

**ESTIMATION OF SMALL DENSE LDL-CHOLESTEROL FROM
CALCULATED AND DIRECT LDL-CHOLESTEROL**

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Thesis
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ESTIMATION OF SMALL DENSE LDL-CHOLESTEROL FROM CALCULATED AND DIRECT LDL-CHOLESTEROL

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ABSTRACT

Small dense low-density lipoprotein (sdLDL) particles are a powerful predictor of atherogenesis. However, most sdLDL methodologies are expensive, time consuming and technically demanding, making them too laborious for routine clinical practice. Calculated low-density lipoprotein cholesterol (cLDL-C) may differ from direct measurement (dLDL-C), and this difference may depend on the presence of sdLDL particles in addition to variation in triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) concentrations. The presence of such dependence would offer a simple means to estimated sdLDL.

We measured glucose, creatinine, total cholesterol, TG, HDL-C, and dLDL-C using standardized methods (n = 297). For sdLDL cholesterol (sdLDL-C), a novel homogeneous assay was used. The cLDL-C was calculated using the Friedewald formula for 220 subjects after excluding for liver or renal disease. Using stepwise regression analysis to predict sdLDL-C we identified non-HDL-C, cLDL-C, and dLDL-C as significant variables ($P < 0.001$; $R^2 = 0.88$). The regression equation was $\text{sdLDL-C (mmol/L)} = 0.575(\text{NonHDL-C}) + 0.417(\text{dLDL-C}) - 0.724(\text{cLDL-C}) - 0.306$.

The sdLDL-C concentration can be estimated from non-HDL-C, dLDL-C, and cLDL-C to provide a cost-effective method for screening patients for the risk of cardiovascular disease. Moreover, identification of a simple, inexpensive marker for sdLDL particles may pre-select patients who would most benefit from a more definitive subfraction workup.

**KEY WORDS: SMALL DENSE LDL CHOLESTEROL / NON-HDL CHOLESTEROL /
LOW DENSITY LIPOPROTEIN CHOLESTEROL/ LIPOPROTEIN
SUBCLASS/ CARDIOVASCULAR DISEASE / ESTIMATED
CALCULATED SLDL-C**

88 pages

การประมาณค่า small, dense LDL-Cholesterol จากผลการทดสอบ LDL-Cholesterol ที่ได้จากวิธีการคำนวณ และวิธีการตรวจวัดโดยตรง

ESTIMATION OF SMALL DENSE LDL-CHOLESTEROL FROM CALCULATED AND DIRECT LDL-CHOLESTEROL

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บทคัดย่อ

Small, dense low density lipoprotein (sdLDL) เป็นไขมันชนิดสำคัญที่ก่อให้เกิดโรคหลอดเลือดแข็ง อย่างไรก็ตามวิธีการตรวจวิเคราะห์ sdLDL มักต้องอาศัยเทคโนโลยีขั้นสูงที่มีขั้นตอนยุ่งยากซับซ้อน เป็นเหตุให้มีค่าใช้จ่ายสูงมากและไม่สามารถนำมาประยุกต์ใช้ในงานประจำวันได้ วิธีทดสอบหาระดับ low density lipoprotein-cholesterol (LDL-C) ที่นิยมใช้คือวิธีตรวจวัดโดยตรง (dLDL-C) และวิธีคำนวณตามสมการของ Friedewald (cLDL-C) ความคลาดเคลื่อนระหว่างวิธีทั้งสองจะแปรตามความเข้มข้นของ triglycerides (TG) ที่สูงขึ้นและ high density lipoprotein cholesterol (HDL-C) ที่ลดต่ำลง สอดคล้องกับระดับ sdLDL ที่มีแนวโน้มจะพบสูงในผู้ที่มีระดับไขมันในเลือดผิดปกติรูปแบบนี้เช่นกัน จึงอาจประมาณค่า sdLDL-C ในเลือดได้จากค่าความคลาดเคลื่อนของผลการทดสอบ LDL-C

การศึกษานี้ได้ทำการทดสอบวัดระดับ glucose, creatinine, total cholesterol, TG, HDL-C, dLDL-C โดยใช้วิธีมาตรฐาน ในตัวอย่างจำนวน 297 ราย สำหรับ sdLDL-C ตรวจวัดโดยใช้ชุดน้ำยาสำเร็จรูป ค่า cLDL-C คำนวณจากสมการของ Friedewald พัฒนาสูตรคำนวณค่า sdLDL-C โดยใช้สถิติ stepwise regression จากตัวอย่างทั้งสิ้น 220 รายหลังจากคัดตัวอย่างที่มีความผิดปกติจากโรคตับและโรคไตออก พบว่า non-HDL-C, cLDL-C และ dLDL-C เป็นตัวแปรที่มีความสัมพันธ์อย่างนัยสำคัญ ($p < 0.001$) ดังสมการ sdLDL-C (mmol/L) = $0.575 (\text{nonHDL-C}) + 0.417 (\text{dLDL-C}) - 0.724 (\text{cLDL-C}) - 0.306$ โดยค่าสัมประสิทธิ์ความถดถอย (R^2) เท่ากับ 0.88

การประมาณค่า sdLDL-C ในเลือดสามารถคำนวณได้จากค่า non-HDL-C, dLDL-C และ cLDL-C วิธีคำนวณค่า sdLDL-C นี้ น่าจะเป็นเครื่องมือที่มีประโยชน์ต่อการตรวจคัดกรองผู้ป่วยที่มีความเสี่ยงต่อการเกิดโรคหลอดเลือดและหัวใจ ยิ่งไปกว่านั้นยังอาจใช้เป็นแนวทางสำหรับการตรวจวิเคราะห์หา sdLDL โดยวิธีที่มีความแม่นยำถูกต้องต่อไป

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LIST OF ABBREVIATIONS

AHA	American Heart Association
BMI	Body mass index
CVD	Cardiovascular disease
CHD	Coronary Heart disease
CMs	Chylomicron
CE	Cholesterol esters
CETP	Cholesterol ester transfer protein
CKD	Chronic kidney disease
CO	Cholesterol oxidase
cLDL	Calculated low density lipoprotein
dLDL	Direct low density lipoprotein
DM	Diabetes mellitus
ESRD	End stage renal disease
eGFR	Estimated glomerular filtration rate
FFAs	Free fatty acids
GK	Glycerol kinase
HDL-C	High density lipoprotein cholesterol
HL	Hepatic lipase
HPO	Hydrogen peroxidase
H ₂ O ₂	Hydrogen peroxide
HK	Hexokinase
IDL-C	Intermediate density lipoprotein cholesterol
IR	Insulin resistance
IHD	Ischemic heart disease
LDL-C	Low density lipoprotein cholesterol
LPL	Lipoprotein lipase
lbLDL	Large buoyant low density lipoprotein

LIST OF ABBREVIATIONS (cont.)

MI	Myocardial infarction
MS	Metabolic syndrome
mmol/L	Milimole per liter
NO	Nitric oxide
PDGF	Platelet-derived-growth factor
PL	Phospholipids
PCOS	Polycystic ovary syndrome
POD	Peroxidase
SMCs	Smooth muscle cells
sdLDL-C	Small, dense lowdensity lipoprotein cholesterol
TC	Total cholesterol
TG	Tryglyceride
UC	Unesterified cholesterol
VLDL-C	Very low density lipoprotein cholesterol
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Cardiovascular disease (CVD) is a major cause of disability and premature death throughout the world (1). Based on data from the Bureau of Health Policy and Strategy, Ministry of Public Health, Thailand, statistics of 2007 revealed that 901 per 100,000 patients suffered from heart disease and 55.3 per 100,000 patients died from this cause and the ratios are going up every year (2). Therefore, several national and international projects have established to study the level of major cardiovascular risk factor including age, gender, smoking status, hypertension, diabetes and dyslipidemia (3).

Dyslipidemia is a term used to describe disorder of lipid metabolism. A lipid profile including total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, and low density lipoprotein (LDL) cholesterol are well accepted and widely used for the assessment of CVD and its equivalence (4). The lipoproteins comprise a heterogeneous spectrum of particles that differ in size, density, electrophoretic mobility and relative lipid-protein proportions. For LDL particles, they are fractionated according to size and density into two major phenotypes: pattern A, with a -higher proportion of large, buoyant LDL particles (lbLDL), with peak particle diameter ≥ 25.5 nm (density 1.019 – 1.044 g/ml) and pattern B, characterized by predominance of small dense LDL particles (sdLDL), with peak particle diameter < 25.5 nm (density range, 1.044 – 1.060 g/ml) (5-7). The sdLDL particles are believed to be a more atherogenic compared with lbLDL particles. Its particles have clearly shown a higher penetration into the arterial wall, a higher affinity to the intimal proteoglycans, a prolonged plasma half-life, a lower binding affinity for LDL receptor, and a lower resistance to oxidative stress (7-9).

Numerous studies have reported a 2- to 3- folds increase in coronary heart disease risk among patients with the predominance of sdLDL (10, 11). The Quebec cardiovascular study has confirmed that a greater proportion of sdLDL at baseline is a

strong and independent predictor of CHD in the first 7 years of follow up (12). The predominance of sdLDL directly correlates with serum triglyceride and inversely correlates with HDL-C. This combined lipid abnormality has been designated the atherogenic lipoprotein phenotype which plays an important role in the development of atherosclerosis. Moreover, many evidences suggest that an increased production of the hepatic triglyceride-enriched large very low density lipoprotein (VLDL), which is following generation of sdLDL, is an important and early complication of the hepatic insulin resistance. Insulin resistance has a central role in the pathophysiology of metabolic syndrome which promotes the progression to type 2 diabetes mellitus (DM type 2), atherosclerotic cardiovascular disease and other abnormalities such as fatty liver and some type of cancer (13). A recent finding also showed that women with polycystic ovary syndrome have significant qualitative LDL alterations, with increased level of atherogenic sdLDL particle (14). Thus, sdLDL may represent a valuable marker for diagnosis and severity of the metabolic syndrome.

Several experimental and clinical trials indicate that the elevated LDL-C is the major cause of CHD (15, 16). For these reasons, the NCEP Adult Treatment Panel III has recommended to identify elevated LDL-C as the primary target of cholesterol lowering therapy (17). Simplified methods to determine LDL-C concentration have been implanted in the clinical laboratories such as an electrophoresis, a homogeneous enzymatic assay and a calculation by using the Friedewald equation (18). The most common approach for determining LDL-C concentration is the Friedewald calculation (cLDL-C), which estimates LDL-C from measurements of total cholesterol (TC), TG, and HDL-C (19) and the directly measurement (dLDL-C) (20). However, the calculation method cannot be accurately estimated when plasma triglycerides ≥ 4.52 mmol/l (400 mg/dl) or in the presence of chylomicron or type III hyperlipoproteinemia (21). Recently, several direct measurements of LDL-C levels have been developed by different manufacturers. The direct method can offer several advantages over the Friedewald calculation including measurement in the non-fasting state, reduce the influence and variance from plasma triglyceride concentration (22, 23). The difference between the cLDL-C and the dLDL-C has been ascribed to variation in triglycerides and HDL-C concentration.

Problems

Although several methodologies have been developed for the assessment of sdLDL particles such as density gradient ultracentrifugation (24-26), non-denaturing gradient gel electrophoresis (27), tube gel electrophoresis (28) and nuclear magnetic resonance spectroscopy (29), all methods are expensive, time consuming and technically demanding, making them too laborious for routine clinical practice. Recently, the measurement of cholesterol carried of sdLDL (sdLDL-C) by a novel homogenous enzymatic assay (sLDL-EX “SEIKEN”;Denka Seiken, Japan) has been developed (30). This method is applicable to routine clinical examination and allows a rapid measurement of a large number of samples. However, it is still expensive (the reagent cost > 500 bath/test).

Several studies indicated that sdLDL concentration directly correlates with serum TG and inversely correlates with HDL-C. The difference between the cLDL-C, and the dLDL-C, has been described to variation in triglycerides, HDL-C, and potentially presence of sdLDL. We hypothesized that one could use the difference between the calculated and directly measured LDL to estimate the sdLDL-C. If the sdLDL-C concentration can be estimated from the TC, HDL-C, cLDL-C and dLDL-C, it will provide a cost-effective method for screening patients for the risk of cardiovascular disease. Moreover, the identification of a simple inexpensive marker for sdLDL particles may pre-select patients who would most benefit from a more definitive subfraction workup.

CHAPTER II

OBJECTIVES

Small dense low density lipoprotein particles are a powerful predictor of atherogenesis. However, most sdLDL methodologies are expensive, time consuming and technically demanding, making them too laborious for routine clinical practice.

From the above problem, this study was aimed to solve this problem by propose of the objectives are

1. to develop an equation for the estimation of sdLDL-C by using classic lipids usually measured in routine clinical laboratory.
2. to study the relationships between plasma levels of sdLDL-C and other lipid parameters including TC, TG, HDL-C and LDL-C.

CHAPTER III

LITERATURE REVIEW

3.1 Incidence of Cardiovascular Disease

Cardiovascular disease (CVD) and stroke have been the most important public health problems world-wide (31) and major causes of global morbidity and mortality (32). These diseases are estimated to be the first and second leading causes of death in the world today and expected to remain so by the year 2020 (33). In 2009, over 180,000 people died from CVD in the United Kingdom, one in three of all deaths (34). Based on data from the Bureau of Policy and Strategy, Ministry of Public Health, Thailand, statistics of 2009 revealed that 475.84 per 100,000 patients suffered from CVD and 20.78 per 100,000 patients died from this cause and the ratios are going up every year (35). Clinical cardiovascular events such as acute myocardial infarction are believed to result from unstable atherosclerotic plaques that rupture, leading to development of thrombosis and acute occlusion of a critical artery (36). Atherosclerosis also occurs in other blood vessels, such as the carotid artery, which carries blood to the brain, or the arteries that provide blood to the legs, and can lead to similar problems (37). Complications of atherosclerosis are considered the leading cause of death and permanent disability in the world.

3.2 Pathophysiology of Atherosclerosis

In the present, many patients have CVD which most patients have pathogenesis of atherosclerosis. Atherosclerosis occurs due to chronic inflammation of the arterial blood vessel. The signs of inflammation occur from incipient lipid accumulation in the arterial wall. The depositions of lipid debris on the arterial wall then lead to impaired function of endothelial cells (38). Endothelial dysfunction is associated with a reduction in availability of nitric oxide (NO), decrease flow-induced vasodilation, and release of platelet-derived growth factor (PDGF) (39-40). NO is a

powerful inhibitor of platelet aggregation on endothelial cell and it can reduce inflammatory cell recruitment into the intima (41). Endothelium defect initiates nitric oxide reduction of NO. Endothelial cells begin to express on their surface selective adhesion molecule that bind to various classes of leukocytes such as the monocytes and T lymphocytes (42). Once present in the intima, monocytes differentiate into macrophages under the influence of chemokines. Macrophages become foam cells which stimulate a variety of proinflammatory, cytokine, and growth factors that contribute beneficially to the evolution of the plaques. Moreover, augmented wall stresses may also promote the production by vascular smooth muscle cells (SMCs) of proteoglycans that can bind and retain lipoprotein particles, especially small dense LDL (sdLDL) which can be easily oxidized (43). Thus, the activated leucocytes and intrinsic arterial cells can release growth factors that promote replication of SMCs and infiltration of oxidized low density lipoprotein (oxLDL) can promote an inflammatory response at site of lesion formation (44-45).

Myocardial infarction (MI) occurs setting from a diminished blood supply to the heart exceeds a critical threshold and overwhelms myocardial cellular repair mechanisms designed to maintain normal operating function and homeostasis. MI is related to progressive atherosclerosis with increasing occlusion of coronary arteries (46). The progressive luminal narrowing from continued growth of SMCs in the plaques was the main cause of infarction.

3.3 Pathogenesis of Atherosclerosis

The pathophysiology of atherosclerosis is occurring from the accumulation of LDL-C within the circulation, especially, sdLDL-C is easily oxidized by free radical. Moreover, sdLDL particles are inherently more atherogenic than large LDL particles (47). The distinctive properties of the sdLDL-C are small sizes, poorly bound to the LDL receptor (48), prolonged residence time in the plasma (49), and low resistance to oxidative stress (50). OxLDL is capable of wide range of toxic effects and it causes vessel wall dysfunctions which are characteristically and consistently associated with the development of atherosclerosis. OxLDL penetrates into the arterial wall more easily. When it is inside the cell wall, it will stimulate the

macrophage to catch it by scavenger receptor. Once the macrophage get oxidized LDL in the bigger amount, they become foam cell which are in the intimal layer of blood vessels. The foam cells are seen as fatty streaks. When the fatty streaks become bigger, it causes endothelial injury. Platelet adherences are stimulated that releasing cytokine such as PDGF. PDGF which stimulate cell proliferation, especially the smooth muscle cells in the intimal layer. Therefore, the lesion of atherosclerosis will progress.

3.4 Atherosclerosis Risk Factors

The Framingham Heart Study demonstrated the risk factors predisposed to atherosclerosis and resultant ischemic heart disease (51). They established older age, male sex, hyperglycemia, hypertension, dyslipidemia, and smoking as the major risk factors for coronary heart disease (CHD) (51). Following advances in our understanding of the pathogenesis of atherosclerotic vascular disease have stimulated interest in the so-called novel risk factors for CHD (52). Risk factor assessment is an important first step in primary prevention and guides the intensity of effort to reduce a patient's CHD risk. Currently, the National Cholesterol Education Program's (NCEP) Adult Treatment Panel III (ATP III) guidelines were recommended 2 algorithms for the assessing cardiovascular risk (17). The first algorithm concerns counting major risk factors and then estimating the 10-year probability of CHD based on an equation obtain from the Framingham Heart Study (51). The second algorithm identifies the presence of metabolic syndrome (MS) (53). An American Heart Association (AHA) Prevention Conference statement in 1999 classified risk factors into 3 categories as shown in Table 3.1 (54). The conventional risk factors appear to have a direct causal role in atherogenesis. Predisposing factors, including obesity, family history of early-onset CHD, and sedentary lifestyle, mediate some risk through the causal factors but may also have independent effects (55).

Table 3.1 Categories of risk factors for Coronary Heart Disease (CHD)

Conventional	Predisposing	Conditional
Cigarette smoking	Overweight and obesity	Homocysteine
Elevated blood pressure	Physical inactivity	Fibrinogen
Elevated serum cholesterol	Male sex	Lipoprotein (a)
Low HDL-C	Family history of early-onset CHD	Small LDL particle size
DM	Socioeconomic factors	C-reactive protein
	Behavioral factors	
	Insulin resistance	

Source: Smith SC, Greenland P, Grundy SM. Prevention Conference V: Beyond Secondary Prevention: Identifying the High-Risk Patient for Primary Prevention: Executive Summary. *Circulation*. 2000;101(1):111–116.

3.5 Dyslipidemia

Dyslipidemia is a major determinant of atherosclerosis and is concomitantly associated with premature CVD and all-cause mortality (56). The AHA (54) and the NCEP (ATP III) (17) have each issued recommendations designed to identify more people who are asymptomatic and clinically apparently free of CHD. These recommendations are specific risk factors, including total cholesterol (TC), LDL-C and high-density lipoprotein (HDL) cholesterol, that are typically used in risk prediction algorithms, such as the Framingham risk score (57), to estimate a global risk assessment for CVD. Emerging evidence indicates that the difference of lipoprotein particle size may play an important role in the manifestation of the atherogenic potential of a given lipoprotein particle (58-59). Specifically, the accumulation of sdLDL and HDL particles is associated with increased risk for atherosclerosis and premature CVD (10, 60).

3.6 Lipoproteins

The lipoprotein particles are composed of core containing triglyceride (TG) and cholesteryl esters (CE) and surface containing apoprotein, phospholipids and unesterified cholesterol (61-62). Lipoproteins comprise a heterogeneous

spectrum of particles that differ in size, density, electrophoretic mobility and relative lipid-protein proportions. There are primarily classified as chylomicrons, very low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL and HDL as demonstrated in Table 3.2 (63).

Table 3.2 Lipoprotein classification

Variable	CM	VLDL	IDL	LDL	HDL
Density (g/mL)	< 0.95	0.95 – 1.006	1.006 – 1.019	1.019 - 1.063	1.063 – 1.210
Electrophoretic mobility	Origin	Pre-beta	Between beta and pre-beta	Beta	Alpha
Molecular weight (daltons)	0.4 -30 x 10 ⁹	5 – 10 x 10 ⁶	3.9 – 4.8 x 10 ⁶	2.75 x 10 ⁶	1.8 – 3.6 x 10 ⁵
Diameter (nm)	> 70	25 -70	22 - 24	19 - 23	8 - 12
Major lipids	Exogenous TGs	Endogenous TGs	Endogenous TGs, CEs	CEs	Phospholipids, CEs
Major proteins	A-I, 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

Source: Saland JM, Ginsberg HN. Lipoprotein metabolism in chronic renal insufficiency. *Pediatric nephrology* (Berlin, Germany). 2007;22(8):1095–1112.

3.6.1 Chylomicrons

Chylomicrons (CMs) are produced in the intestinal lumen following the absorption of digested fat. They are the largest lipoprotein and are rich in TG. CMs are transported in the blood to tissues such as skeletal muscle, fat, and the liver. The capillary beds of these tissues contain high concentrations of lipoprotein lipase (LPL). LPL hydrolyzes TG in the CMs into free-fatty acids that are either oxidized by the muscle cells to generate energy, stored in adipose tissue, oxidized in the liver, or used in hepatic VLDL synthesis (64). Once the CMs have been processed by LPL, the TG-depleted CMs are called a remnant particle, which is then transported to the liver for further processing.

3.6.2 Very Low Density Lipoprotein

VLDL is a lipoprotein particle similar to CMs. It contains a high concentration of TG. VLDL is synthesized from free-fatty acids formed in the catabolism of CM in the liver, or from endogenous production of TG (65). The TG component of VLDL also undergoes hydrolysis by capillary LPL to provide fatty acids to adipose and muscle tissue. The remaining lipid portion is called IDL. IDL is then converted to LDL by enzymatic action of hepatic lipase or is taken up by the liver via the LDL receptor.

3.6.3 Intermediate Density Lipoprotein

Intermediate density lipoprotein (IDL) is one of the five major groups of lipoprotein that enable fats and cholesterol to move within the water-based solution of the bloodstream. IDLs belong to the lipoprotein particle family and are formed from the degradation of very low density lipoproteins. Their size is, in general, 25 to 35 nm in diameter, and they contain primarily a range of triacylglycerols and CE. There are usually cleared from the plasma into the liver by receptor-mediated endocytosis, or further degraded to form LDL particles (66). In general, IDL, somewhat similar to LDL, transports a variety of TG and TC which can also promote the growth of atheroma. Several studies showed that increases IDL – C were associated with atherosclerosis (67-68).

3.6.4 Low Density Lipoprotein

Low density lipoprotein (LDL) particles contain a core of CE, lesser amounts of TG, and a single molecule of apolipoprotein B-100, which is the ligand for binding to the apo B/E receptor. The structural changes that occur during the conversion of VLDL to IDL and from IDL to LDL, Apo B expose a domain that can interact with LDL receptors. LDL is the carriers which transport TC in the circulation and to the other organs. Liver have specific apo B receptors, thus, enhanced uptake of TC from the circulation and a reduction in the plasma cholesterol concentration. Circulating LDL can also enter macrophages and some other tissue through the unregulated scavenger receptors. This pathway can result in excess

accumulation of intracellular cholesterol and the formation of foam cells which contribute to the formation of atherosclerotic plaques (69).

In addition, the heterogeneity of LDL particle sizes, due to differences in the amount of cholesterol per particle, suggests that the particle size is an important consideration in the atherogenic potential of the LDL. The study of Austin et. al. and Lamarche et. al. suggested that high levels of sdLDL particles are predictive of an increased risk of CHD (59,70).

3.6.5 High Density Lipoprotein

High density lipoprotein (HDL) is the smallest of the lipoprotein particles. It contains a lipid core of CE and TG surrounded by phospholipids and apolipoprotein apo A-I and apo A-II (71). HDL particles are synthesized and catabolized in the liver and intestines. Nascent HDL obtains free cholesterol from peripheral tissues. The free cholesterol in HDL is an esterified CE by lecithin cholesterol acyltransferase. CE may be removed by several different pathways, including selective uptake by the liver and scavenger receptors for HDL (72). Another important pathway of reverse cholesterol transport involves the action of plasma cholesteryl ester transfer protein (CETP). CE can be transferred from HDL to apoB containing proteins, such as VLDLs and LDLs, by CETP (73-74).

3.7 Lipoprotein Phenotype

All lipoproteins are actually heterogeneous, consisting of subclasses of particles with differing density, size, electrophoretic mobility, lipid composition, and binding affinity (75). Table 3.3 presents the physicochemical properties of lipoprotein subfraction (47). For LDL particles, they are fractionated according to size and density into two major phenotypes: pattern A, with a -higher proportion of large, buoyant LDL particles (lbLDL), with peak particle diameter ≥ 25.5 nm (density 1.019 – 1.044 g/ml) and pattern B, characterized by predominance of sdLDL, with peak particle diameter < 25.5 nm (density range, 1.044 – 1.060 g/ml) (5-7). In most healthy people, the major LDL subclasses are those of pattern A. Moreover, at any level of LDL-C, individuals with an elevated proportion of small

LDL particles size have been reported to be at greater risk for CHD compared with those with the same LDL-C level, but composing of larger LDL particles (76-77).

Several studies have reported a 2- to 3- folds increase in CHD risk among patients with the predominance of sdLDL (10, 11). These differences are at least in part, responsible for the differences observed in the biological behavior of LDL subfractions and more specifically in their ability to promote atherosclerosis (78). Experimental studies suggest that the sdLDL particles may exhibit greater atherogenic potential compared to lbLDL subfractions (78). SdLDL particle size is associated with several other cardiovascular risk factors, including MS, type 2 diabetes mellitus (DM Type 2) and postprandial hypertriglyceridemia (76).

Table 3.3 Physicochemical properties of lipoprotein subfraction

	Peak S _f	Density Peak (gm/ml)	Diameter (A°)	%PR	%CE	%UC	%TG	%PL
VLDL								
VLDL-1	60 - 400	< 1.006	330 - 700	11	8	6	58	17
VLDL-2	20 - 60	1.006-1.010	300 - 330	18	24	9	29	22
IDL								
IDL 1	12 -20	1.008-1.022	285 - 300	17	35	10	16	21
IDL 2	10 - 16	1.013-1.019	272 - 285	17	37	11	13	21
LDL								
LDL-I	7 -12	1.019-1.023	272 - 285	18	43	9	7	22
		1.023-1.028	265 – 272	19	45	10	4	23
LDL-II	5 - 7	1.028-1.034	256 - 265	21	45	9	3	22
		1.034-1.041	247 – 256	22	46	8	3	21
LDL-III	3 - 5	1.041-1.044	242 -247	24	44	7	3	21
		1.044-1.051	233 – 242	26	42	7	5	19
LDL-IV	0 -3	1.051-1.06	220 - 233	29	40	7	6	18

PR, protein; TG, triglycerides; CE, cholesteryl ester; PL, phospholipids; UC, unesterified cholesterol.

Source: Berneis KK, Krauss RM. Metabolic origins and clinical significance of LDL heterogeneity. *Journal of Lipid Research*. 2002;43 (9):1363–1379.

3.8 Small Dense Low-density Lipoprotein

The sdLDL is an emerging CVD risk factor by the NCEP (ATP III) (79). The individuals who have sdLDL carry a 3-fold increase CHD risk (12, 80-81). Furthermore, several prospective studies have shown that a predominance of sdLDL particles can predict an increase risk of subsequent CAD and myocardial infarction (59, 80-81, 70). Zhao et al. indicated that the prevalence of sdLDL was 3.1 –fold higher among stroke patients compared with healthy controls (82). Data from the Québec Cardiovascular Study showed that men with an LDL particle size < 25.6 nm had a significant 2.2-fold increase in the 5-year rate of ischemic heart disease (IHD) compared with men having an LDL particle size > 25.6 nm (12).

The predominance of sdLDL directly correlates with serum TG and inversely correlates with HDL-C. This combined lipid abnormality has been designated the atherogenic lipoprotein phenotype which plays an important role in the development of atherosclerosis. Atherogenic lipoprotein phenotype is characteristically seen in patients with obesity, the MS, insulin resistance (IR), and DM Type 2 (76). A recent finding also showed that women with polycystic ovary syndrome have significant qualitative LDL alterations, with increased level of atherogenic sdLDL particle (14)

3.8.1 Metabolism of small dense LDL particles

Small dense particles production is increasingly found among patients with high plasma TG. High level of TG will cause the overproduction of VLDL in the liver and spread out to the bloodstream. Then, TGs in this VLDL will be hydrolyzed by lipoprotein lipase (LPL) until they become IDL and LDL respectively (83). At the same time, high level of TG is an important cause of exchange of cholesteryl esters on LDL for TGs on VLDL through the Enzymatic Reaction mechanism, which is called cholesteryl ester transfer protein (CETP) (84-85). Then, they become LDL with high TG levels. These LDL are small lipoprotein particles with high TG levels. These LDL are small lipoprotein particles with high level of TG. They are good precursors for hepatic lipase enzyme, which is called lipase-mediated triglyceride hydrolysis. One of the functions of hepatic lipase is to hydrolyze the TG in lipoprotein into small LDL particles, with decreased cholesterol

esters (86-87). The particles are transformed into sdLDL particles as shown in Figure 3.1(88).

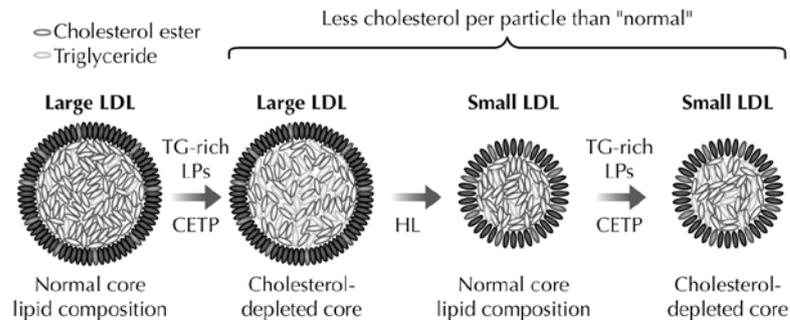


Figure 3.1 Schematic representations of the metabolic origins LDL particles containing less cholesterol than normal

Source: Cromwell WC, Otvos JD. Low-density lipoprotein particle number and risk for cardiovascular disease. *Current atherosclerosis reports*. 2004;6 (5):381–387.

3.8.2 Clinical Significant for small dense LDL Cholesterol

Several studies review the evidence suggesting that the sdLDL phenotype, beyond its own atherogenic properties could also be an additional marker of an athero-thrombotic profile associated with hypertriglyceridemia, low HDL-C level, abdominal obesity, insulin resistance (IR) and other features of the metabolic syndrome (MS) (11). Patient with chronic kidney disease (CKD) (89) and menopausal women also have abnormalities of lipoprotein metabolism that may contribute to the high incidence of CVD (90).

3.8.2.1 Atherosclerosis

SdLDL particles differ from normal-sized LDL particles in terms of metabolism and atherogenicity. SdLDL particles penetrate more easily into the arterial intima (8). They also have a prolonged plasma half-life because of their lower binding affinity for the LDL receptor (91). The particle appears to interact more strongly with increased binding to arterial wall proteoglycans (92), and they are more susceptible to oxidative modification than medium-sized or large LDL particles (92). The oxidation of LDL occurs when the LDL particles react with free radicals. The oxidized LDL itself then becomes more reactive with the surrounding tissues,

which can produce tissue damage (93). Therefore, sdLDL particles are easily oxidized and engulfed by macrophages through injured endothelial cells, resulting in the formation of atherosclerotic lesion (93). Atherosclerosis can affect any artery in the body, including arteries in the heart, brain, arms, legs and pelvis. As a result, different disease may develop based on which arteries are affected. Figure 3.2 shows the progression of atherosclerosis (94).

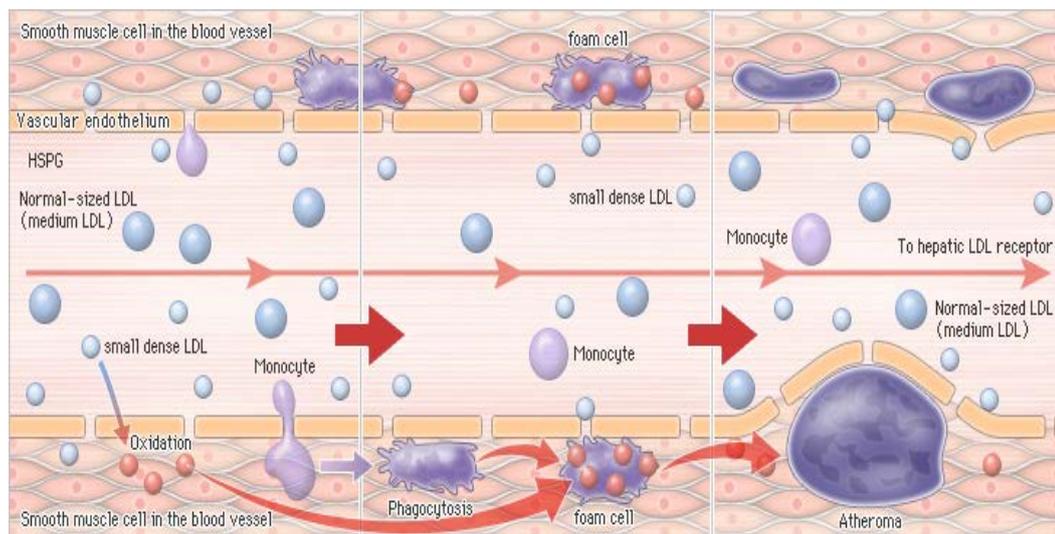


Figure 3.2 The progression of atherosclerosis

Source: <http://www.lipo-search.com/eng/lipo.html>

Accessed date: 21/06/2012

3.8.2.2 Insulin resistance

Hypertriglyceridemia, low HDL and sdLDL particles are common lipid abnormalities in individuals with IR. Therefore, CVD risk factor profiles of subject with sdLDL compose of intrinsically the same factors as those associated with an increased risk for the IR (78, 95-96). IR is a condition in which defect in the action of insulin are such that normal levels of insulin do not trigger the signal for glucose absorption (97). The pancreas compensates for the decreased insulin response by increasing insulin secretion until reserve capacity is exceeded by metabolic demands and insulin secretion is no longer adequate (98). Thus, blood glucose levels are high, impaired glucose tolerance and then type 2 diabetes develops. Several recent studies finding in subjects with normal glucose tolerance,

impaired fasting glucose of DM Type 2 shown that among risk factors used in the World Health Organization (WHO) clinical definition of MS, dyslipidemia was significantly associated with risk of CHD in subjects with DM (99). Interestingly, subjects with predominance of sdLDL have a greater than 2 fold increased risk for developing DM Type 2, independent from age, sex, glucose tolerance, and body mass index (97). SdLDL is also associated with early vascular dysfunction in the form of impaired endothelial response in patients with diabetes independent of other risk factor variables, including lipid levels, body mass index (BMI), blood pressure and intima-media thickness, as well as HbA_{1c} and total peroxy radical-trapping capacity (TRAP) (100-101). From the Skaraborg Hypertension and Diabetes Project, HbA_{1c} and duration of diabetes were positively related to plasma TG concentrations (102) that are often attributed to IR. Pathophysiology of insulin involves free fatty acids (FFAs) released from adipose tissue which increase production of glucose and TGs and secretion of VLDL in the liver. Associated lipoprotein abnormalities induce decreased levels of HDL-C and increased levels of sdLDL particles. FFAs also reduce insulin sensitivity in muscle by inhibiting insulin-mediated glucose uptake. Increases in circulating glucose increase pancreatic insulin secretion, resulting in hyperinsulinemia as shown in (Figure 3.3) (103).

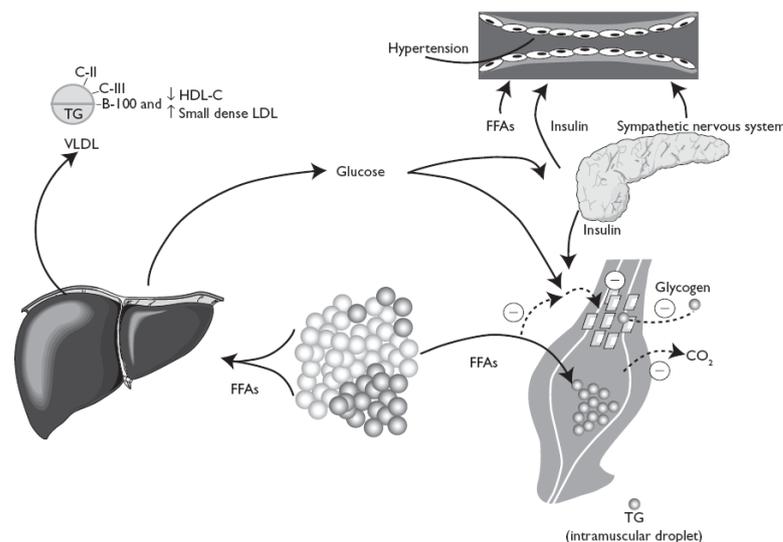


Figure 3.3 Pathophysiology of insulin resistance.

Source: Jellinger PS. Metabolic consequences of hyperglycemia and insulin resistance. *Clinical cornerstone*. 2007;8 Suppl 7:S30-S42.

3.8.2.3 Metabolic syndrome

MS is known as the cluster of changes associated with IR, which plays an important role in patients with CHD (104-105). The large meta-analyses systemically had shown that people with MS are at increased risk of cardiovascular events (106). The investigators concluded that the MS is associated with a 2-fold increase in cardiovascular outcomes and a 1.5-fold increase in all-cause mortality rates. However, the certainty of pathogenesis of IR and the predictive value for CVD of MS has been challenged because of the insufficiency of conclusive evidence (107). From the study of Tenenbaum and Fisman concluded that a 2-fold increase in cardiovascular outcomes associated with the MS (108).

According to the American Heart Association (AHA), the risk factors for MS including, abdominal obesity (excessive fat tissue in and around the abdomen), atherogenic dyslipidemia, elevated blood pressure, IR, prothrombotic state, and proinflammatory state (109). Because the synthesis and degradation of lipoproteins are dependent on insulin action, it has been suggested that the size and composition of lipoproteins are related to IR, a hallmark of MS (110). Reavan et al, coined the phrase MS to characterize patients with dyslipidemia with high level of TGs and low HDL-C (111). The patient with MS found to have a high prevalence of sdLDL. Figure 3.4 shows that the metabolic complications (106).

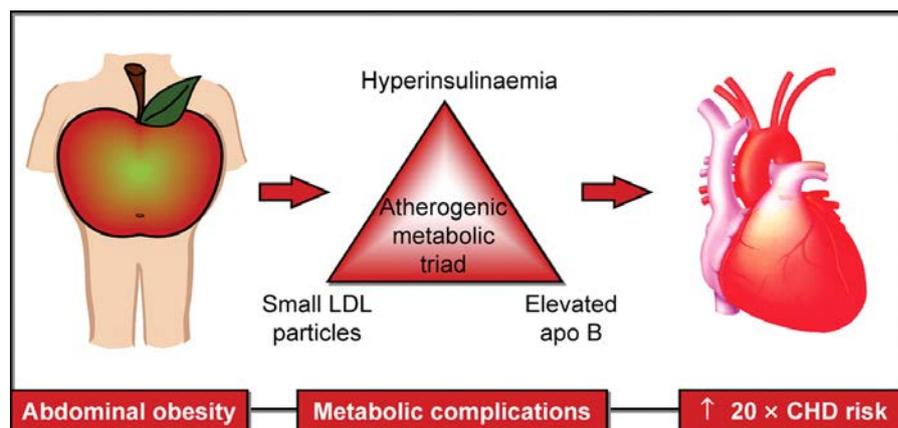


Figure 3.4 Metabolic syndromes

Source: http://eurheartjsupp.oxfordjournals.org/content/10/suppl_B/B24/F3.expansion

Accessed date: 21/6/2012

3.8.2.4 Polycystic ovary syndrome

CVD represent the major cause of death in both sexes, but women have hormonal protection before menopause, and the onset of CVD are usually delayed by 10-15 years in comparison with men (17). Polycystic ovary syndrome (PCOS) are characterised by chronic anovulation and hyperandrogenism (Figure 3.5) (113-114). There is a common diagnosis made in up to 10% of women of reproductive age (115). However, young women may show increased cardiovascular risk if affected by a common endocrine disease (116). Women with PCOS are more likely than normally cycling women to have IR, central adiposity, hypertension, and the MS (114). In addition, several markers of clinical atherosclerosis are altered in women with POCS (113). Dyslipidemia may represent the most common metabolic abnormality in POCS, with prevalence of up to 70% according to the NCEP criteria (117). POCS is classically associated with an atherogenic lipoprotein profile including elevated TG concentrations, accumulation of sdLDL and decrease HDL (115). The risk factors for CVD are more prevalent in women with POCS. The other metabolic condition including insulin resistance and dyslipidemia seems to play a major role on cardiovascular risk in PCOS (118).

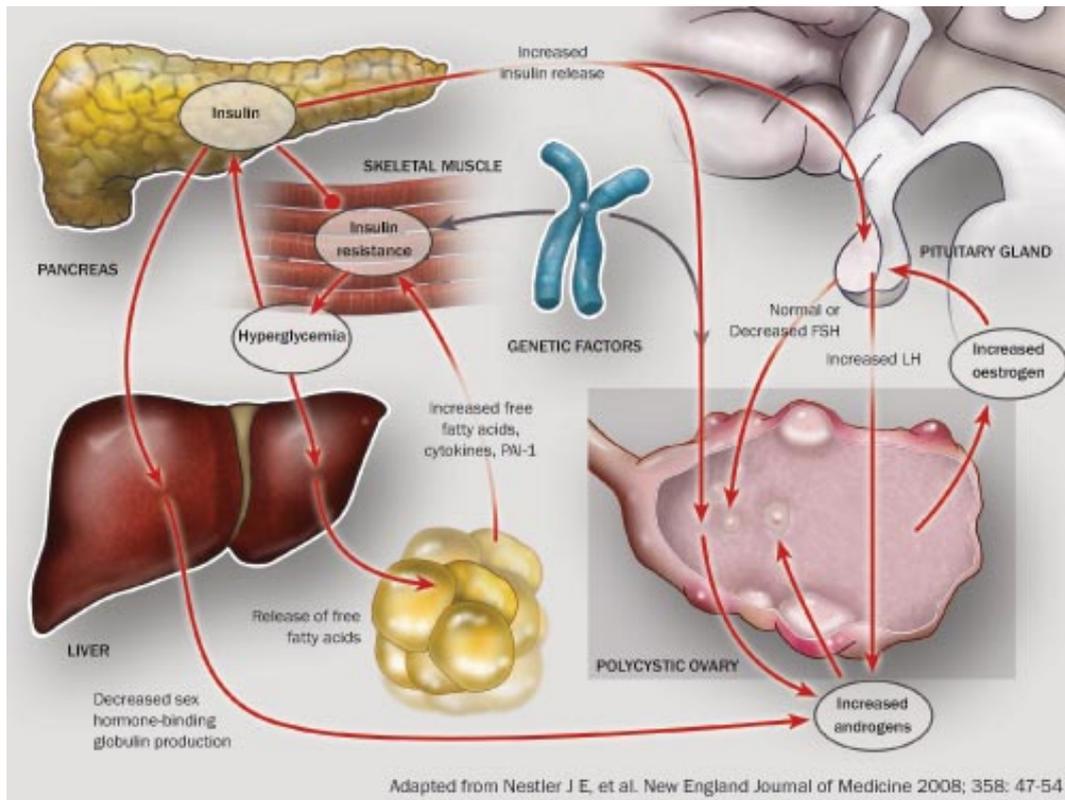


Figure 3.5 Polycystic ovary syndromes

Source: Nestler J E, et al. New England journal of medicine 2008; 358: 47-54.

Accessed date: 21/06/2012

3.8.2.5 Chronic kidney disease

Accelerated atherosclerosis and CVD are the main cause of death in patients with chronic kidney disease (CKD) (120). Atherosclerosis and CVD as well as many other complication of CKD are primarily driven by oxidative stress, inflammation, and lipid disorders (121-123). Mild chronic impaired renal function contributes to the development of CVD, so the AHA has recommended that these patients should be classified in the highest risk group for developing cardiovascular event (124).

In patients who advance to end stage renal disease (ESRD), the prevalence of clinical CHD is 40% and CVD mortality is 10 to 30 times higher than in the general population of the same gender, age and race (124-126). In addition, it is well known that patients with impaired renal function exhibit significant alterations in lipoprotein metabolism, which in their most advanced form

may result in the development of severe dyslipidemia. The plasma lipid profile frequently evolves during the course of progression of CKD. Serum TGs and VLDL levels are elevated, and clearance of VLDL and chylomicrons and their atherogenic remnants is impaired in patients with advanced CKD or ESRD. This is accompanied by presence of sdLDL and accumulation of oxLDL, IDL and chylomicron remnants (127-130). In fact, lipoprotein metabolism appears to be substantially influenced by the severity of renal dysfunction and proteinuria. The predominant mechanism responsible for increased TG-rich lipoproteins concentration in predialysis patients is one of delayed catabolism (131). In the low catabolic rate, the increased hepatic production of TG-rich lipoproteins may also play a contributory role in the pathogenesis of dyslipidemia in renal disease (131). It is well known that CKD causes IR which can promote hepatic VLDL production (132-134). Thus, it could be presumed that the IR-driven overproduction of VLDL may significantly contribute to the development of Hypertriglyceridemia in patients with CKD. CKD patients display important qualitative alterations in LDL metabolism. The proportion of sdLDL particles, which is considered to be highly atherogenic, is increased (135-136).

3.9 Low Density Lipoprotein Measurement

Plasma concentrations of LDL-C are directly related to atherosclerosis risk. Modifications in the structure of native LDL that are capable of inducing aggregation and fusion of the particles are currently recognized to be a prerequisite for the initiation of lipid accumulation (136). The NCEP-ATPIII continues to identify elevated LDL-C as the primary target of cholesterol-lowering therapy for reduces the risk of CVD. Then, the measurement of LDL-C is important for primary criteria in patients with hyperlipemia.

3.9.1 LDL cholesterol measurement

Nowadays, there is accumulating evidence that reduction of plasma LDL concentrations could provide additional benefit in CHD prevention (137). According to the NCEP-ATPIII, the diagnosis and management of patients with hypercholesterolemia is largely based on LDL-C concentration (17). A wide variety of methods have been used for determining LDL-C in serum. These methods include sequential and density-gradient ultracentrifugation (138), β -quantification (139-140), Friedewald equation (19), electrophoresis (141), HPLC (142), and homogeneous assay (143). The reference method for determining LDL-C is β -quantification (144), which combines separation by ultracentrifugation and chemical precipitation. However, the β -quantification method requires a relatively large volume of serum, special equipment, and is a time-consuming procedure; therefore, it is not well suited for routine testing in hospitals and clinics (145). Therefore, alternative approaches have been proposed, including the traditional estimation from Friedewald formula and innovative direct, homogenous assays (23).

3.9.1.1 Calculation method

LDL is most commonly evaluated using the Friedewald formula (19). The advantage of this calculated method is easy, convenient, and low cost. Although the estimation method correlated highly with β -quantification, it has certain limitations. The calculation requires a fasting specimen because nonfasting specimens often contain traces of CMs. Friedewald equation not valid in specimens with chylomicrons because CMs contain proportionately less cholesterol relative to TGs than VLDL, their presence leads to overestimation of VLDL-C and underestimation of LDL-C. As the TGs > 4.52 mmol/L, the proportion of cholesterol to TGs in VLDL decrease, giving rise to errors. Therefore, calculation was recommended only for serums with TGs < 4.52 mmol/L. Patients with hyperlipoproteinemia type III or dysbetalipoproteinemia, characterized by accumulation of cholesterol relative to TGs, leading to underestimation of VLDL-C and overestimation of LDL-C (19,146).

3.9.1.2 Direct measurement assay

The Friedewald equation, the most commonly used method in clinical laboratories, may not always meet the performance criteria of total error of 12% or less established by the NCEP, because it requires the determination of three different measurements, each with its own analytical CV. Therefore, the NCEP working Group on Lipoprotein Measurement recommended the development of direct methods for LDL-C measurement (21).

To provide more efficient measurement of LDL-C in clinical laboratories, new fully automated homogeneous methods have been developed and are commercially available (23,147). Each method generally has a first and a second reagent, which eventually produces a colored product derived specifically from LDL-C which is measured spectrophotometrically (143). In particular, new homogeneous methods using novel surfactants have been found to be simple and reliable for measuring LDL-C, using only a small sample volume and being easily applied to automatic analyzer (148). Most evaluations of the homogeneous methods for LDL-C have involved comparisons with the β -quantification (149). The homogeneous methods do appear to be significantly less susceptible to interference from increased TGs than Friedewald calculation (23). Our study measured LDL-C concentration by homogeneous enzymatic colorimetric assay. This method has been certified using the LDL-C method evaluation protocol by the Cholesterol Reference Method Laboratory Network (CRMLN). Our aim was to estimate sdLDL-C recognized as LDL-C by use the difference between the cLDL-C and the dLDL-C which has been ascribed to variation in TGs and HDL-C concentration.

3.9.2 Determination of LDL subclass

Several methods have been developed for the assessment of sdLDL particles as a basis for dietary counseling, lifestyle adjustments, and the effectiveness of pharmacologic agents in reducing the risk of CVD. These could help guide potential treatment interventions (150). The most widely used technique in clinical studies was the determination of the peak LDL particle diameter by gradient gel electrophoresis. Other methodologies such as density gradient ultracentrifugation and nuclear magnetic resonance spectroscopy allow the direct determination of the

concentrations of LDL subfractions and they could be more feasible for the study of LDL subfraction profiles. Since most of the methodologies that have been used so far for the characterization of lipoprotein subfraction profile are expensive, time consuming and technically demanding, their application in every day clinical practice remains limited (145).

3.9.2.1 Gradient Gel Electrophoresis (GGE) is most often used to measure the LDL particle size. GGE separates LDL particles based on the principle that the particles migrate through the gradient gel until further penetration is restricted by their size (151). This method determines the distribution of LDL size phenotype by proprietary segmented polyacrylamide gradient gels, which separate lipoproteins in a gradient gel on the basis of their size and, to lesser extent, their charge (152). With this method, the size of major peaks and percent distribution of 7 LDL subclasses can be determined (152). Figure 3.6 shows that the gradient gel electrophoresis method (153).

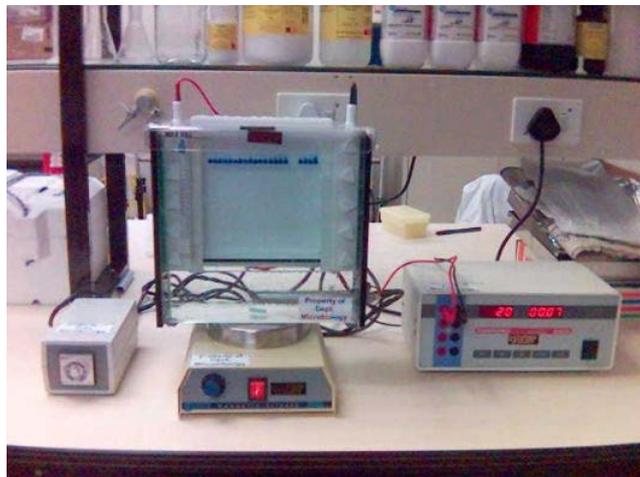


Figure 3.6 Gradient gel electrophoresis.

Source: <http://web.up.ac.za/default.asp?ipkCategoryID=2502>

Accessed date: 21/06/2012

3.9.2.2 Tube Gel Electrophoresis has been available for the separation of LDL subfractions. Tube gels have an advantage in that the movement of molecules through the gels is less prone to lateral movement and thus there is a slightly improved resolution of the band. The VLDL band (slowest migrating) was

assigned an R_f (ratio of distance moved by band relative to marker) value of zero, and the HDL band (fast migrating) was assigned an R_f value of 1. The LDL subfraction bands migrated between the VLDL and HDL bands. The method permits separation of LDL into 7 subfractions (154). LDL subclasses were designated as small ($R_f > 0.40$), intermediate ($R_f = 0.38 - 0.40$), and large ($R_f < 0.38$). The LDL-1 and LDL-2 bands correspond to large buoyant LDLs, whereas bands LDL-3 to LDL-7 comprise sdLDL particles. Figure 3.7 illustrated that the tube gel electrophoresis method (155).

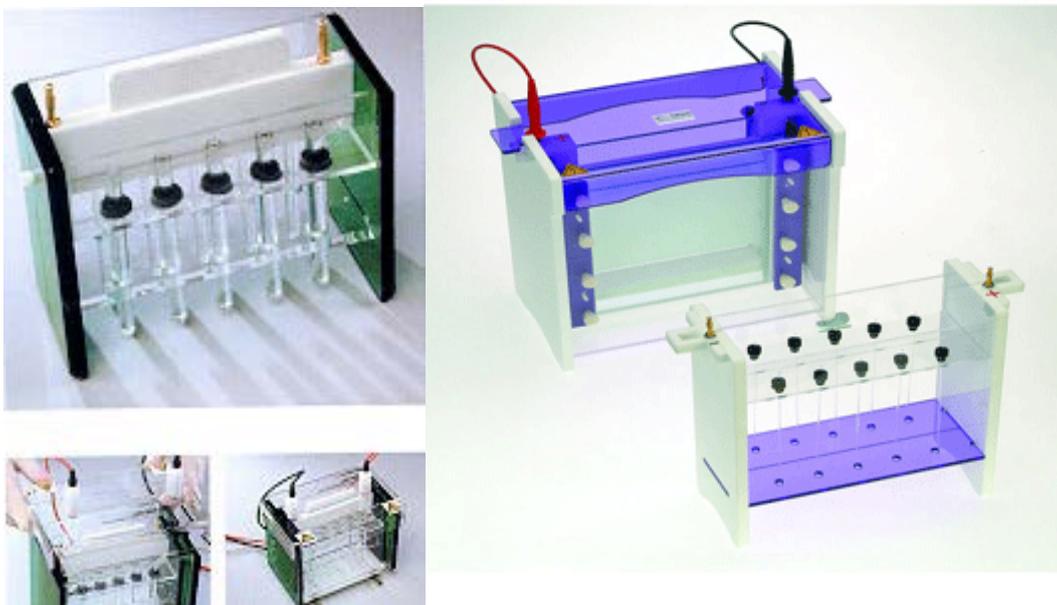


Figure 3.7 Tube gel electrophoresis.

Source: http://www.topac.com/2D_electrophoresis.html

Accessed date: 21/06/2012

3.9.2.3 Density Gradient Ultracentrifugation is performed on plasma samples to calculate the relative LDL flotation rate and density distribution of lipoprotein cholesterol (156). The relative flotation rate (R_f), which characterizes LDL peak buoyant as a continuous variable, is calculated as the fraction number containing the LDL-C peak divided by the total number of fractions collected. This method also determines the predominant LDL size distribution but does not provide concentrations of the lipoprotein particles themselves (157). With this method, 4

distinct LDL subclasses can be identified as LDL-I (largest and most buoyant), LDL-II, LDL-III, and LDL-IV (the smallest and most dense) (24). Figure 3.8 represent the density gradient ultracentrifugation method (158).

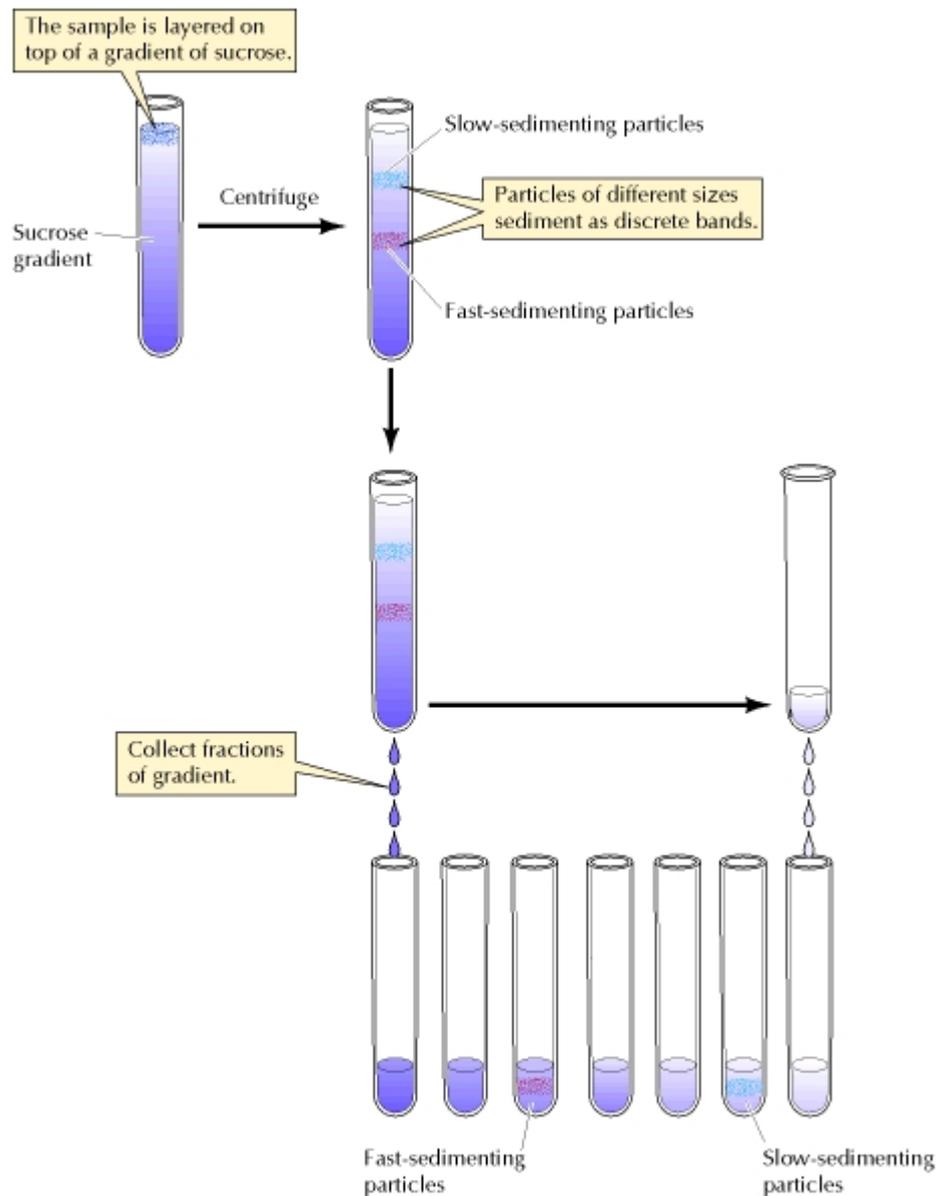


Figure 3.8 Density gradient ultracentrifugation.

Source: <http://www.ncbi.nlm.nih.gov/books/NBK9941/>

Accessed date: 21/06/2012

3.9.2.4 Nuclear Magnetic Resonance Spectroscopy (NMR) is based on the concept that each lipoprotein particle in plasma of a given size has its

own characteristic lipid methyl group NMR signal. Signals are derived from methyl groups on phospholipids, TC, CE, and TGs (159). Lipoprotein particle sizes are then derived from the sum of the diameter of each subclass multiplied by its relative mass percentage based on the amplitude of its methyl NMR signal. NMR LipoProfile-II simultaneously quantifies lipoprotein concentrations of VLDL, IDL, LDL and HDL particles and their subfractions, each expressed as a lipoprotein particle concentration or as an average particle size for each of VLDL, LDL, and HDL (29,160). This method can separate LDL-C into 3 subclasses. Figure 3.9 demonstrated the nuclear magnetic spectroscopy method (161).



Figure 3.9 Nuclear magnetic resonance spectroscopy.

Source: http://www.bruker.com/bas_nmr.html

Accessed date: 21/06/2012

3.9.2.5 Homogeneous assays for sdLDL-C

Recently, a modified photometric test procedure becomes commercially available as a direct homogeneous method for the quantification of sdLDL-C. The method was developed by Denka Seiken (Tokyo, Japan). This novel procedure is based on a simple, two-reagent enzymatic assay that is fully applicable

to an automated chemistry analyzer without manual pretreatment of the sample, reducing the assay time to 10 min (30). This method is applicable to routine clinical examination and allows a rapid measurement of a large number of samples. However, it is still expensive.

From the study of Chung M. et al. reported the comparing ultracentrifugation and GGE methods measured different components of the LDL particle. Therefore, the wide range of agreement and correlation coefficient between these methods is unique to each of the individual reports and laboratories and the results do not lend themselves to easy generalizable to other laboratories using the same methods. This highlights the important need for standardization if these measurements are to be more widely used in clinical practice, especially given the fact that the methods use different principles for subfractionation of lipoproteins (162). Although the importance of measurement of sdLDL is well recognized, it requires special equipment that is expensive, complicated techniques, time consuming procedures and is too difficult to use in daily clinical practice. Figure 3.10 showed that the homogeneous assays for sdLDL-C (163).



Figure 3.10 Homogeneous assays for sdLDL-C (Denka Seiken)

Source: http://www.aacc.org/publications/cln/2009/july/Pages/newproducts2_0709.aspx

Accessed date: 21/06/2012

CHAPTER IV

MATERIALS AND METHODS

4.1 Equipments

In the present work, all experimental studied were performed using facilities, and analytical instruments provided by the Clinical Chemistry laboratory in Ramathibodi Hospital.

4.1.1 The Siemens Dimension RxL Max (Siemens Medical Solution Diagnostics, Tarrytown, NY 10591-5097) for the implementation of sdLDL-C assay and the determination of all biochemical assays as shown in Figure 4.1 (153).

4.1.2 Semi-automate HYDRASYS System (Sebia Inc.,CA30093, USA) for the qualitative determination of major serum lipoproteins as shown in Figure 4.2 (154).



Figure 4.1 Siemens Dimension RxL Max

Source: http://www.diamonddiagnostics.com/equipment/Chem/Siemens_Dimension_RXL.htm

Accessed date: 11/01/2012



Figure 4.2 Semi-automate HYDRASYS System

Source: <http://www.sebia-usa.com/products/hydrasys2.html>

Accessed date: 11/01/2012

4.2 Chemical reagents

4.2.1 Biochemical reagent kits

All biochemical reagent kits were of listed in Table 4.1

Table 4.1 List of biochemicals and suppliers

Reagent kits	Reference	Suppliers
Cholesterol Flex® reagent cartridge	Product # DF27	Siemens Medical Solution Diagnosis, USA
Triglyceride Flex® reagent cartridge	Product # DF69A	Siemens Medical Solution Diagnosis, USA
AHDL Flex® reagent cartridge	Product # DF48A	Siemens Medical Solution Diagnosis, USA
ALDL Flex® reagent cartridge	Product # DF131	Siemens Medical Solution Diagnosis, USA
Glucose Flex® reagent cartridge	Product # DF39A	Siemens Medical Solution Diagnosis, USA
Creatinine Flex® reagent cartridge	Product # DF33A	Siemens Medical Solution Diagnosis, USA
SLDL-EX “SEIKEN”	CAT. NO. 56261	Randox Laboratories, Antrim, UK

4.2.2 HYDRAGEL 7, 15 and 30 LIPO + Lp (a) kits. The reagent were listed in Table 4.2

Table 4.2 Reagents and materials supplied in the hydragel 7, 15 and 30 LIPO + Lp (a) kits

Items	Supplied	Reference
Agarose Gels (ready to use)	10 gels contains: agarose, 0.8 g/dL buffer pH 7.5 ± 0.1	PN 4104
Buffered Strips (ready to use)	10 packs of 2 each contains: buffer pH 7.5 ± 0.1 0.3% sodium azide	PN 4104
Sudan Black Stain (stock solution)	1 vial, 20 mL contains: (1) Pure ethanol (96%) 120 ml + Sudan black stain (6.6 g/dL in dimethylformamind) 1.45 ml + Deionized water 100 mL : (2) Pure isopropanol (100%) 100 mL + Sudan black stain (6.6 g/dL in dimethylformamind) 1.45 ml + Deionized water 120 mL	PN 4104
Applicators (ready to use)	1 pack of 10 (7 teeth)	PN 4104
Filter Papers - Thin	1 pack of 10	PN 4104

4.3 Methods

4.3.1 Study Samples

The study has been approved by the Institutional Review Board (IRB) committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand. A total of 329 individual patients including those attending the outpatient clinics of Ramathibodi Hospital, Bangkok, Thailand from April 2009 to March 2010

who was requested for the lipid profile testing (TC, TG, HDL-C and LDL-C) were included in the study. A blood sample was collected from each subject after 10-12 hours overnight fast. The blood collected tubes were centrifuged and the serum and the plasma were separated from the cells. Using lipoprotein electrophoresis, patient sera with presenting of chylomicron or TG level > 4.52 mmol/L were excluded.

All sera were analyzed for TC, TG, HDL-C, dLDL-C, sdLDL-C and creatinine, within 3 hours after sampling and for lipoprotein electrophoresis within a day kept at 2-8°C. Sodium fluoride plasma samples were used for glucose analysis.

4.3.1.1 Classification of Age

For age group, the data were stratified by decade into 4 groups. Group 1 included 71 patients with age <50 years; group 2 included 115 patients with age between 51 and 60 years; group 3 included 74 patients with age between 61 and 70 years; group 4 included 74 patients with age >70 years.

4.3.1.2 Classification of Chronic Kidney Disease

The renal function status of individual patients was classified by the estimated GFR (eGFR) into 5 stages of renal dysfunction, ranging from I to V: normal, minimally impaired, moderately impaired, severely impaired, and failure. The eGFR was calculated from the serum creatinine concentration, age, and sex (166).

$$\text{GFR (mL/min/1.73 m}^2\text{)} = 141 \times \min(\text{Scre}/\kappa, 1)^\alpha \times \max(\text{Scre}/\kappa, 1)^{-1.209} \times 0.993^{\text{Age}} \\ \times 1.018 \text{ [if female]} \times 1.159 \text{ [if African American]},$$

where Scre is serum creatinine, κ is 0.7 for women and 0.9 for men, α is -0.329 for women and -0.411 for men, min indicates the minimum of Scre/ κ or 1, and max indicates the maximum of Scre/ κ or 1. GFR was categorized as > 120, 90 – 119, 60 – 89, 45 – 59, 30 – 44, and < 30 mL/min/1.73 m². The categories are based on National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) CKD stages. CKD stage 1 had eGFR greater than 90 mL/min/1.73 m² that kidney damage with normal or increased GFR. CKD stage 2 had eGFR between 60 to 89 mL/min/1.73 m² that mild decrease in GFR. CKD stage 3 had eGFR between 30 to 59 mL/min/1.73 m² that moderate decreased in eGFR. CKD stage 4 had eGFR

between 15 to 29 mL/min/1.73 m² that severe decreased in eGFR. Finally, CKD stage 5 had eGFR less than 15 mL/min/1.73 m² that a kidney failure.

4.3.1.3 Classification of Glucose Metabolism

To study the effect of impaired fasting plasma glucose, this study divided the data into three groups according to criteria of plasma glucose level for the diagnosis of diabetes mellitus (167). Group 1 includes patients with normal glucose regulation (plasma glucose concentrations < 5.5 mmol/L). Group 2 includes patients with impaired fasting glucose level (plasma glucose concentrations between 5.5 to 7.0 mmol/L). Group 3 includes patient with diabetes mellitus (plasma glucose concentrations > 7.0 mmol/L).

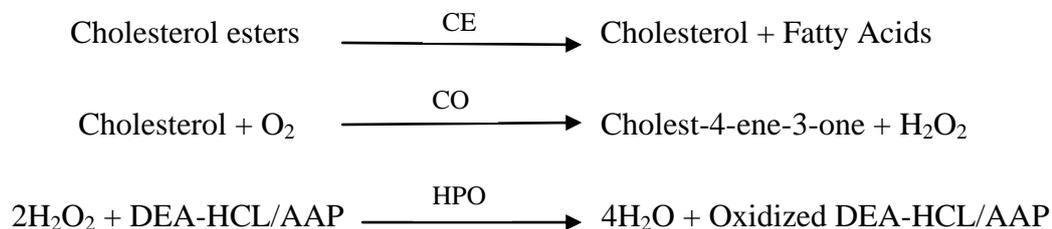
4.3.2 Procedures

All biochemical analyses were measured on the Siemens Dimension RxL Max by using the commercial enzymatic methods. All assays were performed exactly as directed by the manufacturer's recommendations. For the Dimension RxL analyzer, it was operated according to the manufacturer's specifications. The determination of lipids and lipoproteins analysis used in this study was standardized by the Centers for Disease Control and Prevention (CDC) National Heart Lung and Blood Institute Lipid Standardization Program.

4.3.2.1 Assays for Glucose

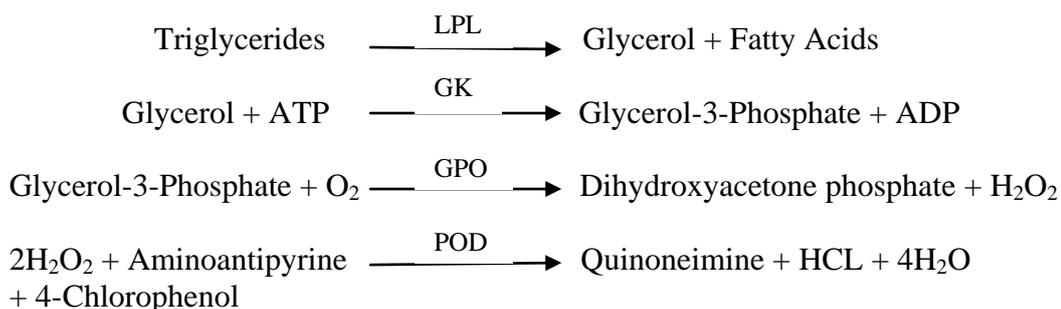
Hexokinase (HK) catalyzes the phosphorylation of glucose by adenosine-5'-triphosphate (ATP) to glucose-6-phosphate which is oxidized to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase (G-6-PDH) with simultaneous reduction of nicotinamide-adenine dinucleotide phosphate (NADP). One mole of NADP was reduced to one mole of NADPH for each mole of glucose presented. The absorbance due to NADPH (and thus the glucose concentration) is determined using a bichromatic (340 and 383 nm) endpoint technique (157).

The analytical reaction of enzymatic cholesterol determination was showed in the following reactions:



4.3.2.4 Assay for Triglyceride

Lipoprotein lipase (LPL) converted triglycerides into free glycerol and fatty acids. Glycerol kinase (GK) catalyzes the phosphorylation of glycerol by adenosine-5-triphosphate to glycerol-3-phosphate. Glycerol-3-phosphate-oxidase (GPO) oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate and H₂O₂. The catalytic action of peroxidase (POD) forms quinoneimine from H₂O₂, aminoantipyrine and 4-chlorophenol. The change in absorbance due to the formation of quinoneimine was directly proportional to the total amount of glycerol and its precursors in the sample and was measured using a bichromatic (510, 700 nm) endpoint technique (160). The analytical reaction of enzymatic triglycerides determination is showed in the following reactions:

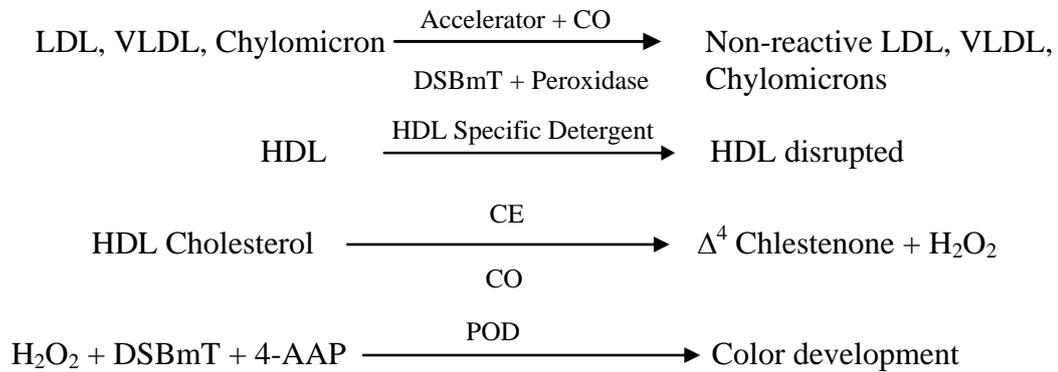


4.3.2.5 Assay for HDL-C

The method was in a two-reagent format and depends on the properties of a unique detergent. The assay is based on accelerating the reaction of cholesterol oxidase (CO) with non-HDL unesterified cholesterol and dissolving HDL selectively using a specific detergent. In the first reagent, non-HDL unesterified cholesterol was subjected to an enzyme reaction and the peroxide generated was

consumed by a peroxidase (POD) reaction with N, N-bis (4-sulfobutyl)-m-toluidine, disodium salt (DSBmT) yielding a colorless product. The second reagent consists of a detergent capable of solubilizing HDL specifically, cholesterol esterase (CE) and chromogenic coupler to develop color for the quantitative determination of HDL-C (161).

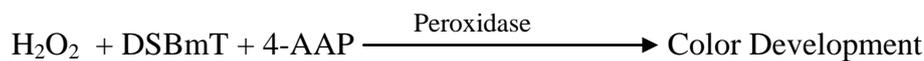
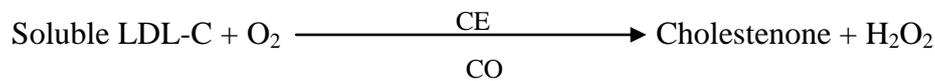
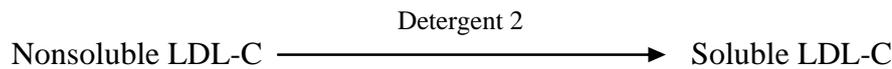
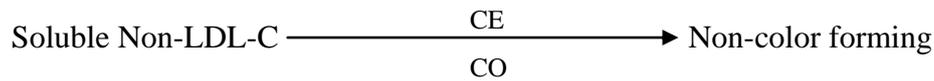
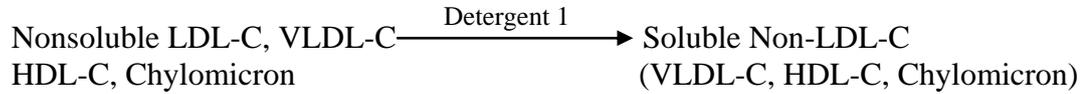
The analytical reaction of enzymatic HDL-C determination was shown in the following reactions:



4.3.2.6 Assays for LDL-C

For direct LDL-C assay, the method was a two-reagent format and depends on the properties of detergent 1 which solubilizes only non-LDL particles. The cholesterol released was consumed by cholesterol esterase (CE) and cholesterol oxidase (CO) in a non-color forming reaction. Detergent 2 solubilizes the remaining LDL particles. The soluble LDL-C was then oxidized by the action of CE and CO forming cholestenone and H₂O₂. The enzymatic action of peroxidase on H₂O₂ produces color in the presence of DSBmT and 4-aminoantipyrine (4-AAP) that was measured using a bichromatic (540 nm, 700 nm) endpoint technique. The color produced was directly proportional to the amount of LDL-C present in the sample (162).

The analytical reaction of enzymatic LDL-C determination was shown in the following reactions:



For calculated LDL-C assay, we calculated using the Friedewald formula: $\text{cLDL-C (in millimole per liter)} = \text{TC} - (\text{HDL-C}) - (\text{TG} / 2.2)$.

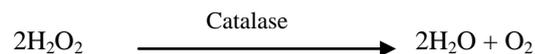
4.3.2.7 Assay for small, dense LDL-C

The assay consists of two steps and was based on the technique using well-characterized surfactants and enzymes that selectively react with certain groups of lipoproteins. In the first step, non-sd LDL (chylomicron, VLDL, IDL, lbLDL, and HDL) are decomposed by a surfactant and sphingomyelinase in the Reagent-1. The cholesterol ester from non-sdLDL lipoproteins was hydrolyzed by the cholesterol esterase (CE) and then oxidized by the cholesterol oxidase (CO). The hydrogen peroxides produced are finally decomposed to water and oxygen by the catalase.

In the second step, another surfactant in the Reagent-2 releases cholesterol only from sdLDL particles which was then subjected to the enzymatic reactions. H_2O_2 produced from the reaction with the CE and CO develops a purple-red color with the coupler in the presence of peroxidase (POD) (163).

The analytical reaction of enzymatic sdLDL-C determination was shown in the following reactions:

1st step reaction



2nd step reaction



* N-Ethyl-n-(2-hydroxy-3-sulfopropyl)-3-methylaniline

4.3.2.8 Principle of HYDRAGEL 7 LIPO + Lp(a)

The analysis is performed by electrophoresis on buffered (pH 7.5) agarose gels on the semi-automated HYDRASYS instrument. The separated lipoproteins are stained with a lipid-specific Sudan black stain. The excess of stain was removed with an alcoholic solution. The resulting electrophoregrams could be evaluated for pattern abnormalities or by densitometry to obtain relative quantification of individual zones.

Densitometry of stained electrophoregrams at 570 nm yields relative concentrations (percentages) of each fraction. The ranges of normal values (mean \pm SD) for individual zones on HYDRAGEL 7 LIPO + Lp(a) is

Beta lipoproteins (LDL) : 38.6 – 69.4%

Pre-beta lipoproteins (VLDL) : 4.4 – 23.1 %

Alpha lipoproteins (HDL) : 22.3 – 53.5%

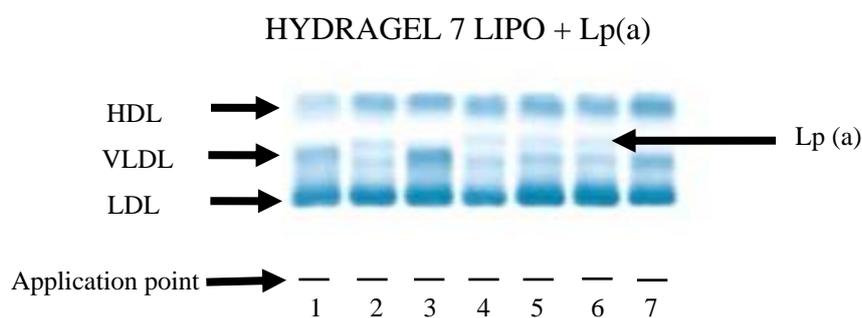


Figure 4.3 Migration patterns

Source: <http://www.sebia-usa.com/products/pn4114.html>

Accessed date: 11/01/2012

Analysis pattern of lipoprotein has become an integral part of the diagnosis and treatment of hyperlipidemia state and other disorders of lipid metabolism (165). The lipoprotein pattern of a clinical sample must be interpreted visually by comparing it with a control or a normal serum pattern. Densitometry may be useful for the follow up of the patient to see which fraction will increase or decrease. Densitometry provides accurate relative percentage of individual lipoprotein fractions. Qualitative (presence of abnormal or absence of normal fractions) or semi-quantitative (relative increase or decrease of fractions) abnormalities necessitate further lipoprotein analyses.

4.4 Statistical analysis

Statistical analysis was carried out using SPSS 16.0 software (SPSS, Chicago, IL). Descriptive statistics mean and standard deviation for continuous variables or proportions for categorical variables were computed for all study variables and all study groups. The study used the univariable analysis to study the effect of sex, age, renal dysfunction and impaired fasting plasma glucose on sdLDL-C concentrations. The stepwise multivariate regression analysis was performed to elucidate factors related to sdLDL-C concentrations in all subjects and by gender. Pearson's correlation coefficient was used to examine the relationship of sdLDL-C and lipid parameters. A paired Student t-test and Bland-Altman plot was used to test the significance of differences between the two homogeneous methods. This study

compared the slope and intercept of the regression equations between measured and calculate sdLDL-C to study the influence of individual subgroup such as sex, age, renal function, and glucose metabolism. Outcomes were considered statistically significant when P values < 0.05.

4.5 Experimental Design

The subjects were screened from 329 consecutives who attending the outpatient clinics of Ramathibodi Hospital. Among the 329 participants, 32 were excluded because samples were presented with chylomicron or TG > 4.53 mmol/L by using lipoprotein electrophoresis methods. The remaining 297 participants were analyzed for TC, TG, HDL-C, dLDL-C, sdLDL-C, glucose, and creatinine.

The effect of liver and kidney diseases may interfere the assays for dLDL-C and HDL-C. The 67 participants who had alanine aminotransferase concentrations more than twice the upper limit of normal and an eGFR less than 60 mL/min/1.73 m² were excluded. The final number of patient sample was 220. Then, all data were analyzed and the equations were generated

The diagrammatic of the design is shown in Figure 4.4.

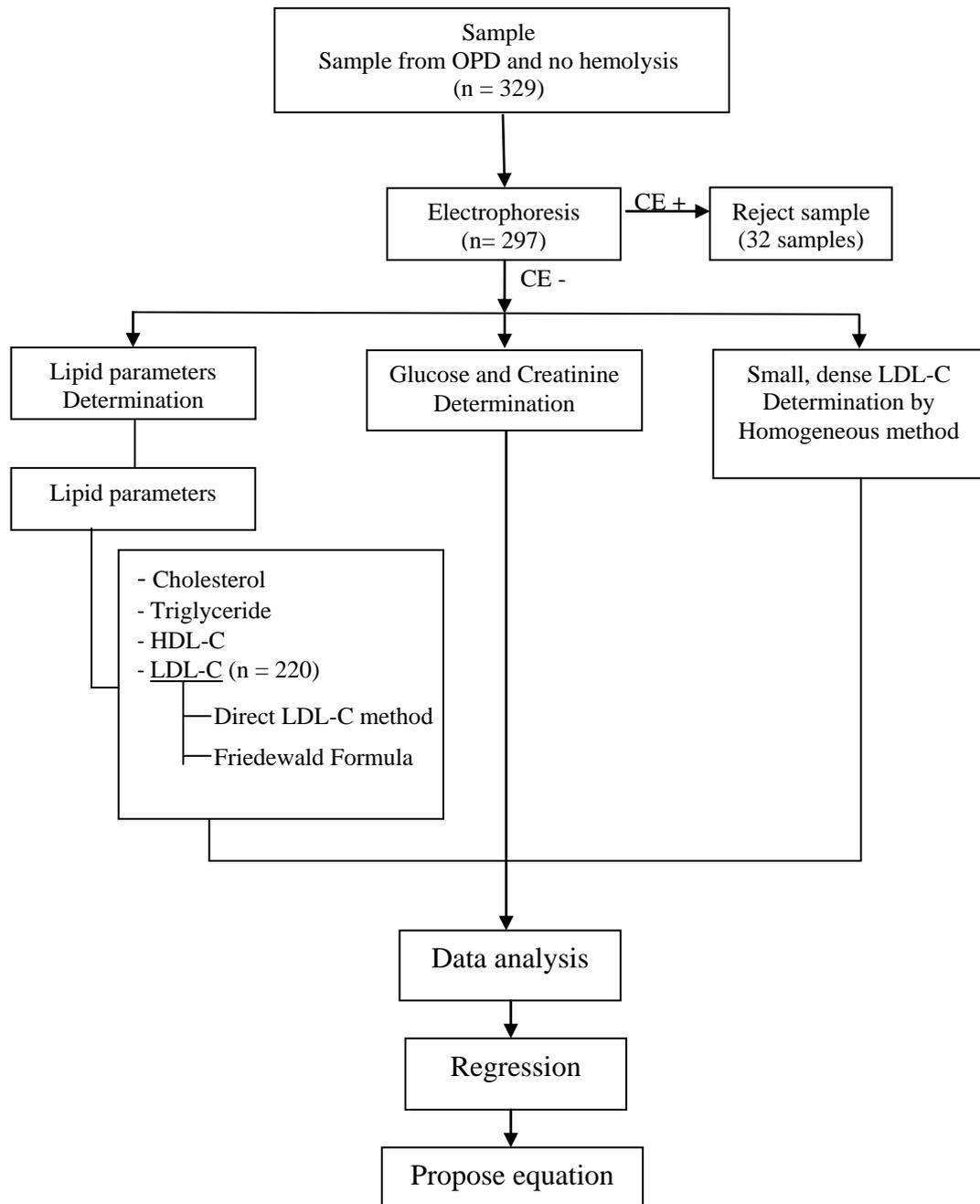


Figure 4.4 Diagrammatic representation of the study design.

CHAPTER V

RESULTS

5.1 Characteristics of the Study Sample

The biochemical characteristics of individual patients were summarized in Table 5.1. A total of 297 patient samples (115 males and 182 females) were included in the study. The mean age of the patients was 59.5 (ranging from 25 to 86 years) and 59.8 years (ranging from 24 to 86 years) for male and female, respectively.

The lipids and lipoproteins concentrations among the male and female patients in the present study were ranging from 2.00 to 10.60 and 2.90 to 14.90 mmol/L, respectively for TC; 0.42 to 4.47 and 0.42 to 4.48 mmol/L, respectively for TG. The ranging 0.47 to 2.02 and 0.52 to 2.91 mmol/L, respectively for HDL-C; 0.90 to 9.00 mmol/L and 1.30 to 13.50 mmol/L, respectively for direct LDL-C and 0.18 to 3.42 and 0.36 to 4.84 mmol/L, respectively for sdLDL-C.

Mean sdLDL-C concentration was 1.24 and 1.35 mmol/L for male and female, respectively. All biochemical tests and the lipid ratio have no significant difference between male and female ($p = 0.07$) except HDL-C ($p < 0.001$).

Table 5.1 Characteristics of the Study Samples

Characteristics	Men (n= 115)		Women (n= 182)		p - value ^a
	Mean ± SE	Range	Mean ± SE	Range	
Age (years)	59.5 ± 1.3	25 – 86	59.8 ± 0.9	24 – 86	0.023
Biochemical measures					
Glucose (mmol/L)	6.6 ± 0.2	3.5 – 18.9	6.1 ± 0.2	3.2 – 20.7	0.273
eGFR (mL/min/1.73 m ²) ^b	66.8 ± 2.6	3.0 – 120.0	77.1 ± 1.8	6.0 – 124.0	0.333
TC (mmol/L)	5.47 ± 0.16	2.00 - 10.60	6.50 ± 0.15	2.90 – 14.90	0.349
TG (mmol/L)	2.04 ± 0.10	0.42 – 4.47	1.88 ± 0.08	0.42 – 4.48	0.406
HDL-C (mmol/L)	1.05 ± 0.02	0.47 - 2.02	1.43 ± 0.04	0.52 – 2.91	< 0.0001
cLDL-C (mmol/L)	3.50 ± 0.14	0.71 – 8.46	4.22 ± 0.15	1.12-13.57	0.082
dLDL-C (mmol/L)	3.72 ± 0.15	0.90 - 9.00	4.36 ± 0.15	1.30 – 13.50	0.078
lbLDL-C ^c	2.49 ± 0.11	0.53 - 6.80	3.01 ± 0.19	0.78 – 10.66	0.120
sdLDL-C	1.24 ± 0.06	0.18 - 3.42	1.35 ± 0.05	0.36 – 4.84	0.070
NonHDL-C (mmol/L) ^d	4.57 ± 0.14	1.47 – 10.36	5.16 ± 0.15	1.96 – 14.13	0.095
TC/HDL-C ratio	5.40 ± 0.16	2.47 – 12.02	5.03 ± 0.20	2.00 – 23.16	0.086

^a Statistical significance of differences determined using two-tailed t-test

^b eGFR values were calculated from the new Chronic Kidney Disease Epidemiology Collaboration equation. ⁽¹⁶⁶⁾

^c Large buoyant LDL values were calculated by subtracting the sdLDL-C from the dLDL-C concentrations.

^d NonHDL-C values were calculated by subtracting the HDL-C from the TC concentrations.

eGFR = estimated glomerular filtration rate; TC = total cholesterol; TG = triglyceride; HDL-C = high density lipoprotein cholesterol; dLDL-C = direct measured low density lipoprotein cholesterol; cLDL-C = calculated low density lipoprotein cholesterol; sdLDL-C = small dense low density lipoprotein cholesterol.

5.2 Univariable analysis of sdLDL-C Level

The study performed a univariable analysis to examine the effect of sex, age, renal dysfunction and impaired fasting plasma glucose on sdLDL-C concentrations. Table 5.2 tabulated the univariable analyses of sdLDL-C. The estimated marginal means were adjusted for each of the other factors. The mean values for sdLDL-C (1.237 and 1.347 mmol/L for male and female, respectively) were unaffected by gender ($p = 0.07$). For age group, the data were stratified by decade into 4 groups. Group 1 included 63 patients with age ≤ 50 years; group 2 included 103 patients with age between 51 and 60 years; group 3 included 66 patients with age between 61 and 70 years; group 4 included 65 patients with age >70 years. The estimated marginal mean values for sdLDL-C were 1.310, 1.452,

1.283 and 1.086 mmol/L, for age group 1 to 4, respectively. The mean values for sdLDL-C were affected by age group ($p = 0.007$).

To study the effect of renal function on sdLDL-C concentrations, this study divided the data into five groups according to baseline stage of eGFR (166). Group 1 included 93 patients with chronic kidney disease (CKD) stage 1 who had a normal or high eGFR (greater than 90 mL/min/1.73 m²); group 2 included 129 patients with CKD stage 2 who had a mild decreased eGFR (between 60 to 89 mL/min/1.73 m²); group 3 included 50 patients with CKD stage 3 who had a moderate decreased eGFR (between 30 to 59 mL/min/1.73 m²); group 4 included 15 patients with CKD stage 4 who had a severe decreased eGFR (between 15 to 29 mL/min/1.73 m²); group 5 included 10 patients with CKD stage 5 who had a kidney failure (eGFR; less than 15 mL/min/1.73 m²). The estimated marginal mean values for sdLDL-C were 1.262, 1.421, 1.098, 1.241 and 1.326 mmol/L for CKD stage I, II, III, IV and V, respectively. The presence or absence of renal dysfunction did not statistically affect the sdLDL-C concentrations ($p = 0.058$).

To study the effect of impaired fasting plasma glucose, the study divided the data into three groups according to criteria of plasma glucose level for the diagnosis of diabetes mellitus (167). Group 1 included 146 patients with normal glucose regulation (plasma glucose concentrations <5.5 mmol/L); group 2 included 89 patients with impaired fasting glucose level (plasma glucose concentrations between 5.5 to 7.0 mmol/L); group 3 included 62 patients with diabetes mellitus (plasma glucose concentrations >7.0 mmol/L). The estimated marginal mean values for sdLDL-C were 1.280, 1.304 and 1.362 mmol/L, respectively. The presence or absence of impaired fasting glucose did not statistically affect the sdLDL-C concentrations ($p = 0.727$). However, the estimated marginal means were more likely to elevate with increasing degree of impaired fasting glucose.

Table 5.2 Univariable analysis of sdLDL-cholesterol levels

	Number of samples	Estimated mean (SE) ^a of sdLDL-cholesterol (mmol/L)	F test	p-value
Sex			1.876	0.172
Male	115	1.237 (0.055)		
Female	182	1.347 (0.053)		
Age (year)			4.091	0.007
≤ 50	63	1.310 (0.0718)		
51 – 60	103	1.452 (0.078)		
61 – 70	66	1.283 (0.642)		
> 70	65	1.086 (0.528)		
CKD stages ^b			2.306	0.058
I	93	1.262 (0.0718)		
II	129	1.421 (0.059)		
III	50	1.098 (0.078)		
IV	15	1.241 (0.164)		
V	10	1.326 (0.294)		
Fasting glucose categories ^c			0.319	0.727
< 5.5 mmol/L	146	1.280 (0.057)		
5.5 to 7.0 mmol/L	89	1.304 (0.068)		
> 7.0 mmol/L	62	1.362 (0.089)		

^a Estimated marginal means are adjusted for each of the other factors.

^b CKD = Chronic Kidney Disease (see Materials and Methods section for the definition of stage I through V)

^c Fasting glucose categories are defined according to a criteria for the diagnosis of diabetes mellitus (167)

5.3 Relationship between sdLDL-C and Lipids Concentrations

The associations between the TC, TG, HDL-C and LDL-C concentrations and the sdLDL-C concentrations obtained from the study samples were performed. Figure 5.1 showed the association between the TC concentration (x) and the sdLDL-C concentrations (y). The linear regression equation was $y = 0.273x - 0.362$ with the correlation coefficient (r) of 0.808 ($p < 0.001$).

Figure 5.2 showed the association between the TG concentration (x) and the sdLDL-C concentrations (y). The linear regression equation was $y = 0.330x + 0.665$ with $r = 0.523$ ($p < 0.001$).

Figure 5.3 showed the association between the HDL-C concentration (x) and the sdLDL-C concentrations (y). The linear regression equation was $y = -0.220x + 1.586$. It has been found that the sdLDL-C concentration inversely correlated with HDL-C level ($r = -0.150$, $p < 0.01$).

Figure 5.4 showed the association between the LDL-C concentration (x) and the sdLDL-C concentrations (y). The linear regression equation was $y = 0.296x + 0.089$ with $r = 0.827$ ($p < 0.001$).

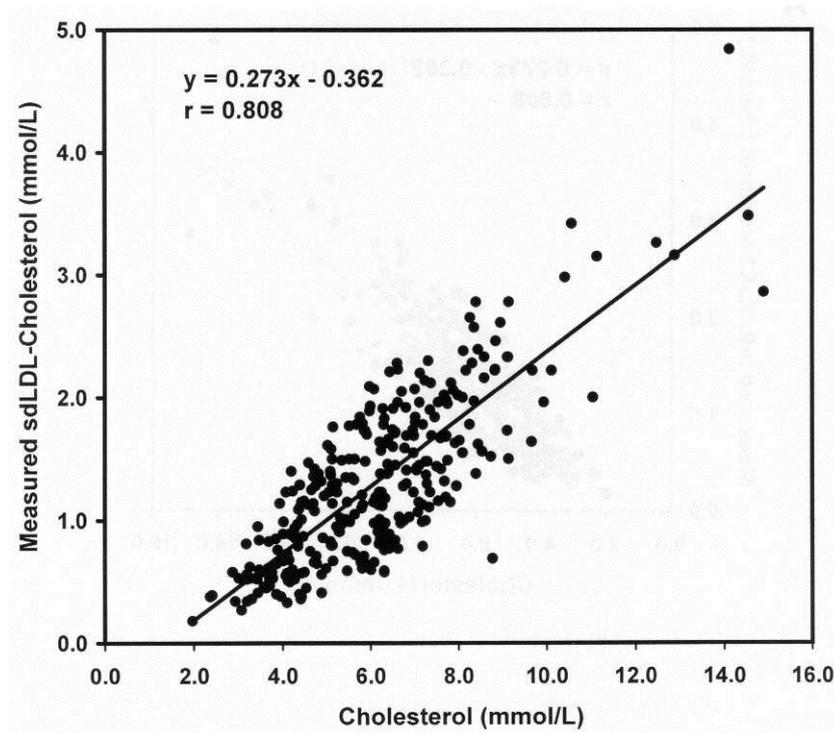


Figure 5.1 Correlation between the cholesterol concentrations (x) and the sdLDL-C (y). Linear regression was $y = 0.273x - 0.362$, $r = 0.808$.

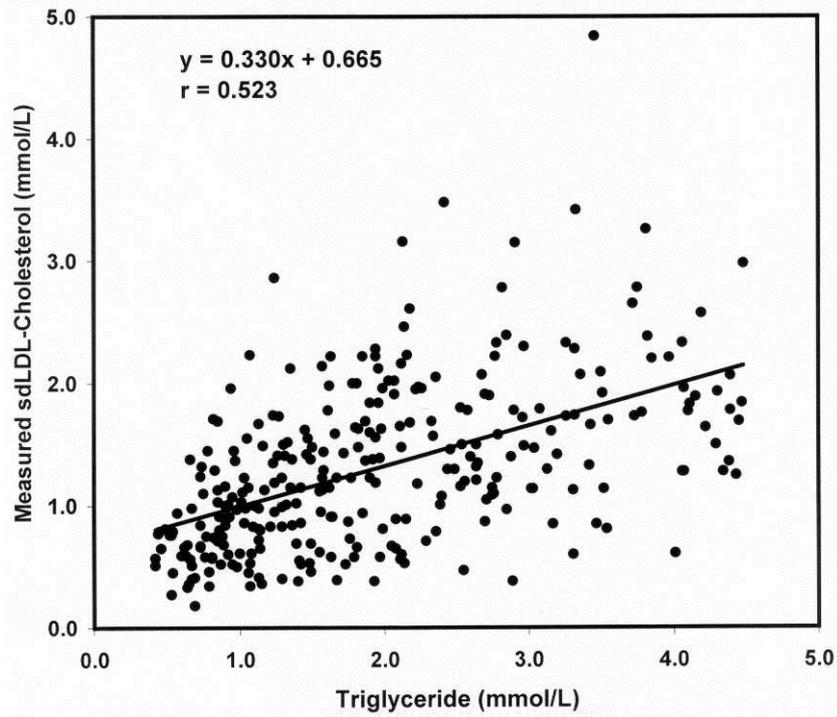


Figure 5.2 Correlation between the triglyceride concentrations (x) and the sdLDL-C (y). Linear regression was $y = 0.330x + 0.665$, $r = 0.523$.

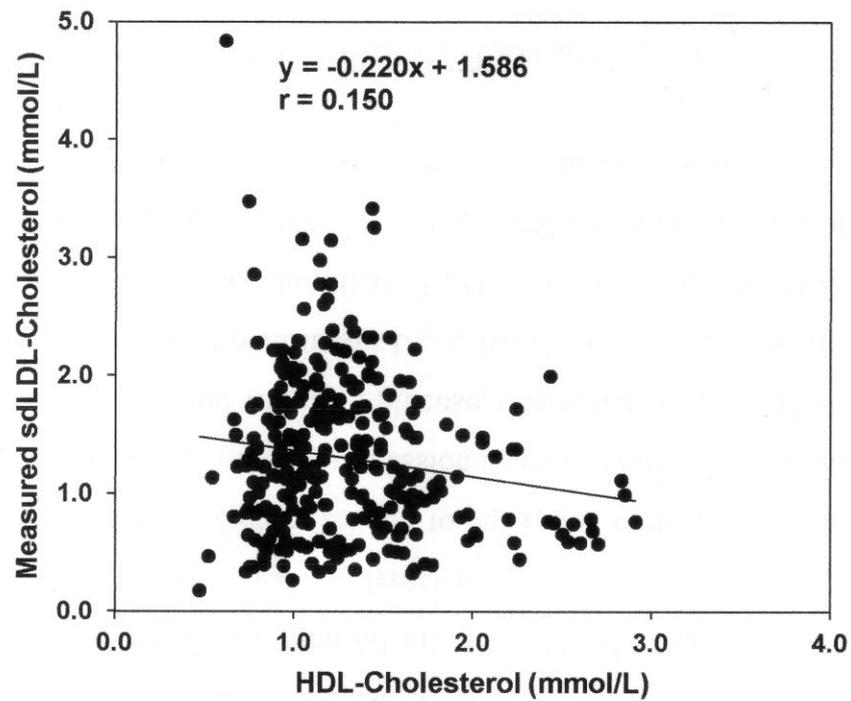


Figure 5.3 Correlation between the HDL-C concentrations (x) and the sdLDL-C (y). Linear regression was $y = -0.220x + 1.586$, $r = 0.150$.

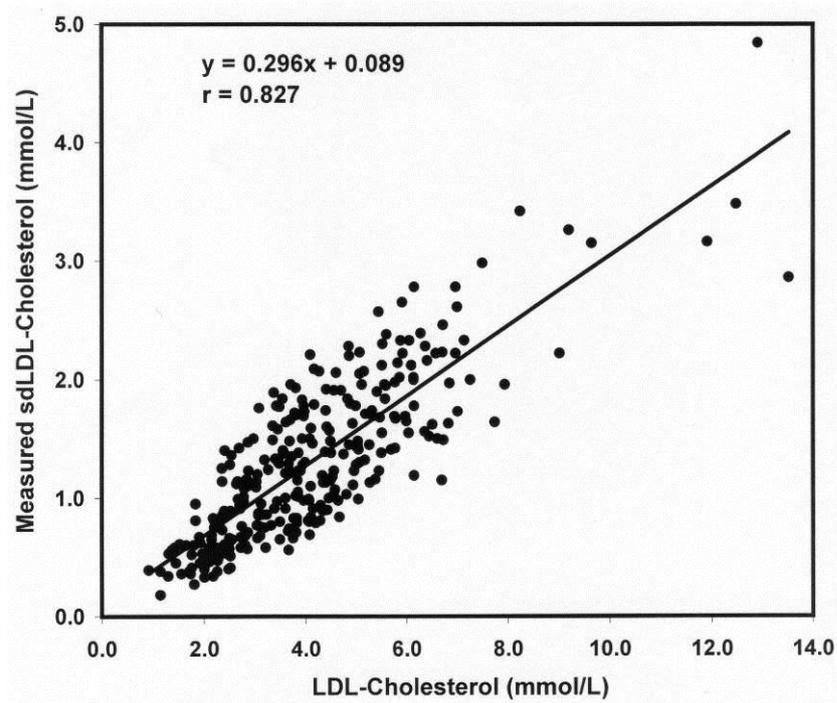


Figure 5.4 Correlation between the LDL-C concentrations (x) and the sdLDL-C (y). Linear regression was $y = 0.296x + 0.089$, $r = 0.827$.

5.4 Multiple Regression Analysis of sdLDL-C Concentrations

The presence of liver and kidney diseases may interfere with dLDL-C and HDL-C assays. To reduce the effect of both diseases and to exclude the serum samples with alanine aminotransferase concentration more than twice the upper limit of normal and an eGFR less than $60 \text{ ml/min/1.73 m}^2$. The total number of patient samples was 220 (78 males and 142 females).

To elucidate factors related to sdLDL-C concentrations in all subjects, this study performed the multiple regression analysis with sdLDL-C concentration as the dependent variable and with age, glucose, creatinine, TC, TG, HDL-C, calculated LDL (cLDL-C) and direct measured LDL-C (dLDL-C) as independent variables. The stepwise regression analysis identified TC, HDL-C, dLDL-C, and cLDL-C (Model I) as significant variables ($p < 0.001$), $R^2 = 0.88$, and the standard error of the estimated (SE) of 0.236 mmol/L as shown in Table 5. 3. The best fit of the linear regression equation was as follows:

$$\text{sdLDL-C}_{\text{mmol/L}} = 0.594 (\text{TC}) - 0.512 (\text{HDL-C}) + 0.457 (\text{dLDL-C}) - 0.784 (\text{cLDL-C}) - 0.436.$$

Table 5.3 Multiple Regression Parameters for sdLDL-C concentrations (Model I)

Predictors	Regression parameters ^a				r (zero order)
	β^b	Std. Error	t-value	p-value	
β_0 , Constant	-0.436	0.081	-5.409	< 0.0001	
β_1 , (TC)	0.594	0.044	13.471	< 0.0001	0.808
β_2 , (HDL-C)	-0.512	0.053	-9.594	< 0.0001	-0.150
β_3 , (dLDL-C)	0.457	0.068	6.688	< 0.0001	0.827
β_4 , (cLDL-C)	-0.784	0.059	-13.253	< 0.0001	0.763
R^2_{adj} (SE) ^c	0.883 (0.236)				

^a The multiple regression formula is

$$\text{sdLDL-C} = \beta_0 + \beta_1[\text{TC}] + \beta_2[\text{HDL-C}] + \beta_3[\text{dLDL-C}] + \beta_4[\text{cLDL-C}]$$

^b Coefficient of variable

^c Adjusted R-squared (Standard error of the estimate)

The coefficients for TC and HDL-C were near the same magnitude but with the opposite signs. Therefore, these studies substitute non-HDL-cholesterol (TC minus HDL-C) for the independent variables TC and HDL-C. By using non-HDL-C concentration (Model II), the stepwise regression analysis identified non-HDL-C, cLDL-C, and dLDL-C as significant independent variables (p -value < 0.0001) $R^2 = 0.88$ and the SE of 0.238 mmol/L as shown in Table 5.4.

The best fit of the linear regression equation was as follows:

$$\text{sdLDL-C}_{\text{mmol/L}} = 0.575 (\text{non-HDL-C}) + 0.417 (\text{dLDL-C}) - 0.724 (\text{cLDL-C}) - 0.306$$

In addition, non-HDL-C provided the strongest relationship ($r = 0.864$) with sdLDL-C followed by dLDL-C ($r = 0.827$), TC ($r = 0.808$), cLDL-C ($r = 0.763$) and HDL-C ($r = -0.150$). Therefore, the linear equation by using the non-HDL-C (Model II) was selected for calculating of sdLDL-C concentration.

Table 5.4 Multiple Regression Parameters for sdLDL-C concentrations (Model II)

Predictors	Regression parameters ^a				r (zero order)
	β^b	Std. Error	t-value	p-value	
β_0 , Constant	-0.306	0.048	-6.420	< 0.0001	
β_1 , (NonHDL-C)	0.575	0.043	13.268	< 0.0001	0.864
β_2 , (dLDL-C)	0.417	0.066	6.341	< 0.0001	0.827
β_3 , (cLDL-C)	-0.724	0.051	-14.120	< 0.0001	0.763
R^2_{adj} (SE) ^c	0.880 (0.238)				

^a The multiple regression formula is

$$\text{sdLDL-C} = \beta_0 + \beta_1[\text{NonHDL-C}] + \beta_2[\text{dLDL-C}] - \beta_3[\text{cLDL-C}]$$

^b Coefficient of variable

^c Adjusted R-squared (Standard error of the estimate)

5.5 Relationship between Measured and Calculated sdLDL-C Values

The association between the measured and the calculated sdLDL-C concentrations obtained from overall study samples were shown in Figure 5.5. For the scatter plot (Figure 5.5A), the linear regression equation obtained between the calculated (y) and the measured (x) sdLDL-C were $y = 0.867x + 0.198$ (95% confidence interval, 0.829 to 0.904 for the slope and 0.143 to 0.253 mmol/L for the y-intercept) with a correlation coefficient of 0.936. The mean bias and the standard deviation of the residuals (Sy/x) were -0.0001 and 0.220 mmol/L, respectively. Moreover, the paired Student *t*-test revealed no significant mean difference between the measured and the calculated sdLDL-C concentrations (p -value = 0.072).

Besides the use of simple linear regression to evaluate correlation, this study also analyzed the other regression statistics, Deming and Passing-Bablok as displayed in Table 5.5. Deming regression statistics were $y = 0.921x + 0.129$ (95% CI, 0.867 to 0.974 for the slope and 0.063 to 0.194 mmol/L for the y-intercept). Passing-Bablok regression equations were $y = 0.918x + 0.129$ (95% CI, 0.875 to 0.962 for the slope and 0.074 to 0.173 mmol/L for the y-intercept). All regression

analyses showed a high correlation between the measured and the calculated sdLDL-C concentrations.

To assess the degree of agreement between the calculated and measured sdLDL-C concentrations, the study performed the Bland – Altman plot as shown in Figure 5.5B. The average difference reported from the calculated sdLDL-C minus the measured sdLDL-C and the standard deviations (SD) of the difference were 0.02 mmol/L, 0.49 and -0.44 mmol/L, respectively.

Table 5.5 Correlation between Calculated versus Measured sdLDL-C values

Type	Coefficient (95% CI)	
	Slope	Intercept (mmol/L)
Linear regression	0.867 (0.829 to 0.904)	0.198 (0.143 to 0.253)
Deming regression	0.921 (0.867 to 0.974)	0.129 (0.063 to 0.194)
Passing-Bablok regression	0.918 (0.875 to 0.962)	0.129 (0.074 to 0.173)

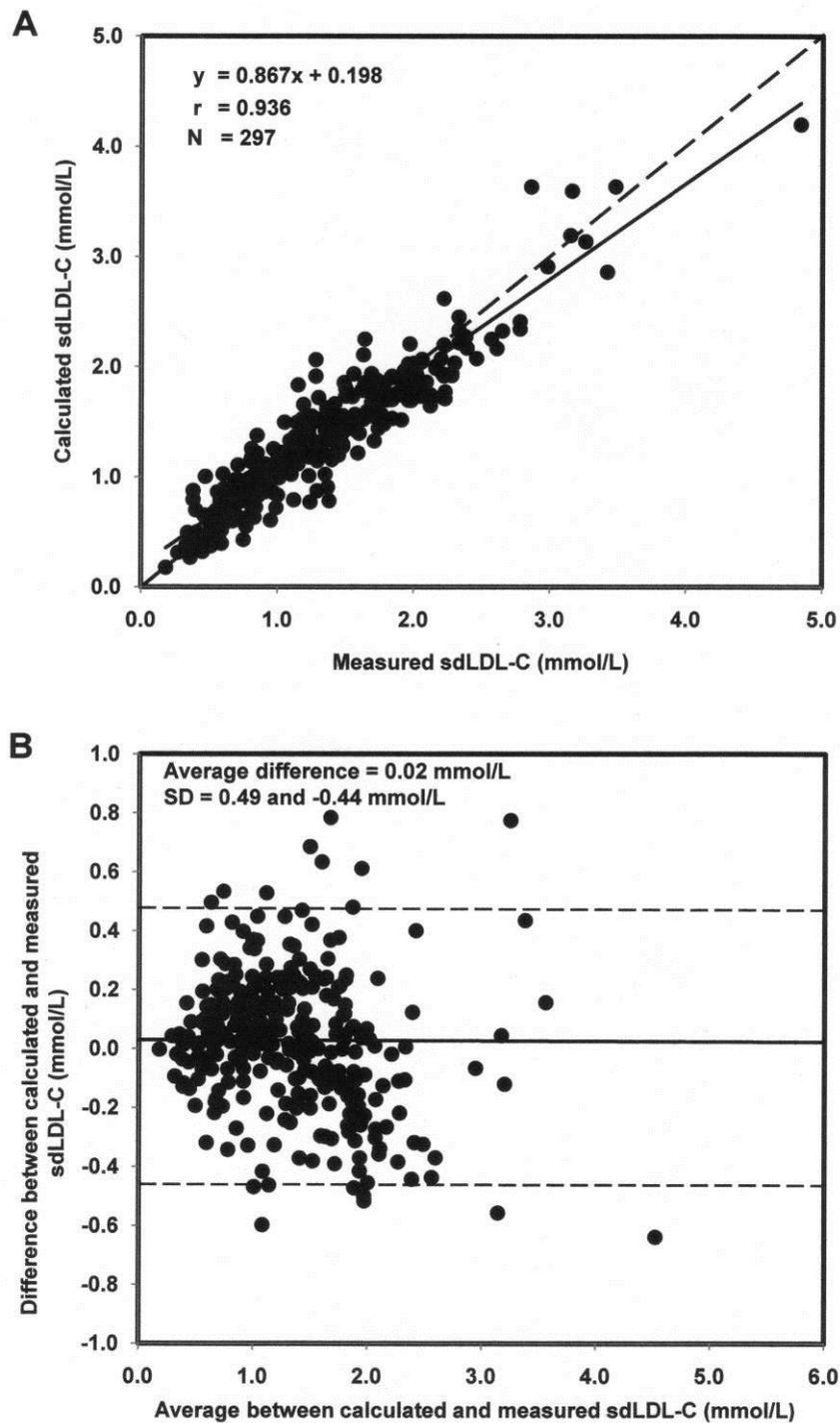


Figure 5.5 (A) Correlation graph of the calculated and measured sdLDL-C values.

Linear regression of the measured (x) and the calculated (y) sdLDL-C concentrations, $y = 0.867x + 0.198$; $r = 0.936$.

(B) Bland – Altman difference plot of the calculated and measured sdLDL-C values. The average difference from the calculated sdLDL-C minus the measured sdLDL –C and the SD of the difference were -0.44 and 0.49 mmol/L, respectively.

5.6 Effect of TC, TGs, HDL-C and LDL-C Concentrations on the estimated sdLDL-C

The study used the difference plot to examine the effect of TC, TG, HDL-C and LDL-C concentrations on the estimated sdLDL-C concentration.

The difference between the calculated sdLDL-C and the measured sdLDL-C against the independent variable of TC concentrations ranging from 2.00 to 14.90 mmol/L. These experimental TC concentrations did not significantly affect the bias error of calculated sdLDL-C; $r = 0.063$, $p = 0.268$ (Figure 5.6). The difference between the calculated sdLDL-C and the measured sdLDL-C against the independent variable of TG concentrations ranging from 0.42 to 4.48 mmol/L. These TG concentrations did not significantly affect the bias error of calculated sdLDL-C; $r = 0.04$, $p = 0.492$ (Figure 5.7).

The difference between the calculated sdLDL-C and the measured sdLDL-C against the independent variable of HDL-C concentrations ranging from 0.47 to 2.91 mmol/L. These HDL-C concentrations did slightly significant affect the bias error of calculated sdLDL-C; $r = 0.126$, $p = 0.030$ (Figure 5.8). For LDL-C concentrations, the difference between the calculated sdLDL-C and the measured sdLDL-C against the independent variable of LDL-C concentrations ranging from 0.90 to 13.50 mmol/L. These LDL-C concentrations did not significantly affect the bias error of calculated sdLDL-C; $r = 0.052$, $p = 0.371$ (Figure 5.9).

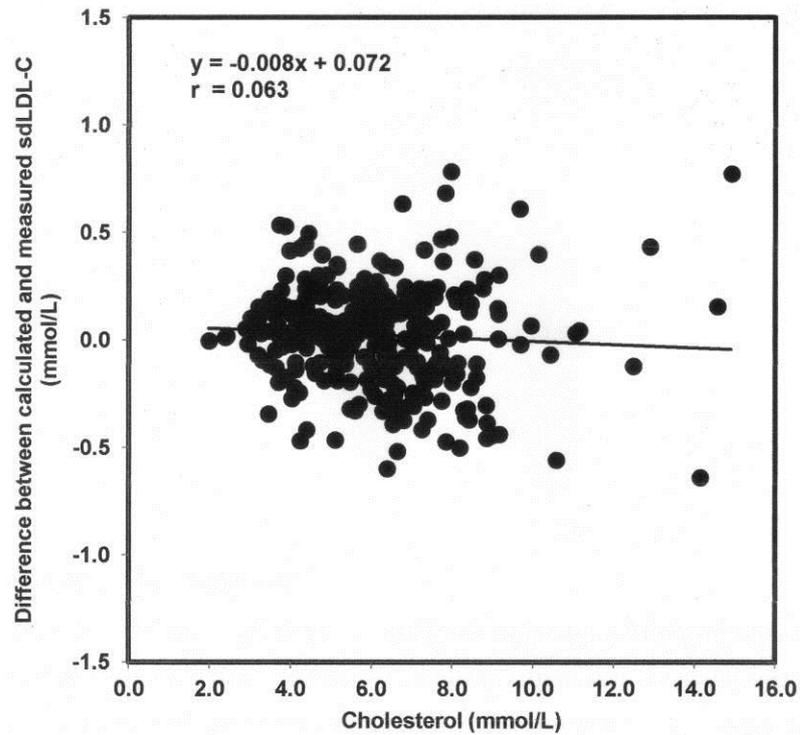


Figure 5.6 Calculation of the difference between the calculated sdLDL-C minus measured sdLDL-C against the concentrations of TC. Equation for the line: $y = -0.008x + 0.072$.

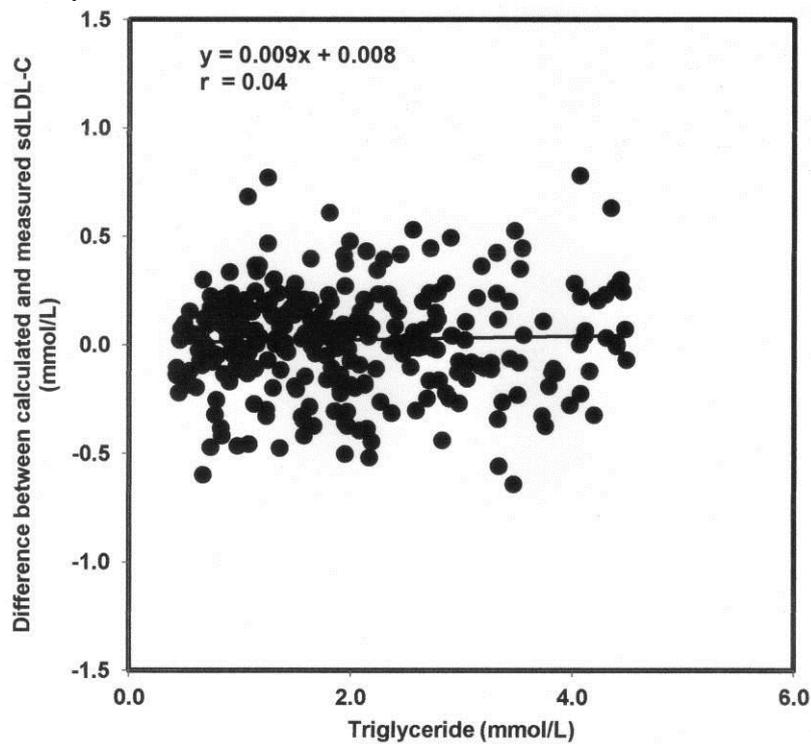


Figure 5.7 Calculation of the difference between the calculated sdLDL-C minus measured sdLDL-C against the concentrations of TG. Equation for the line: $y = 0.009x + 0.008$.

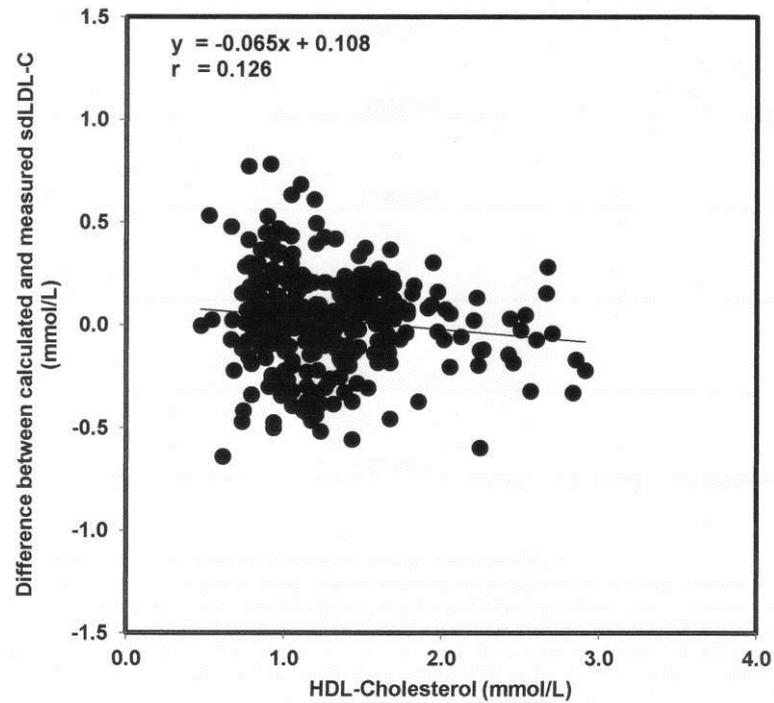


Figure 5.8 Calculation of the difference between the calculated sdLDL-C minus measured sdLDL-C against the concentrations of HDL-C. Equation for the line: $y = -0.065x + 0.108$.

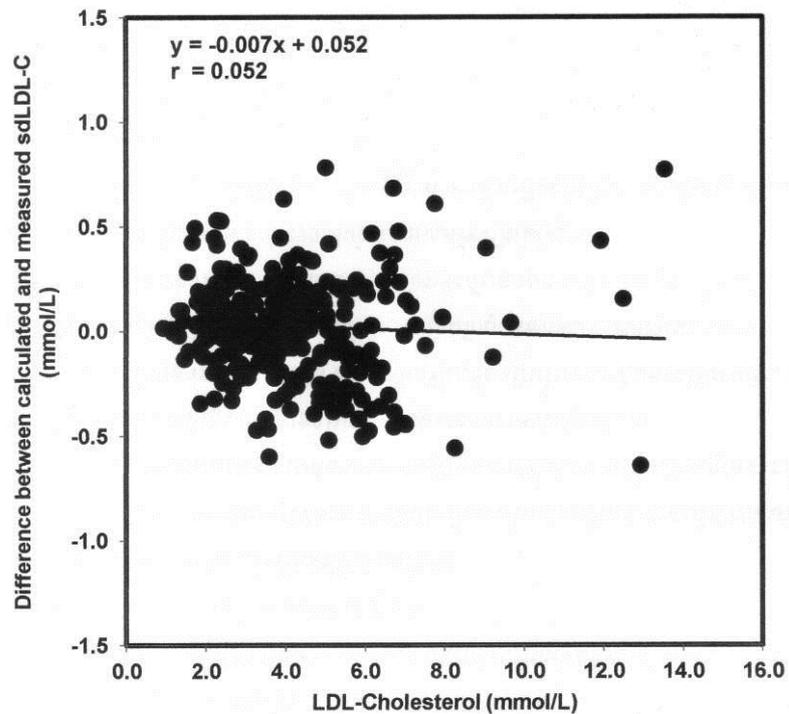


Figure 5.9 Calculation of the difference between the calculated sdLDL-C minus measured sdLDL-C against the concentrations of LDL-C. Equation for the line: $y = -0.007x + 0.052$.

5.7 Regression Analyses for Subgroups

The regression analyses between the measured and the calculated sdLDL-C values obtained from the non-HDL-C equation for subgroups sex, age, chronic kidney disease stages and fasting plasma glucose categories were analyzed.

The association between the measured (x) and the calculated (y) sdLDL-C concentrations for male and female were shown in Figure 5.10A and 5.10B, respectively. The linear regression statistics for male was $y = 0.825x + 0.239$ and $r = 0.919$. The mean bias and the Sy/x were -0.215 and 0.211 mmol/L, respectively. For female, the linear regression equation was $y = 0.884x + 0.184$ with $r = 0.942$. The mean bias and the Sy/x were -0.027 and 0.226 mmol/L, respectively.

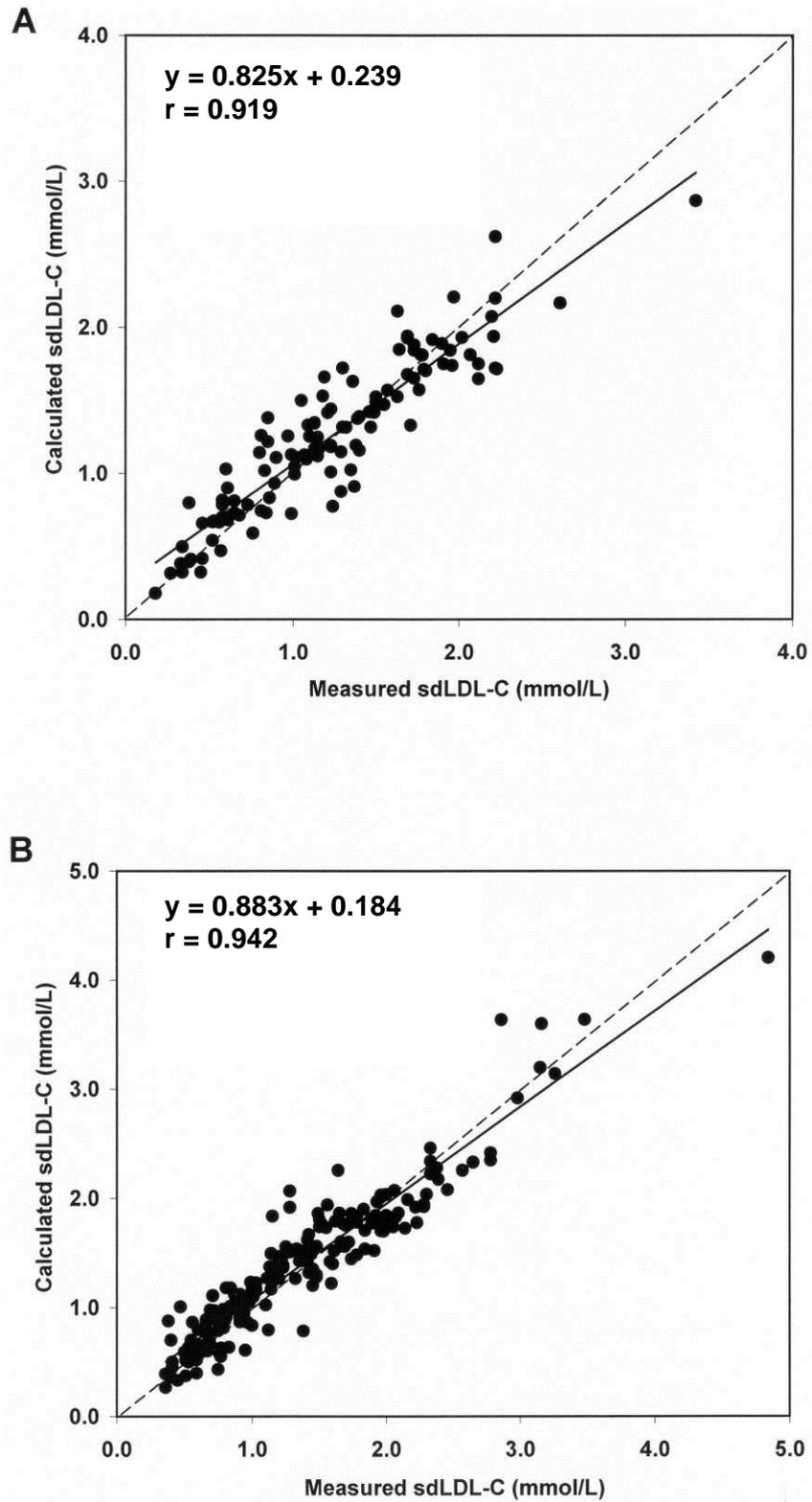


Figure 5.10 Correlation graphs of the calculated and measured sdLDL-C values in sex (A: male, B: female).

The associations between the measured (x) and the calculated (y) sdLDL-C concentrations for age groups stratified by decade. The linear regression equation for age < 50 years (Figure 5.11A), between 51 and 60 years (Figure 5.11B), between 61 and 70 years (Figure 5.11C) and age > 70 years (Figure 5.11D) were $y = 0.791x + 0.280$ with $R^2 = 0.819$, $y = 0.861x + 0.190$ with $R^2 = 0.906$, $y = 0.966x + 0.858$ with $R^2 = 0.892$ and $y = 0.870x + 0.227$ with $R^2 = 0.816$, respectively. The mean bias and the Sy/x were -0.006 and 0.213 mmol/L, 0.012 and 0.221 mmol/L, -0.042 and 0.218 mmol/L, and -0.085 and 0.220 mmol/L, respectively (Figure 5.11).

The associations between the measured (x) and the calculated (y) sdLDL-C concentrations for CKD stage ranging from I to V. The linear regression equation for CKD stage I (Figure 5.12A) was $y = 0.871x + 0.169$ and $R^2 = 0.907$. The mean bias and the Sy/x were -0.007 and 0.195 mmol/L, respectively. The linear regression equation for CKD stage II (Figure 5.12B) was $y = 0.881x + 0.161$ and $R^2 = 0.857$. The mean bias and the Sy/x were 0.007 and 0.242 mmol/L, respectively. The linear regression equation for CKD stage III (Figure 5.12C) was $y = 0.865x + 0.246$ and $R^2 = 0.854$. The mean bias and the Sy/x were -0.097 and 0.199 mmol/L, respectively. Using CKD stage IV (Figure 5.12D), the linear regression equation was $y = 0.795x + 0.353$ and $R^2 = 0.956$. The mean bias and the Sy/x were -0.098 and 0.112 mmol/L, respectively. For CKD stage V (Figure 5.12E), the linear regression equation was $y = 0.881x + 0.298$ and $R^2 = 0.872$. The mean bias and the Sy/x were -0.140 and 0.334 mmol/L, respectively (Figure 5.12).

The association between the measured (x) and the calculated (y) sdLDL-C concentrations for fasting plasma glucose categories divided by the criteria of plasma glucose level for the diagnosis of diabetes mellitus (156). The linear regression equation obtained from normal glucose regulation group (Figure 5.13A) was $y = 0.883x + 0.149$ with $R^2 = 0.892$. The mean bias and the Sy/x were 0.0007 and 0.212 mmol/L, respectively. The linear regression statistics obtained from impaired fasting glucose level group (Figure 5.13B) were $y = 0.892x + 0.21$, $R^2 = 0.855$. The mean bias and the Sy/x were -0.069 and 0.236 mmol/L, respectively. For the group of diabetes mellitus (Figure 5.13(C)), the linear regression equation was $y = 0.797x + 0.298$, $R^2 = 0.881$. The mean bias and the Sy/x were -0.022 and 0.206 mmol/L, respectively (Figure 5.13).

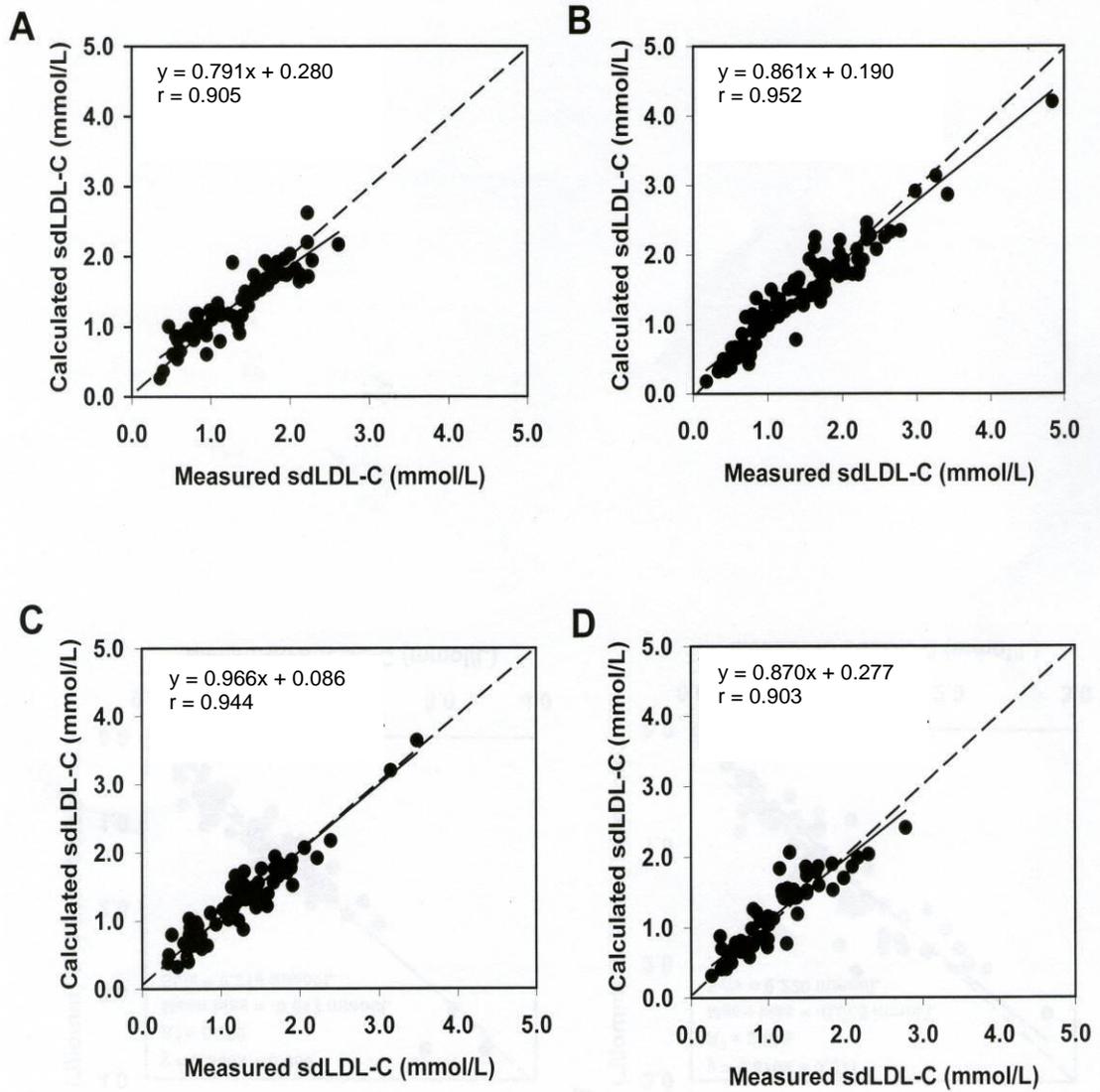


Figure 5.11 Correlation graphs of the calculated and measured sdLDL-C values in age group

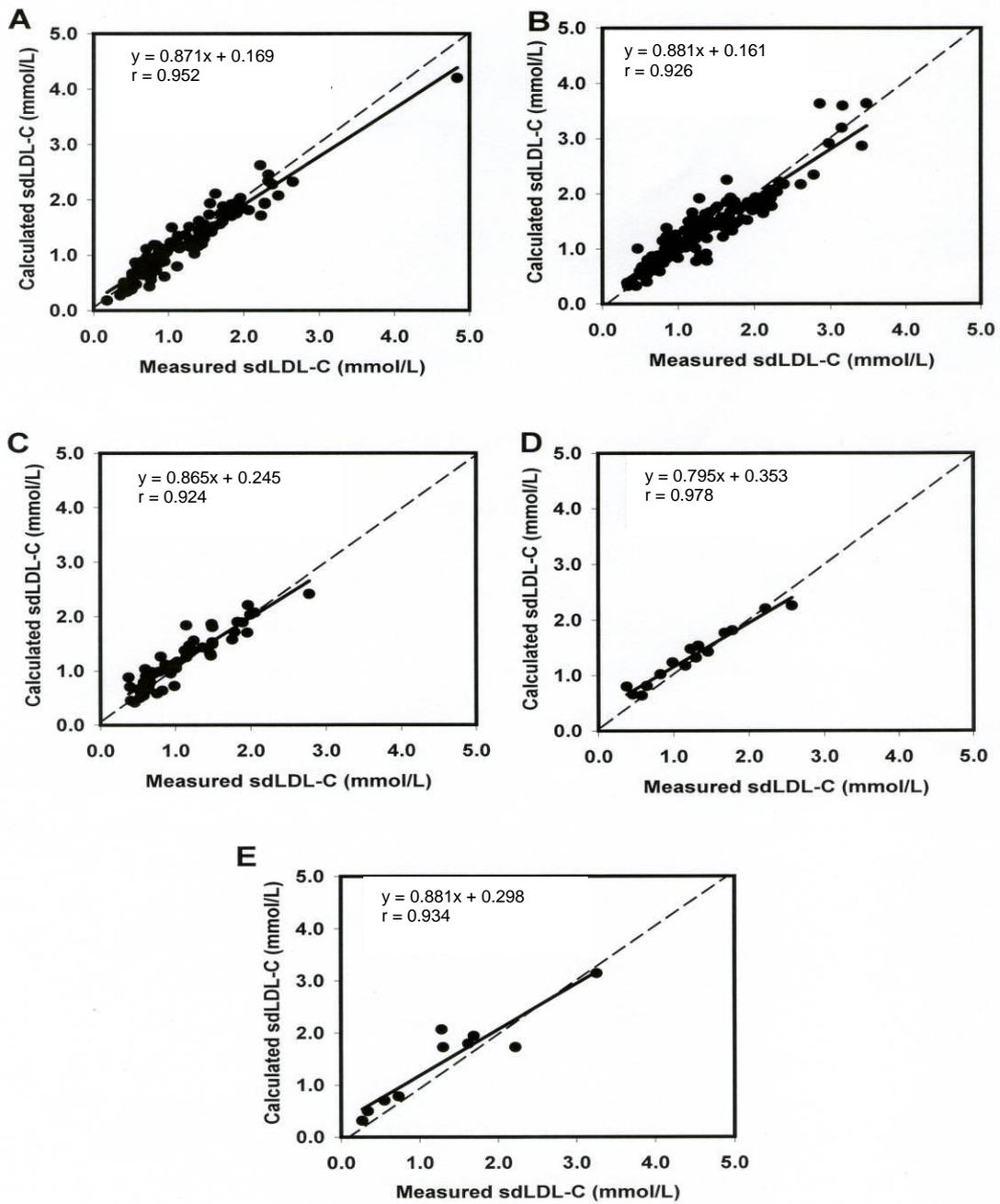


Figure 5.12 Correlation graphs of the calculated and measured sdLDL-C values in CKD stage.

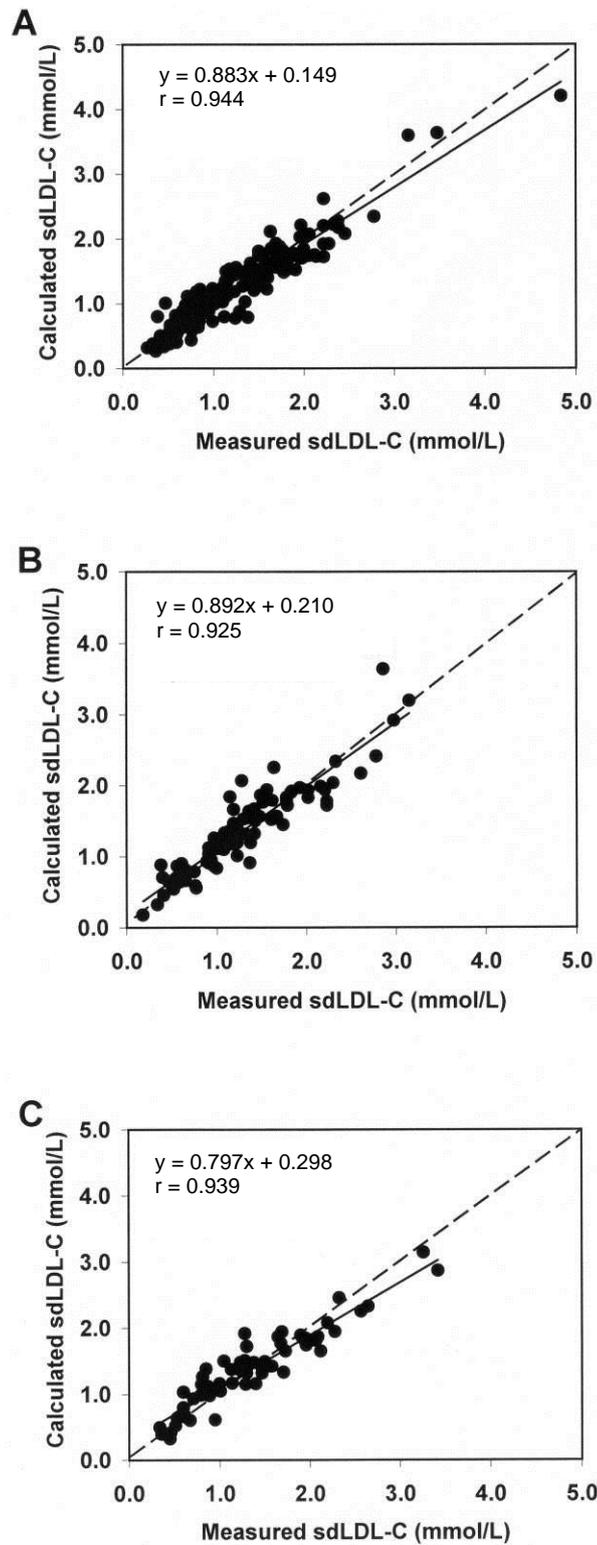


Figure 5.13 Correlation graphs of the calculated and measured sdLDL-C values in levels of plasma glucose

Table 5.6 tabulates the regression analyses between the measured and the calculated sdLDL-C values obtained from the non-HDL-C equation for subgroups. The relationship by least-squares regression analysis was tight and consistent across subgroups of gender, age, chronic kidney disease stages and fasting plasma glucose categories. Here the slopes and the y-intercepts showed consistent direction, ranging from 0.791 to 0.966 and 0.149 to 0.353 mmol/L, respectively. There were no significant differences in the slope or intercept for the regression equations for any of the subgroup comparisons ($p > 0.37$). All correlation coefficient values were greater than 0.85. The mean bias between measured and calculated sdLDL-C values was small, ranging from -0.215 to 0.012 mmol/L.

Table 5.6 Regression analyses between Measured and Calculated sdLDL-C for subgroups

	n	r	SE	Bias (mmol/L)	Coefficient (95% CI)	
					Slope	Intercept
Gender						
Male	115	0.919	0.052	-0.215	0.825 (0.759 - 0.891)	0.239 (0.148 - 0.329)
Female	182	0.942	0.053	-0.027	0.844 (0.838 to 0.930)	0.184 (0.114 to 0.254)
<i>p</i> -value					0.914	0.993
Age (years)						
≤ 50	63	0.905	0.066	-0.006	0.791 (0.696 - 0.886)	0.280 (0.144 - 0.416)
51 – 60	103	0.952	0.074	0.012	0.861 (0.806 - 0.915)	0.190 (0.099 - 0.281)
61 – 70	66	0.944	0.080	-0.042	0.966 (0.882 - 1.05)	0.086 (-0.035 - 0.206)
> 70	65	0.903	0.071	-0.085	0.870 (0.766 - 0.974)	0.227 (0.101 - 0.352)
<i>p</i> -value					0.447	0.553
CKD stage						
I	93	0.952	0.075	-0.007	0.871 (0.813 - 0.929)	0.169 (0.085 - 0.253)
II	129	0.926	0.055	0.007	0.881 (0.818 - 0.944)	0.161 (0.062 - 0.260)
III	50	0.924	0.088	-0.097	0.865 (0.761 - 0.969)	0.245 (0.118 - 0.373)
IV	15	0.978	0.132	-0.098	0.795 (0.693 - 0.897)	0.353 (0.212 - 0.494)
V	10	0.934	0.287	-0.140	0.881 (0.738 - 1.156)	0.298 (-0.141 - 0.737)
<i>p</i> -value					0.568	0.544
Fasting glucose (mmol/L)						
< 5.5	146	0.944	0.057	0.0007	0.883 (0.832 - 0.933)	0.149 (0.076 - 0.223)
5.5 to < 7.0	89	0.924	0.066	-0.069	0.892 (0.813 - 0.970)	0.210 (0.096 - 0.324)
> 7.0	62	0.939	0.080	-0.022	0.797 (0.722 - 0.873)	0.298 (0.183 - 0.414)
<i>p</i> -value					0.492	0.708
All Subject	297	0.917	0.277	-0.025	0.842 (0.802 - 0.882)	0.217 (0.156 - 0.278)

CHAPTER VI

DISCUSSION

Several epidemiological studies have been demonstrated that many patients with cardiovascular disease had LDL-C levels in the same range compared to healthy subjects, whereas the distribution of LDL particle size shift toward smaller (9,11,12,166). The recent report from the Québec cardiovascular study has confirmed that a predominance of sdLDL is a strong and independent predictor of coronary heart disease in the first 7 years of follow-up (73). Thus, sdLDL particles are believed to be a more atherogenic compared with large buoyant LDL (lbLDL) particles. The sdLDL particles more readily penetrate the arterial wall, and show a higher affinity to the intimal proteoglycans, a more prolonged plasma half-life, a lower binding affinity for LDL receptor, and a lower resistance to oxidative stress than lbLDL. Koba et al identified that the cholesterol concentration carried on sdLDL was significantly higher in severe coronary heart disease than in mild disease and its concentrations were associated with the severity of coronary atherosclerosis independently of the levels of LDL-C, HDL-C, apolipoprotein B, and non-HDL cholesterol in the overall cardiovascular disease patients (167). Furthermore, sdLDL-C may be a good biomarker to assess response to therapeutic interventions in type 2 DM patients with dyslipidemia (168).

The results indicated that the mean values for sdLDL-C were likely elevated with increasing degree of impaired glucose metabolism (Table 5.2). These results confirm the Nozue et al study that sdLDL-C levels were significantly higher in the patients with metabolic syndrome and insulin resistance (169). Therefore, sdLDL-C may be a potential factor for screening patients with type DM.

LDL-C can be determined by calculation using the Friedewald formula (cLDL-C) (19) and directly measured (dLDL-C) by specially designed assays (23,170). Using Friedewald equation, $cLDL-C \text{ (in mg/dL)} = TC - (HDL-C) - (TG / 5)$, a value of TG dividing by 5 represents a VLDL-C concentration. An

overproduction of the TG-enriched large VLDL causing high generation of sdLDL might lead to overestimation of VLDL-C and underestimation of cLDL-C concentrations. The difference between the cLDL-C, and dLDL-C, has been ascribed to variation in TG, HDL-C, and potentially presence of sdLDL. The study determined that one can easily estimate sdLDL-C from the equation as $\text{sdLDL-C} = 0.575 (\text{non-HDL cholesterol}) + 0.417 (\text{dLDL-C}) - 0.724 (\text{cLDL-C}) - 0.306$. The estimated sdLDL-C appeared reliable across a wide spectrum of sdLDL-C from 0.18 to as high as 4.84 mmol/L. The results clearly support a strong linear relationship between measured and calculated sdLDL-C values, with R^2 of 0.88 (Figure 5.5A). The paired *t*-test revealed no significant mean difference (p -value = 0.072). The difference between measured and calculated sdLDL-C values were independent of the TC ranging from 2.00 to 14.90 mmol/L (Figure 5.6), TG ranging from 0.42 to 4.48 mmol/L (Figure 5.7), HDL-C ranging from 0.47 to 2.91 mmol/L (Figure 5.8), and dLDL-C ranging from 0.90 to 13.5 mmol/L (Figure 5.9). Moreover, the differences between various sub-group of individuals such as gender, age-group, chronic kidney disease stages and fasting plasma glucose categories were not statistically significant (Table 5.6). The mean bias between measured and calculated sdLDL-C values was small, ranging from -0.44 to 0.49 mmol/L, which demonstrated that the equation may be reliable for the general population.

The results of study showed that non-HDL cholesterol levels gave the strongest relationship ($r = 0.864$) with sdLDL-C levels followed by dLDL-C ($r = 0.827$), TC ($r = 0.808$), cLDL-C dLDL-C ($r = 0.763$), and HDL-C ($r = -0.150$) (Table 5.3 – 5.4). Non-HDL-cholesterol is define as the difference between TC and HDL-C which represents the amount of cholesterol carried on all proatherogenic apo-B-containing particles, chylomicrons, VLDL, VLDL remnants, IDL, LDL, and lipoprotein (a). It has been known that the large triglyceride-rich VLDL particles can be ultimately catabolized to sdLDL particles (171). Thus, the level of non-HDL cholesterol shows a strong positive correlation with the level of sdLDL-C.

LDL-C is the primary target of lipid-lowering therapy and is used the classify patients into various cardiovascular disease risk categories (172). Using only LDL-C level for risk classification may limit the number of otherwise undertreated

high risk patients with atherogenic lipoprotein phenotype (173). Recent observational and intervention studies suggest that the predictive value of non-HDL cholesterol for cardiovascular risk and mortality is better than LDL-C (171,174-175). In addition, the close association between non HDL-C and sdLDL-C add additional support for using non-HDL – C level as predictor of CVD mortality.

Many available measurements of sdLDL such as density gradient ultracentrifugation (24-26), gradient gel electrophoresis (27), tube gel electrophoresis (28) and nuclear magnetic resonance (29) require special equipments and a lengthy analytical time and are, therefore, still unsuitable for general clinical use and screening. Moreover, currently available methods considerably vary limiting their clinical usefulness (176). The assessment of patient's cardiovascular disease risk based on LDL phenotype was different depending on the method used. Complete agreement among LDL subclass phenotypic occurred in only 8% of cases (176). Therefore, a standardization program for measuring LDL subclasses should be considered. The sdLDL-C assay is not more technically demanding than that for LDL-C. The method is typically based on the technique to use well-characterized surfactants and enzymes that selectively react with certain groups of lipoproteins. Because sdLDL-C is one part of the LDL-C typically use to assess the risk for CVD, it may be easier to standardize than those measurements for lipid subclasses.

In calculating sdLDL-C, three of the parameters (TC, TG, and HDL-C) are currently standardized, while that of LDL-C is not. Although most direct measurement methods for LDL-C have received Centers for Disease Control and Prevention certification from the Cholesterol Reference Method Laboratory Network (CRMLN) (133), the accuracy may vary. Miller WG et al found that seven direct methods for measuring LDL-C failed to meet the NCEP total error goals especially for samples from patients with CVD and/or dyslipidemia (177). Variation in the results for LDL-C may have contributed to the variation found in our study for the calculated sdLDL. The coefficients from this study are applicable to the dLDL-C based on the particular liquid selective detergent method used in the study. Other methods, such as elimination, selective solubilization, and enzyme selective protecting methods, for dLDL-C may show different results. Extending these results to other method for dLDL-C would require performance with those reagents.

Standardization of all the assays would reduce the error in the estimation of sdLDL-C using our method.

Although the importance of measurement of sdLDL has been well recognized, there has been no standard assay procedure in general clinical use. This study developed a convenient equation of calculating sdLDL-C in serum from commonly available measurement of non-HDL-C and both direct and calculated LDL-C. Additionally a simple method for the calculation of sdLDL-C in serum without requiring specialized laboratory measurement will provide a cost-effective method for screening patients for the risk of CVD. Furthermore, it may be a particular importance in clinical practice and public-health consequences for screening of abnormalities in the metabolism of lipoproteins. The identification of a simple inexpensive marker for sdLDL particles may pre-select patients who would most benefit from a more definitive subfraction workup. This study can play an important role in helping clinicians to embrace a calculated sdLDL-C as a new addition approach to CVD risk.

The study has not evaluated the performance of our proposed equation in specific patient groups having abnormal lipoprotein metabolism such as CVD, kidney disease, DM type 2, MS, and type III hyperlipoproteinemia. Future studies are needed to add confirmation that the equation can provide the potential application in all population.

Small dense LDL-C appears to be an independent risk factor for CVD, independent of LDL-C, because patients with CVD may have LDL-C within the reference interval, but increased sdLDL (9, 11, 12, 166). Non-HDL and apoB may represent improved measurement over LDL-C, but they do not necessarily capture the increased risk associated with increased sdLDL (167).

CHAPTER VII

CONCLUSION

Numerous studies have indicated that an increased small dense LDL (sdLDL) resulting from changes in abnormality of lipoprotein metabolism closely associates with increased risk of CVD and cerebrovascular disease. In addition, atherogenic dyslipidemia, as demonstrated by sdLDL, is closely associated with the MS and IR.

This study determined that one can easily estimate sdLDL-C from measurements of classic plasma lipids. The calculated sdLDL-C was developed on the hypothesis that the calculated LDL-C (cLDL-C) by using the Friedewald formula may differ from the direct measurement (dLDL-C), and this difference may depend on presence of sdLDL particles in addition to variation in TGs and HDL-C concentrations. Using stepwise regression analysis identified non-HDL cholesterol, cLDL-C and dLDL-C as significant variables ($p < 0.001$), $R^2 = 0.88$ and the SE of 0.238 mmol/L. The best fit of the linear regression equation was

$$\text{sdLDL-C} = 0.575 (\text{non-HDL-C}) + 0.417 (\text{dLDL-C}) - 0.724 (\text{cLDL-C}) - 0.306.$$

Non-HDL cholesterol provided the strongest relationship ($r = 0.866$) with sdLDL-C followed by dLDL-C ($r = 0.827$), TC ($r = 0.808$), cLDL-C ($r = 0.763$) and HDL-C ($r = -0.150$). Our results clearly support a strong linear relationship between measured and calculated sdLDL-C values, with R^2 of 0.88. The paired t -test revealed no significant mean difference (p -value = 0.072). The difference between the measured and the calculated sdLDL-C values was independent of the TC, TG, HDL-C and dLDL-C concentrations. Moreover, the differences between various sub-group of individuals such as gender, age-group, CKD stages and fasting plasma glucose categories were not statistically significant which demonstrate that this equation may be reliable for the general population.

A simple method for the calculation of sdLDL-C in serum without requiring specialized laboratory measurement will provide a cost-effective method for screening patients for the risk of CVD. Furthermore, it may be a particular importance in clinical practice and public-health consequences for screening of abnormalities in the metabolism of lipoproteins. The identification of a simple inexpensive marker for sdLDL particles may pre-select patients who would most benefit from a more definitive subfraction workup. This study can play an important role in helping clinicians to embrace a calculated sdLDL-C as a new addition approach to CVD risk which should be considered worldwide in clinical practice.

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