serum samples



- Compare and assess agreement between pairs of HDL, calculated LDL and hs-CRP results
- Compare the overall risk of CHD using combinations of calculated LDL and hs-CRP methods.

CHAPTER 4
RESULTS

4.1 Comparison of two homogeneous HDL cholesterol methods in a large population study

The mean concentration of Dade [1.23 ± 0.33 mmol/L (48 ± 13 mg/dL)] was significantly lower when compared with those of Roche [1.29 ± 0.33 mmol/L (50 ± 13 mg/dL)], $p < 0.001$. The ranges from Dade and Roche were 0.19 to 2.86 mmol/L (7 to 110 mg/dL), and 0.30 to 3.05 mmol/L (12 to 118 mg/dL), respectively. Figure 1 shows the correlation of the HDL-C results between the Dade and Roche methods. According to the Figure 1A, the slope [1.009; 95% confidence interval (CI) = 1.002 to 1.016] of the regression line was close to 1.00 with a small positive intercept [0.048 mmol/L (2 mg/dL); 95%CI = 0.039 to 0.057 mmol/L (1.51 to 2.20 mg/dL)], and $S_{y/x}$ [0.080 mmol/L (3 mg/dL)], respectively. The correlation coefficient (r) was 0.972. These data show that both methods are highly correlated. However, agreement between methods is not adequately determined by the correlation coefficient alone. Therefore, we assessed the degree of agreement between the two methods using the Bland-Altman graphical technique (Figure 1B). The mean difference was 0.06 mmol/L (2 mg/dL) with a standard deviation of 0.08 mmol/L (3 mg/dL). The 95 percent confidence interval for the mean of the method differences was very low [from -0.10 to 0.22 mmol/L (-4 to 9 mg/dL)]. In addition, clinical concordance were assessed using the Dade method as the comparative method. From Table 1, percentages of low, normal, and high risk results were 15.5, 55.4, 29.0 for the Dade and 19.3, 59.3, 21.4 for the Roche methods. The percentage of concordantly classified subjects at each cut point was 77.1%, 84.4% and 95.5% (Table 2). In Figure 2, concordance for the three risk groups was assessed graphically. The overall consistency was 85.4%. 13% of subjects was discordantly classified into the higher risk group while the 1.6% of subjects was discordantly classified into the lower risk group.

4.2 Comparative study of two automated high sensitivity C-reactive protein methods in a large population

The median hs-CRP value for the N High Sensitivity CRP method (1.23 mg/L) was significantly lower than that for the Tina-quant method (1.50 mg/L), $p < 0.001$. We found the N High Sensitivity CRP method is highly associated with the Tina-quant method (r=0.9916, Fig.3A). The slope (0.958; 95%CI = 0.954 to 0.962) was close to 1.00 with a positive intercept (0.280; 95%CI = 0.268 to 0.292). Although a high correlation coefficient was obtained, this does not mean necessarily that the methods gave identical results. We assessed the degree of agreement between the two methods using Bland-Altman analysis (Fig.3B). The mean of the difference was 0.19 mg/L and the limits of agreement (LOA) which encompass 95% of results were -0.36 to 0.74 mg/L. In addition, clinical concordances were assessed using the N High Sensitivity CRP method as the comparative method. The percentages of low, average, and high risk results were 42.9, 33.8, 23.3 for the N High Sensitivity CRP and 33.2, 41.1, 25.7 for the Tina-quant method (Fig.4). The percentage of concordantly classified subjects at each cut point was 99.8%, 75.7% and 89.9% (Table 3). In Figure 5, concordance for the three risk groups was assessed graphically. The overall agreement was 87.4%. 12.4% of subjects was discordantly classified into the higher risk group while the 0.2% of subjects was discordantly classified into the lower risk group by the Tina-quant method.

4.3 Comparison of risk stratification using combinations of two HDL-cholesterol methods and two high sensitivity C-reactive proteins assays in a large population study

The LDL concentration ranges from the LDL_{DB} and LDL_R methods were 0.20 to 10.49 mmol/L (8 to 405 mg/dL) and 0.16 to 10.38 mmol/L (6 to 401 mg/dL), respectively. The mean of LDL_{DB} [3.55 $\pm$ 1.14 mmol/L (137 $\pm$ 44 mg/dL)] was significantly higher than the mean of LDL_R [3.49 $\pm$ 1.14 mmol/L (135 $\pm$ 44 mg/dL)], $p < 0.001$. Deming regression analysis (Fig. 6A) shows a high correlation between both calculated LDL methods (r = 0.998). The slope (0.998; 95% CI = 0.996 to 1.001) of the regression line was close to 1 with a small negative intercept [-0.052 mmol/L (2.01

mg/dL); 95% CI = -0.06 to -0.04 (-2.32 to -1.54 mg/dL)] and the $S_{y/x}$ was 0.08 mmol/L (3 mg/dL). Agreement between the two LDL methods was assessed using Bland-Altman plots (Fig. 6B). The mean difference was -0.06 mmol/L (-2 mg/dL) with a standard deviation of 0.08 mmol/L (3 mg/dL). The 95% limits of agreement for the mean of the two estimated LDL difference were -0.22 to 0.10 mmol/L (-9 to 4 mg/dL). The ability of the two calculated LDL methods to classify subjects at each NCEP medical decision cut point was determined. The percentages of subjects classified into the low, average and high risk group by the LDL_{DB} and LDL_R methods are shown in Table 4. Additionally, clinical concordance was assessed graphically using the LDL_{DB} as the comparative method (Fig. 7). The overall concordance for risk classification was 94.8%. Only 4.7% and 0.5% of the subjects were discordantly classified into the lower and higher risk group, respectively. Next, we examined the LDL concentration at each hs-CRP cut points using the two hs-CRP methods as shown in Figure 8. The means of calculated LDL concentrations at each tertile of hs-CRP_{DB} were 3.36, 3.62, 3.79 mmol/L (130, 140, 146 mg/dL) for the LDL_{DB} and 3.31, 3.56, 3.73 mmol/L (128, 138, 144 mg/dL) for the LDL_R. For each tertile of hs-CRP_R, the means of LDL concentration were 3.30, 3.61, 3.78 mmol/L (127, 139, 146 mg/dL) for the LDL_{DB} and 3.25, 3.55, 3.72 mmol/L (126, 137, 144 mg/dL) for the LDL_R. We evaluated the percentage of the subjects assigned to nine risk groups according to the LDL cut points and the tertile of hs-CRP. The highest percentage of subjects were classified into the low risk groups for both LDL and hs-CRP (Fig. 9). When comparing the overall risk assessment of the various method combinations, the hs-CRP_R method classifies more subjects in the average risk group and fewer subjects in the low risk group than the hs-CRP_{DB} method. Table 5 shows the percentage of subjects assigned to each relative risk group by LDL and hs-CRP method combinations. At each relative risk level, the percentages of subjects for each method combination showed more variability as a result of hs-CRP method differences than that resulting from method differences in calculated LDL values, especially at the relative risks of 1.0 and 4.7-5.3.

CHAPTER 5
DISCUSSION

5.1 Comparison of two homogeneous HDL cholesterol methods in a large population study

In our study, method comparison studies of the two homogeneous HDL assays were performed with fasting specimens. We used the Dade method as the comparative method because it is certified by the CDC in our laboratory. There was a high correlation between the two assays. The methods show good agreement and the slope of the regression line was very close to the identity line but the Roche method did give slightly higher results than the Dade method. This is similar to reports by Arranz-Pena et al (44) and Nauck et al (97) which found correlation coefficients between 0.922 to 0.994 when a new homogeneous method were compared with a precipitation method and an ultracentrifugation method. The mean difference between the two methods in our study was close to zero. According to the ranges from both methods, the lowest result from Dade method is lower than the Roche method. The difference in the results may be due to a calibration difference. The calibration of Dade requires three concentrations of calibrator while that of the Roche requires only two. The possible reasons such as the difference of calibrator material, analyzer or detergent of each method may be the causes of different results. There were only 0.05% of the paired data (2 samples) that provided difference results larger than 0.50 mmol/L (19mg/dL). We are unable to explain the reason for these highly discrepant samples. To our knowledge, no studies have been published on the performance of homogeneous HDL methods using a large population. We found that the Dade method classified more participants into the high risk group than Roche method. The percentages of concordant classification was increased when the HDL concentration was increased. The concordance between methods is dependent on the HDL concentration and concordance improves as the HDL concentration increases. However, no subject was

discordant by more than one risk group and only 1.6% of results were discordant in the lowest risk group.

5.2 Comparative study of two automated high sensitivity C-reactive protein methods in a large population

Recent studies have shown that C-reactive protein can be used as a prognostic risk marker of cardiovascular disease (21, 144). This new clinical indication requires accurate and precise measurement of CRP at low concentration. For this reason, various hs-CRP assays have been developed and evaluated (29, 30, 32, 33). Our findings are similar to Roberts et al (r=0.997) and Hamwi et al (r=0.995) who found a good correlation of hs-CRP between the N High Sensitivity CRP and the Tina-quant CRP methods (30, 32). The Tina-quant CRP method gave hs-CRP results slightly higher than N High Sensitivity CRP assay with the mean difference >0.1 mg/L for the three lowest quartiles (30). The mean difference between the two methods in our study was close to zero. Most of the points lie within the LOA which encompass 95% of results were very low. However, our study demonstrated larger inter-method differences with increasing hs-CRP concentrations. The major causes for these discrepancies may be due to the inadequacies in calibration curve fitting, inaccurate assignment of values to assay calibrators as well as other factors related to individual samples. Although both assays are calibrated with the same reference material, significant differences still exist. The authors believed it to be related to standardization (170). Other authors have also described this issue and concluded that standardization efforts are needed (29, 30, 171). To address this issue, recent work by the CDC standardization committee on hs-CRP (Phase I) was performed (160) and continuing to the next phase. Other than calibration differences, the differences in methodology between nephelometric and turbidimetric systems may also yield different results (172). Another reason could be the result of different antibody avidities. Moreover, in our study, the Tina-quant CRP values tended to be lower than the N High Sensitivity CRP when CRP concentrations are increased. Data from the College of American Pathologist Proficiency Testing survey for the hs-CRP assay show that the Tina-quant CRP results tend to be higher than the N High Sensitivity CRP when CRP concentrations are increased (173).

Tarkkinen et al found lower concentrations with a noncompetitive microparticle CRP assay when compared with the N High Sensitivity CRP at concentrations >10 mg/L. This may due to the dilution factor used by the N High Sensitivity CRP assay; the hs-CRP is assayed at the initial sample dilution of 1:20. If the hs-CRP is higher than the upper limit of the analytic measurement range (10 mg/L), the samples have to be further diluted 1:100 or 1:400, which may have an effect on assay linearity (174).

For clinical concordance, the percentages of subjects in the average and high risk group obtained from the N high sensitivity method were lower than the Tina-quant method. Most of the participants were classified into the same tertile. Only 0.2% of subjects were discordant into the lower risk group and none of the subjects was discordant by more than one tertile. Our results were comparable to a previous study (30). However, we used the cut-points recommended by CDC/AHA consensus guidelines, whereas the earlier study used quartile cut-points. Hamwi et al found the Tina-quant method gave the best concordance with the N High Sensitivity method and no result differed by more than one quintile. Although agreement has been demonstrated, they still concluded that additional standardization work is needed, especially at the cutpoint values separating risk groups (32). It is noteworthy that 6.36% of results were greater than 10 mg/L. Increased concentrations of hs-CRP may due to the other causes. Therefore, these results were excluded. Values >10 mg/L are not uncommon but may confound use of this assay.

Race and ethnicity are the variables known to affect CRP results (167). Although there have been Japanese, American and European population-based studies of distribution of hs-CRP but the clinical implication from these studies is not indicate to the ethnic specific cutpoints for CRP (162-166). According to the tertile cut-points are derived from a Caucasian population (34). Thus, the chance for over- or under classification the risk of cardiovascular disease using these cut-points may be inappropriate for Asian populations. Further research to determine the utility of hs-CRP measurements for cardiovascular risk prediction in Asian populations and appropriate cutpoint values derived from these populations is needed.

5.3 Comparison of risk stratification using combinations of two HDL-cholesterol methods and two high sensitivity C-reactive proteins assays in a large population study

Several epidemiological studies and prospective clinical trials have reported that an increased LDL concentration is an important risk factor for CHD. According to the NCEP ATP III guidelines, a major goal of primary and secondary prevention for CHD is lowering LDL concentration (79). The most accurate method for determination of LDL is the β-quantification but this method is not applicable to routine analysis. Thus, the estimation of LDL using the Friedewald formula has been recommended as the routine method despite well established limitations (84). Recently, fully automated homogeneous HDL methods have been adopted and may decrease the inaccuracy of the LDL calculations. Bairaktari et al (175) found the homogeneous HDL assay has improved slightly the accuracy of the LDL calculation by the Friedewald formula and the estimated LDL values were correlated with the reference method ($r = 0.95$, $p<0.001$). The recent evaluation of four homogeneous LDL methods by Miller et al (176) found the agreement and imprecision of these methods and the Friedewald-calculated values were similar at TG concentrations up to 400 mg/dL (4.52 mmol/L) when compared with the reference method. In our study, both calculated LDL values using two homogeneous HDL methods were comparable. The slope of the regression line was very close to the identity line. Although the LDL_{DB} values were slightly higher than the LDL_R values, the mean difference between the two calculated LDL values was close to zero and most of the points lie within the 95% limits of agreement. The measurements of TC, TGs and HDL can affect the estimation of LDL. In our study only HDL measurements affected LDL results. Difference between the two homogeneous HDL assays could be due to the differences in calibrator material, calibration method, analyzer or detergents used by each method as discussed previously. According to the NCEP ATP III cutpoints, the LDL_{DB} method tended to classify more participants into the average and high risk group than the LDL_R. However, most subjects were classified into the same risk group and no subject was classified discordantly by more than one risk group. These results are similar to previous studies (108, 177, 178) which compared calculated LDL with the β-

quantification method and found 86-88% of the participants were classified correctly. Misclassification over two medical decision points was also rare.

Ridker et al (25) reported that baseline CRP concentrations adds to the prognosis value of lipid parameters in determining risk of first myocardial infraction and found that CRP is a stronger predictor of cardiovascular events than the LDL (27). As shown in the same study, increasing LDL concentrations were associated with increased risk of cardiovascular events at each tertile of hs-CRP, similar to increasing hs-CRP concentrations in all LDL risk groups. In our study, we found the means of both calculated LDL concentrations were increased for each higher risk group of hs-CRP. The percentages of subjects were assigned to each risk group when using various method combinations were different. The difference between hs-CRP methods influenced the percentages of subjects in each risk group more than differences due to LDL method differences. Ledue et al (170) suggested that the major causes for differences between hs-CRP methods may be due to inadequacies in calibration curve fitting, inaccurate assignment of assay calibrator values as well as other factors related to individual samples. Other authors also found significant differences even though assays are calibrated against the same reference material (29, 30, 171). The other possible explanation is a difference in the methodology (172).

Middleton (179) described the effect of analytical variation in hs-CRP, HDL and TC assays on cardiovascular risk assessment. This study found that imprecision of HDL methods affected risk classification more strongly than other variables and multiple measurements of HDL may reduce misclassification while replication of TC and hs-CRP assays was less important. In contrast, our data show that differences in hs-CRP methods had more effect on the assessment of relative risk classification than differences in calculated LDL values. However, Middleton used the TC:HDL ratio and hs-CRP quintile adapted from Rifai and Ridker (26) for the cardiac risk assessment while we used tertiles of calculated LDL and hs-CRP.

CHAPTER 6
CONCLUSIONS

In our study we compared the two homogeneous HDL assays, estimated LDL by the Friedewald Formula using the two different homogeneous HDL assays and the two hs-CRP methods. Their abilities to classify the subjects into the low, normal and high risk group were also assessed based on the cutpoints of these analytes. Finally, a combination of calculated LDL and hs-CRP for each combination of HDL and hs-CRP methods were used to compare the overall risk of CHD. The results are summarized as following:

1. Both homogeneous HDL assays from the Dade and Roche methods correlate and agree well. Either method can be used for population screening of HDL in large epidemiological studies. However, the Dade method classified a larger percentage of subjects into the high risk group than the Roche method.

2. Both N High Sensitivity CRP and Tina-quant CRP methods for hs-CRP are highly associated and suitable for screening large populations. The high discrepancies of the elevated CRP concentration results and some differences in their ability to classify the subjects into tertile cutpoints were exhibited. Once the standardization is improved, both methods can contribute useful for determining coronary risk stratification in the healthy population. The cutpoint values of hs-CRP for Asian population is needed.

3. Calculated LDL values obtained using two different homogeneous HDL methods correlated well and were suitable for screening large populations. Variations in population risk assessment due to differences in calculated LDL results are considerably less than those due to differences in hs-CRP methods. Some further standardization of hs-CRP methods appears to be necessary to minimize these variations.

CHAPTER 7
TABLES AND FIGURES

Table 1. Percentages of Classified Subjects in the Low, Normal and High Risk Groups Based on Categories Established in the Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

Classification	Percentage of subjects (%)	
	Dade Method	Roche Method
Low risk **HDL ≥ 1.55 mmol/L (60 mg/dL)**	15.5	19.3
Normal **HDL 1.03 - 1.54 mmol/L (40 - 59 mg/dL)**	55.4	59.3
High Risk **(HDL < 1.03 mmol/L) (40 mg/dL)**	29.0	21.4

Table 2. Classification of Subjects Based on Categories Established in the Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). The values presented as number of concordant subjects per total number of subjects in that category.

		Dade Method (mmol/L)		
		<1.03 (40)	**1.03-1.54 (40-59)**	**≥1.55 (60)**
Roche Method mmol/L (mg/dL)	**<1.03 (40)**	627/813 77.1%	186/813 22.9%	0/813 0%
	1.03-1.54 (40-59)	27/2498 1.1%	2109/2498 84.4%	362/2498 14.5%
	≥1.55 (60)	0/903 0%	41/903 4.5%	862/903 95.5%

Table 3. Classification of Subjects Based on Cut-points Recommended in the Centers for Disease Control and Prevention and the American Heart Association Consensus Guidelines

		N High Sensitivity CRP (mg/L)		
		<1.0	**1.0-3.0**	**>3.0**
Tina-quant (mg/L)	**<1.0**	1278/1280 99.8%	2/1280 0.2%	0/1280 0%
	1.0-3.0	378/1585 23.8%	1200/1585 75.7%	7/1585 0.5%
	>3.0	0/991 0%	100/991 10.1%	891/991 89.9%

Table 4. Percentages of Classified Subjects in the Low, Normal and High Risk Groups Based on Categories Established in the Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

Classification	Percentage of subjects (%)	
	LDL_{DB}	LDL_R
Low risk **LDL < 3.36 mmol/L** **(130 mg/dL)**	45.8	48.2
Average risk **LDL 3.36 – 4.13 mmol/L** **(130-160 mg/dL)**	26.2	25.6
High Risk **LDL > 4.13 mmol/L** **(160 mg/dL)**	28.2	26.2

Table 5. Percentages of Subjects Assigned to Each Relative Risk Group by Four LDL/hs-CRP Method Combinations

Relative Risk*	LDL and hs-CRP risk groups†	Percentages of subjects for each method combination			
		LDL_R/ $hs\text{-}CRP_{DB}$	LDL_{DB}/ $hs\text{-}CRP_{DB}$	LDL_R/ $hs\text{-}CRP_R$	LDL_{DB}/ $hs\text{-}CRP_R$
1.0	low LDL - low hs-CRP	24.1	23.0	19.2	18.4
1.7	average LDL - low hs-CRP low LDL - average hs-CRP	25.2	24.7	26.7	25.9
2.8-3.0	high LDL - low hs-CRP average LDL - average hs-CRP	18.3	19.4	17.4	18.2
4.7-5.3	high LDL - average hs-CRP average LDL - high hs-CRP low LDL - high hs-CRP	24.8	24.8	28.4	28.6
8.0	high LDL - high hs-CRP	7.6	8.1	8.3	8.9

*** Adapted from Reference [152].**

† Low, average and high risk groups are based on LDL-cholesterol categories established in the Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) [4] and high sensitivity C-reactive protein cutpoints recommended by CDC/AHA consensus guidelines [34].

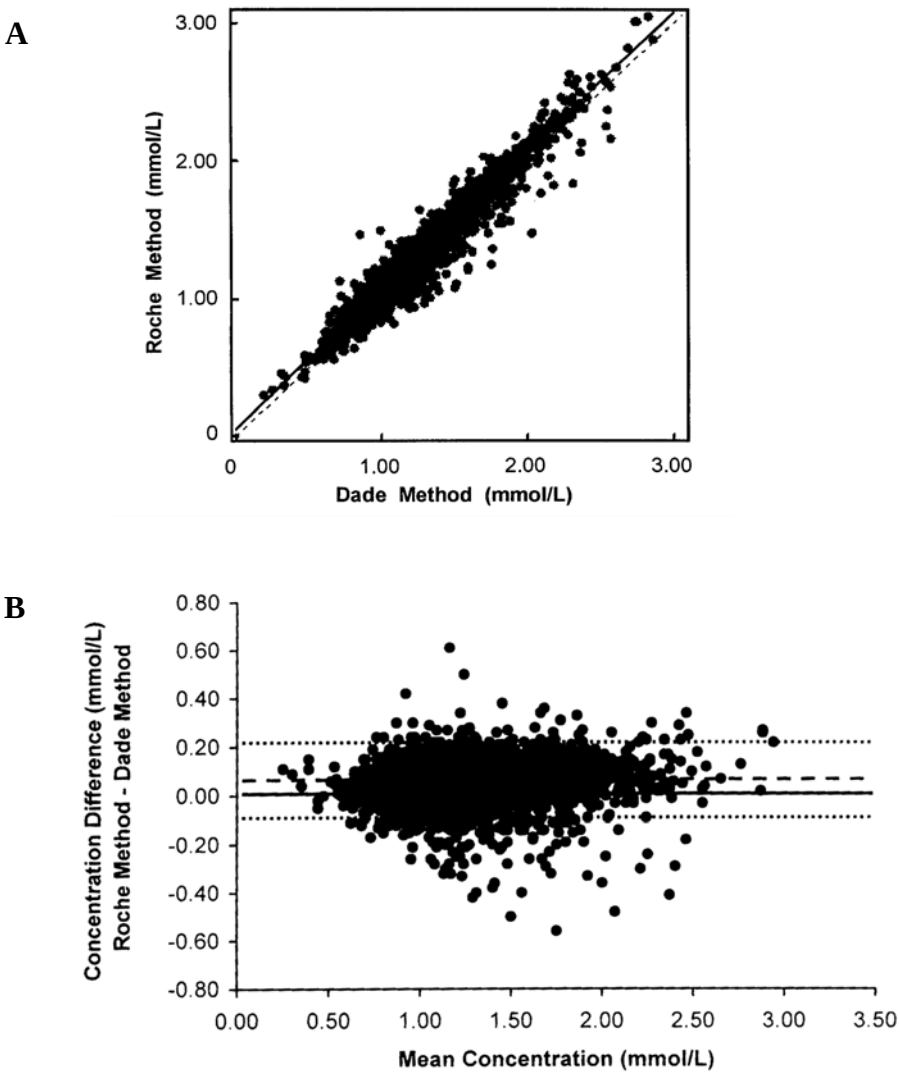


Figure 1. Comparison of the results between the Dade and Roche methods. A, Deming regression analysis. The dashed line represents the line of identity where as the solid line represents the Deming regression line. Slope = 1.009 (95% confidence interval, 1.002 to 1.016); intercept = 0.048 (95% confidence interval, 0.039 to 0.057); Sy/x = 0.08; r = 0.972; n = 4,214. B, Bland-Altman plot. The solid line indicates the zero line. The thick dashed line indicate the mean difference (0.06 mmol/L) and the thin dashed lines indicate the 95% confidence interval of the mean difference (-0.10 to 0.22 mmol/L).

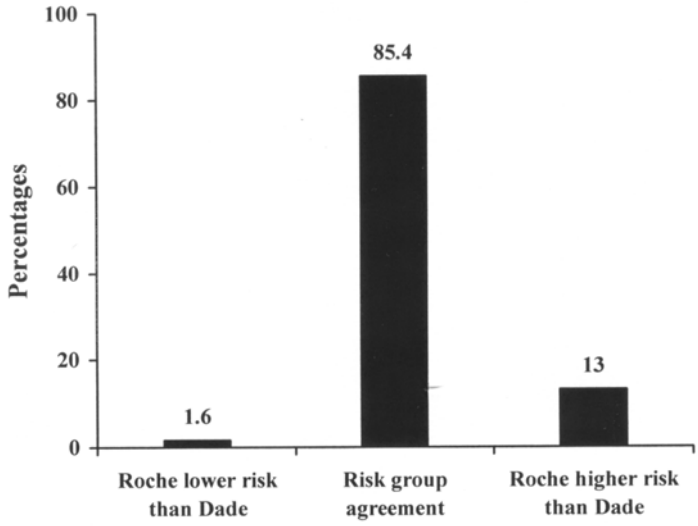


Figure 2. Agreement of Homogeneous HDL Methods According to NCEP Cut-points

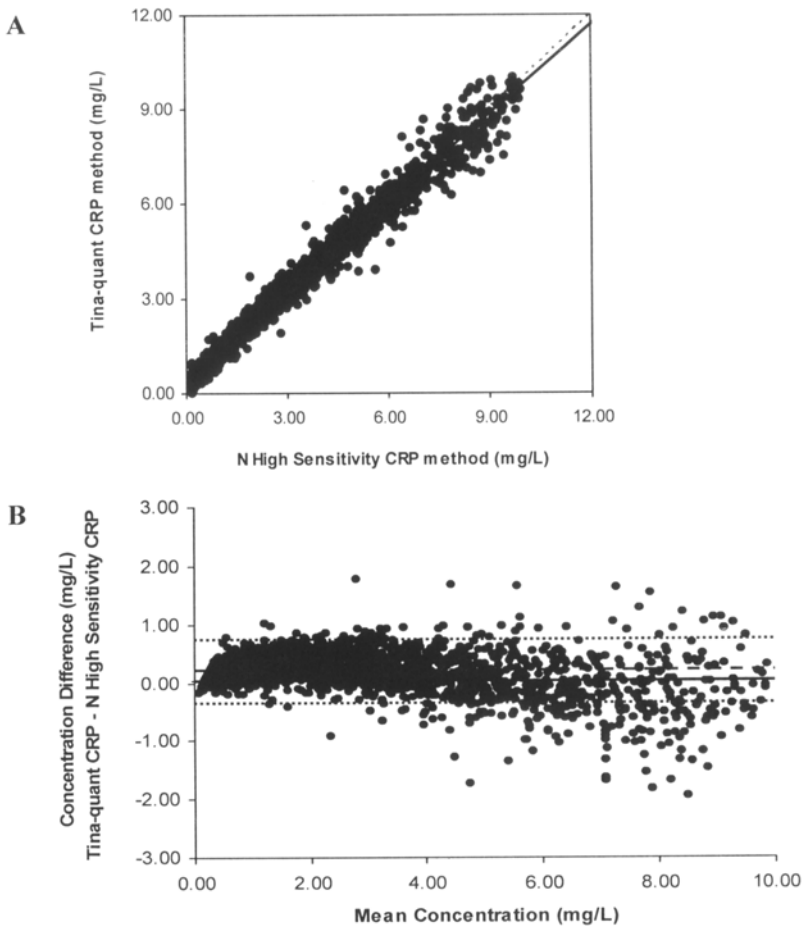


Figure 3. Comparison of the results from the N High Sensitivity CRP and Tina-quant methods. A, Deming regression analysis. The dashed line represents the line of identity where as the solid line represents the Deming regression line. Slope = 0.958 (95% confidence interval, 0.954 to 0.962); intercept = 0.280 (95% confidence interval, 0.268 to 0.292); $S_{y/x}$ = 0.264; r = 0.992; n = 3,856. B, Bland-Altman plot. The solid line indicates the zero line. The thick dashed line indicates the mean difference (0.19 mg/L) and the thin dashed lines indicate the limits of agreement (LOA; -0.36 to 0.74 mg/L).

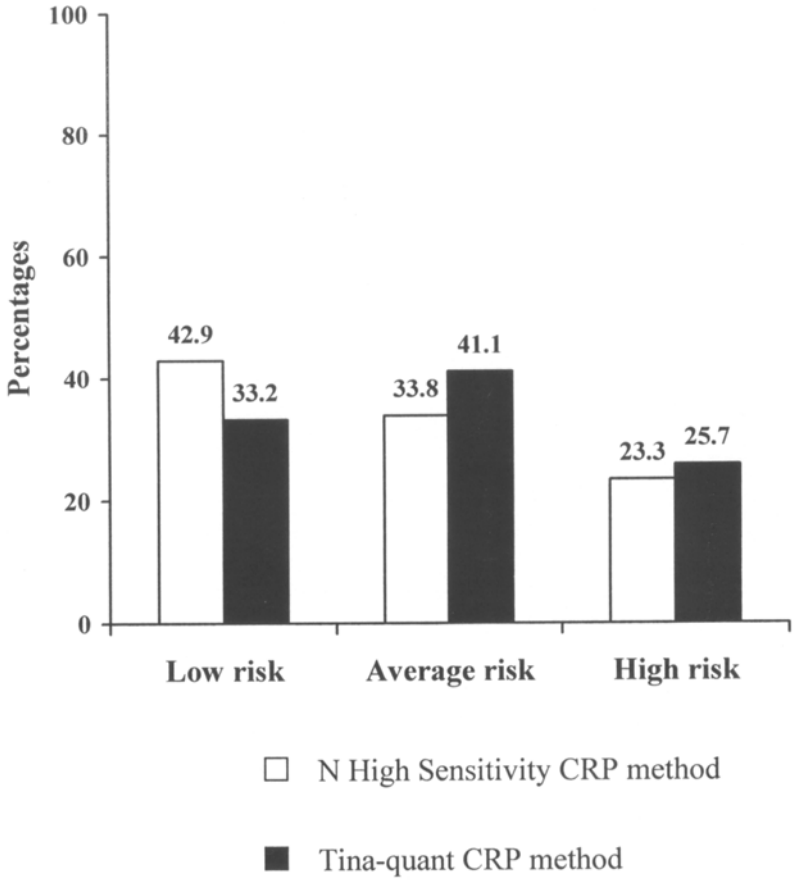


Figure 4. Percentages of Classified Subjects in the Low, Average and High Risk Groups Based on Cut-points Recommended in the Center for Disease Control and Prevention and the American Heart Association Consensus Guidelines

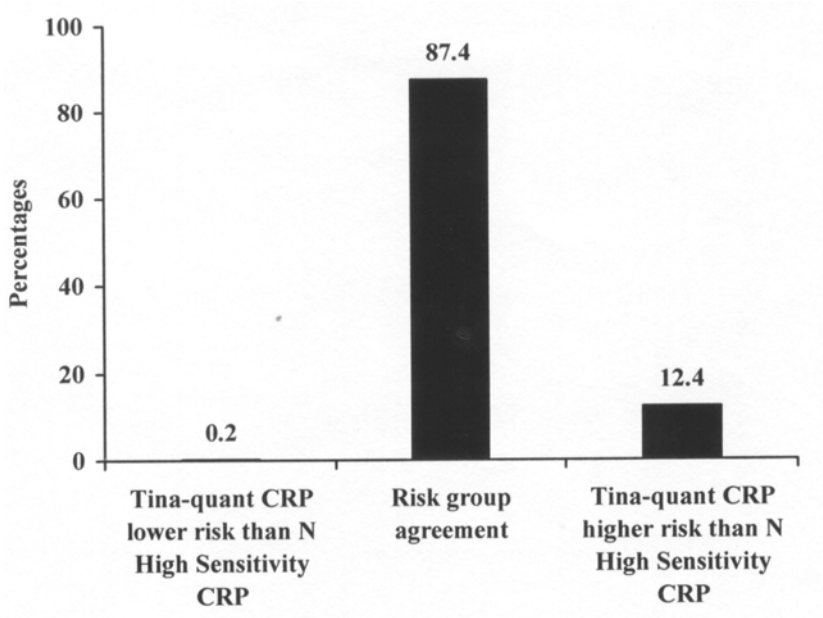


Figure 5. Agreement of Tina-quant CRP and N High Sensitivity CRP Methods According to CDC/AHA Cut-points

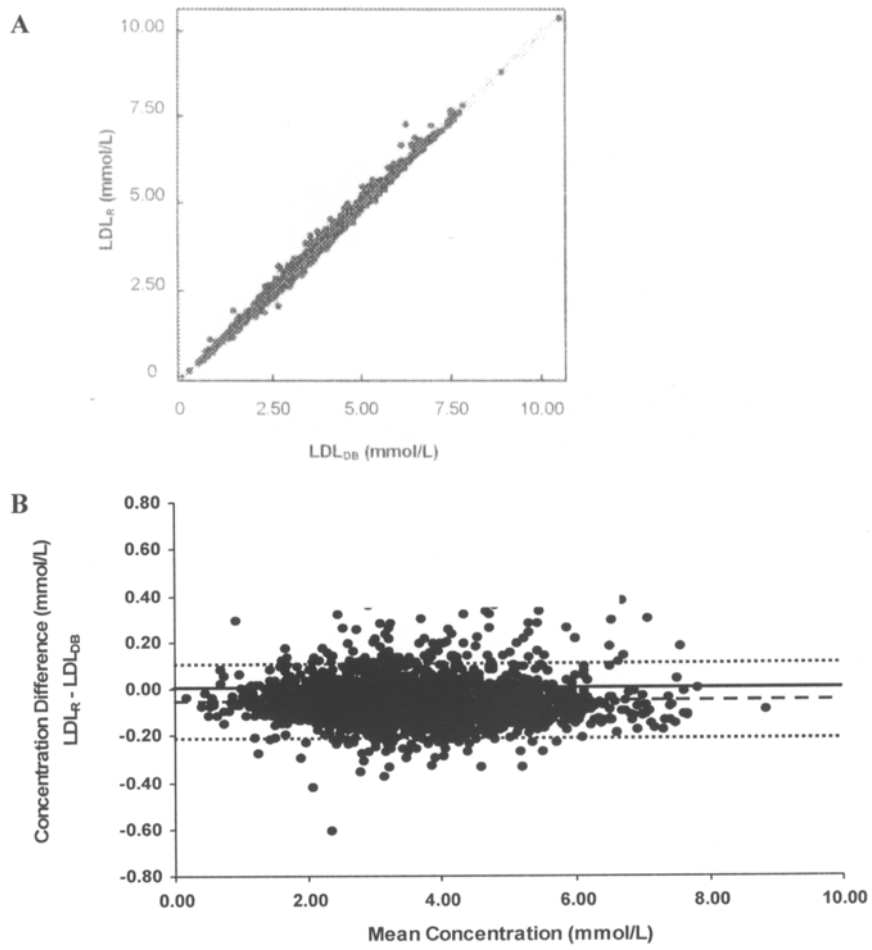


Figure 6. Comparison of the results between the LDL_{DB} and LDL_R. A, Deming regression analysis. The dashed line represents the line of identity where as the solid line represents the Deming regression line. Slope = 0.999 (95% confidence interval, 0.996 to 1.001); intercept = -0.05 mmol/L; 95% confidence interval, -0.06 to –0.04; $S_{y/x}$ = 0.08 mmol/L; r = 0.998; n = 3,728. B, Bland-Altman plot. The solid line indicate the zero line. The thick dashed line indicate the mean difference -0.06 mmol/L and the thin dashed lines indicate the 95% limits of agreement -0.22 to 0.10 mmol/L.

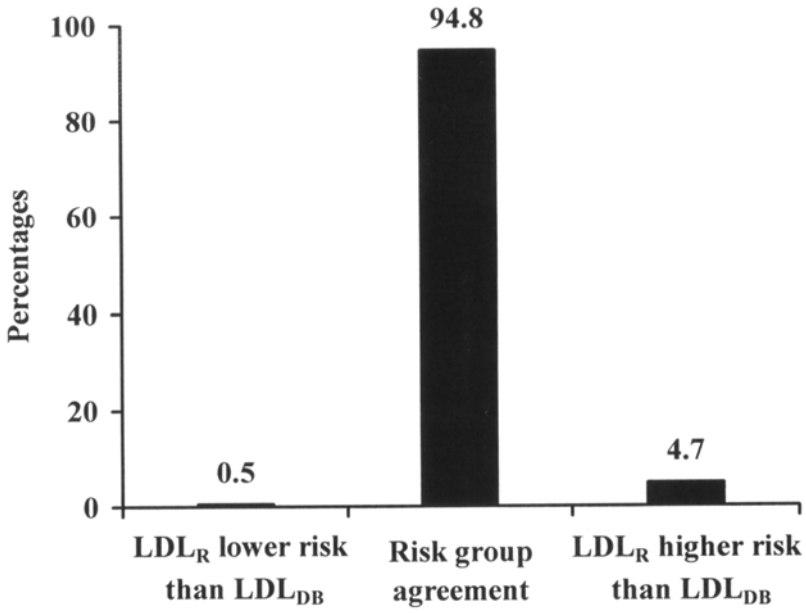


Figure 7. Agreement of Calculated LDL-C Results Based on the Treatment Targets Recommended by the NCEP ATP III Guidelines

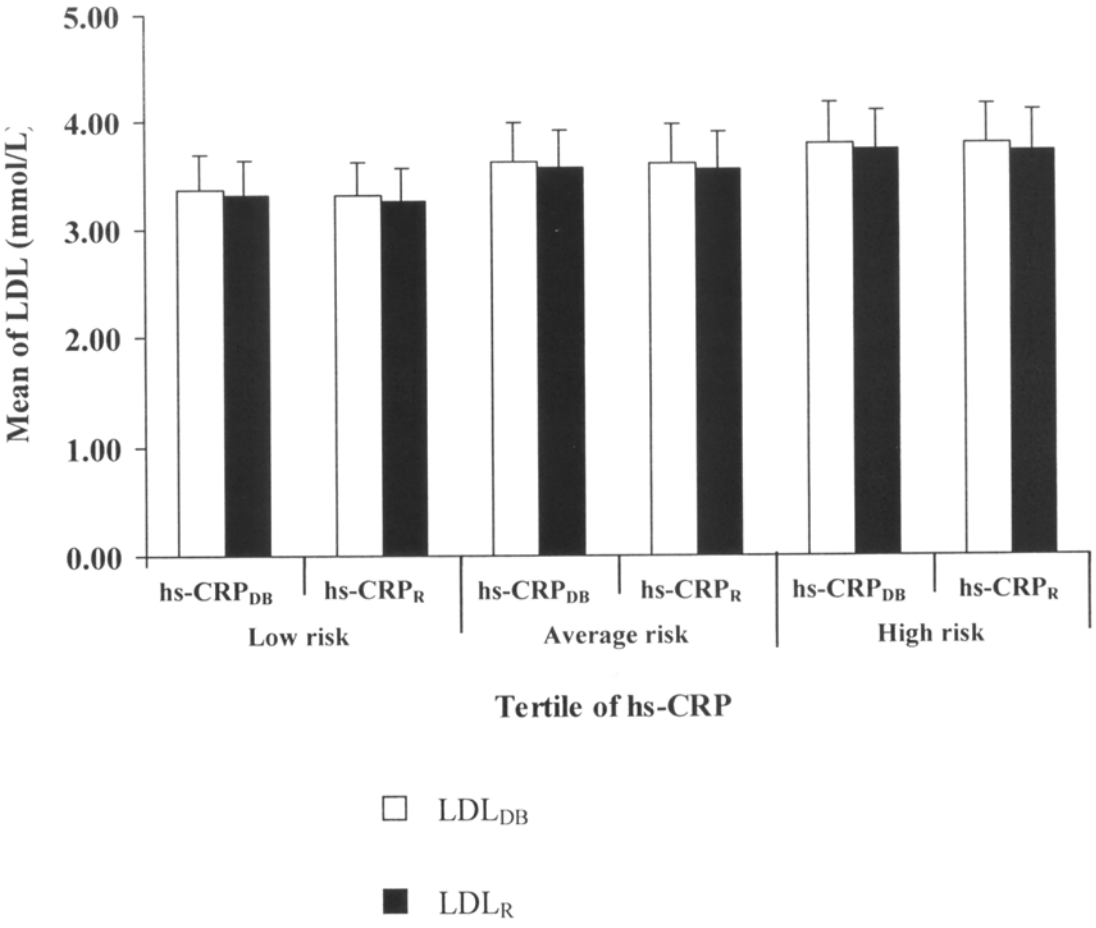


Figure 8. Means of Calculated LDL-cholesterol concentration According to the Tertile of hs-CRP

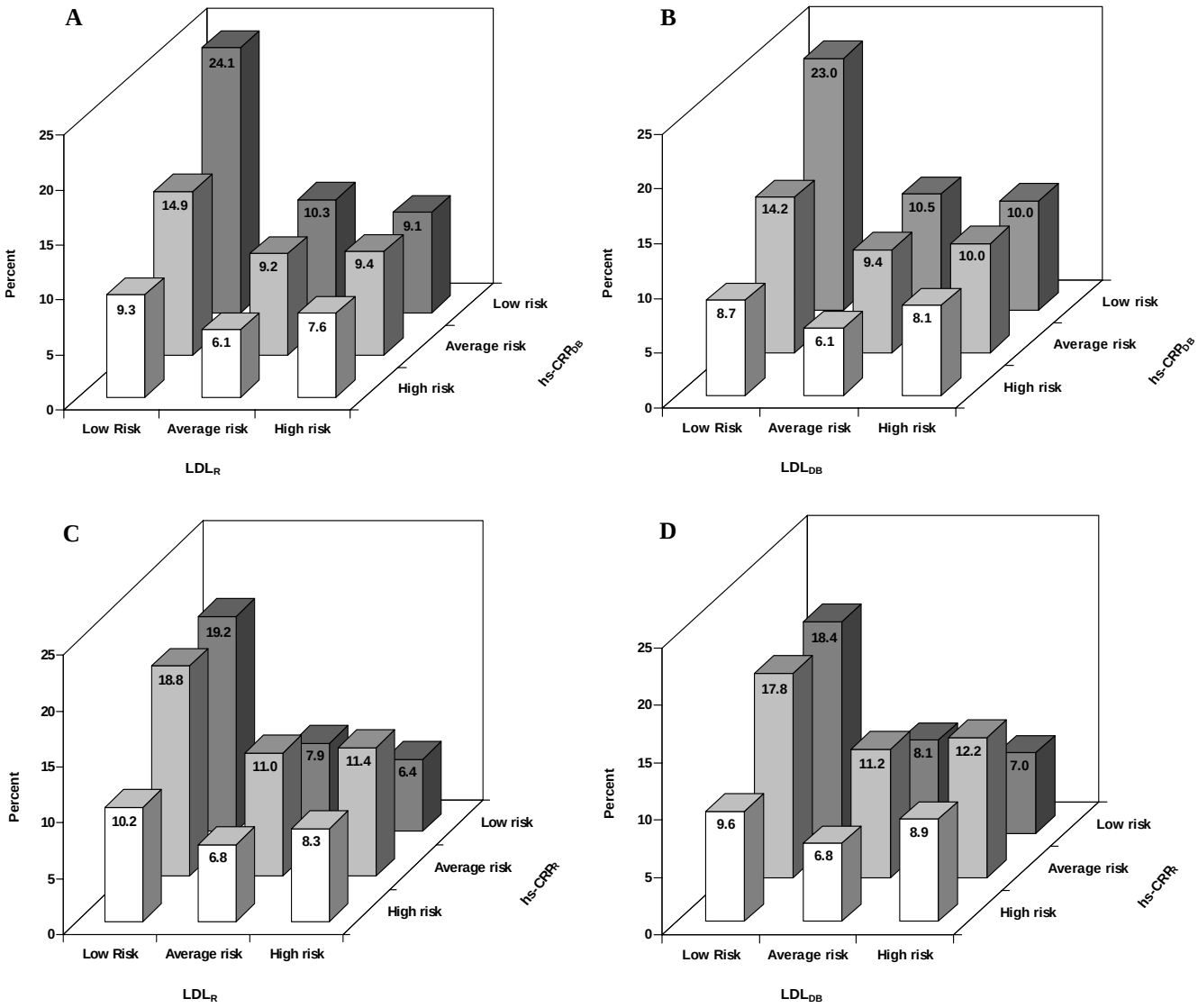


Figure 9. Percentages of Subjects Classified in the Low, Average and High risk Groups Based on LDL Categories Established in the Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel (ATP) III and Tertile of High Sensitivity C-Reactive Protein Recommended by the CDC/AHA Consensus Guidelines

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