

**COMPARISON OF DIRECT AND PRECIPITATION  
METHODS FOR ESTIMATION OF MAJOR SERUM  
LIPOPROTEINS IN HYPERTENSIVE PATIENTS**

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**Comparison of Direct and Precipitation Methods for Estimation of  
Major Serum Lipoproteins in Hypertensive Patients**

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## DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

Signature.....Date.....

**Mbithi Mulinge**

This thesis has been submitted for examination with our approval as University supervisors

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## **DEDICATION**

I dedicate this thesis to my wife Rachael Nzasu, my children Samuel Mulinge and Joan Mutanu who have been a pillar of strength as i undertook the study. May the almighty God bless you all.

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## **LIST OF ABBREVIATIONS**

<b>Apo-A</b>	Apo protein A
<b>Apo-B</b>	Apo protein B
<b>Apo-C</b>	Apo protein
<b>CVD</b>	Cardiovascular disease
<b>CETP</b>	Cholesteryl ester transfer protein
<b>EDTA</b>	Ethylene di amine tetra acetic acid
<b>HDLC</b>	High density lipoprotein cholesterol
<b>HDL-c</b>	High density lipoprotein
<b>HL</b>	Hepatic lipase
<b>IDL</b>	Intermediate density lipoprotein
<b>LCAT</b>	Lecithin-cholesterol acyltransferase
<b>LDLC</b>	Low density lipoprotein cholesterol
<b>LDL</b>	Low density lipoprotein cholesterol
<b>NCEP</b>	National Cholesterol Education Programme
<b>VLDL</b>	Very Low Density Lipoprotein Cholesterol
<b>TC</b>	Total cholesterol
<b>TGs</b>	Triglycerides
<b>sd-LDL</b>	Small, dense low-density lipoprotein
<b>sd-LDL-C</b>	Small, dense low-density lipoprotein cholesterol

### **DEFINITION OF OPERATIONAL TERMS**

- Atherosclerosis** : A disease of the arteries characterized by the deposition of plaques of Fatty material on the inner walls of arteries
- Homogeneous** : Of the same or a similar kind or nature
- Heterogeneous** : Made up of parts that are different or consisting of dissimilar or diverse ingredients or constituents
- Precipitation** : Creation of a solid in a solution or inside another solid during a chemical reaction or by diffusion in a solid.
- Supernatant** : Denoting the liquid lying above a solid residue after crystallization, precipitation, centrifugation, or other process.

## ABSTRACT

Application of automated direct assays for analysis of high density lipoproteins cholesterol is increasing in response to the need by clinical laboratories to cope up with increasing workloads. However, performance characteristics of homogeneous assays often differ in important aspects from those of the earlier precipitation methods. Calculation of low density lipoprotein cholesterol (LDLC) by precipitation method is based on total cholesterol (TC), triglycerides (TG's) and high density lipoproteins (HDL) thus cumulatively carries errors of individual methods. Our study sought to compare the two methods. A total of 384 samples were collected in the medical outpatient clinic, analyzed in clinical chemistry section of Kenyatta National Hospital for TC, TG's, HDL, and LDLC by direct assays. HDL and LDLC analyzed again by precipitation and Friedewalds formulae. The mean HDL concentration by direct and precipitation methods was  $1.52 \pm 0.47$  mmol/L and  $1.51 \pm 0.47$  mmol/L respectively while those of direct and Friedewald's formulae for LDLC was  $2.88 \pm 1.01$  mmol/L and  $2.89 \pm 0.47$  mmol/L respectively. There was no significant difference in mean for HDL by precipitation and direct method at all total cholesterol ranges as well as Friedewalds formulae and direct method for LDLC at all triglyceride ranges  $p > 0.05$ . Paired t test found no significant difference in the mean of HDL estimated by direct analytical methods and precipitation method  $p = 0.93$ . Average time for precipitation/calculated method was 45 minutes and direct was 20 minutes. There was no significant difference in LDLC estimated by direct analytical methods and Friedewald's formulae  $p = 0.91$  both precipitation and precipitation/calculated methods gave similar results. Therefore, Selection should be based solely on workload, availability and technical expertise.

## **CHAPTER ONE**

### **INTRODUCTION**

#### **1.1 Introduction to the study**

Total cholesterol in humans is distributed primarily among three major lipoprotein classes: very low density lipoproteins cholesterol (VLDL-C), low density lipoproteins cholesterol (LDL-C), and high density lipoproteins cholesterol (HDL-C) (Bachorik., & Ross,1995). Smaller amounts of cholesterol are also contained in two minor lipoprotein classes: intermediate density lipoprotein (IDL) and Lipoprotein (a). In normal individuals, the minor lipoprotein classes can be expected to contribute on average about 0.0621mmol/L to the total cholesterol measurement (Bachorik,& Ross, 1995) HDL-C and LDL-C are major lipoproteins of cholesterol in human plasma and major transporters of cholesterol in human plasma (Gordon, Castelli , Hjortland, Kannel & Dawber .1977). Measurements of these markers have been proposed as primary tools for risk assessment and monitoring of patients with risk of developing cardiovascular disease (Gregory et al., 2001).

Several studies have indicated that there is an inverse relationship between the risk for coronary heart disease and the plasma concentration of high density lipoprotein (HDL-C) cholesterol (Grundy,Cleeman,Merz,Brewer,Clark,Hunninghake,... & Stone, N. J. 2004). Implications of recent clinical trials for the national cholesterol education program adult treatment panel III guidelines. *Circulation*, 110(2), 227-239.Grundy et al., 2004). Possible mechanisms by which HDL-C might play a direct protective role have also been studied as well as conditions associated with elevated or depressed HDL-cholesterol levels (Langlois, & Blaton, 2006). These investigations have led to increased interest in the routine determination of HDL-cholesterol levels, aiding the assessment of risk for ischemic heart disease, as well as interest in further studies of the putative protective effect of HDL, and have stimulated efforts to understand better the capabilities and limitations of quantitative HDL methods (Miller, W. G., Myers, G. L., Sakurabayashi, I., Bachmann, L. M., Caudill, S. P., Dziekonski, A., ... & Nakajima, K.2010). Precipitation method involves precipitation of Apo -b lipoproteins followed by centrifugation at 3000 rpm for 10 minutes, the supernatant is used for estimation of HDL-C using a method

similar to that of total cholesterol (Bachorik, and Albers, 1986). LDL-C is estimated by use of Friedewald formulae which is based on total cholesterol (TC), Triglycerides (TG'S) and High density lipoproteins cholesterol (HDL-C) (Friedewald et al., 1972).

Measurements of HDL-C and LDL-C by direct methods offer the potential to improve both analytical and biological variability. The precision of HDL-C and LDL-C measurement would not depend upon the analytical variability in measurement of total cholesterol and low levels cholesterol in supernatants after precipitation (Eckel et al.,2002).Capabilities and limitations of quantitative HDL-C methods is not known, thus this study aimed at comparing quantitative analytical methods for major serum lipoproteins.

## **1.2 Statement of the problem**

In clinical practice, LDL-C is either estimated by the Friedewald formula or directly measured with a direct assay. Since the calculation is based on serum TG, TC, and HDL-C, it necessarily includes the accumulated errors in all three measurements. The validity of calculated LDLC values therefore does not depend on the accuracy of a sole direct assay, but rather on the accuracy of three other assays (TC, HDLC, and TG) with a mathematical calculation formula that estimates the amount of cholesterol in VLDLs, but it has been reported that at higher triglyceride levels, this ratio tends to decrease, rendering the original formula inaccurate (DeLong, Wood, Lippel, & Rifkind, 1986).

The Friedewald formula is nonlinear at triglycerides above 4.5mmol/L due to increased turbidity thus limiting the use in non-fasting specimens (Baruch et al., 2010).

The Friedewald formula assumes that the triglyceride to cholesterol ratio in Very Low Density Lipoprotein (VLDL) is constant. This ratio is altered in non-fasting samples (containing chylomicrons and chylomicron remnants). Consequently, the Friedewald's formula cannot be used for LDL-C calculation when the subject is not fasting, when serum TG >400 mg/dl or < 100mg/dL (Ahmadi et al., 2008) or in

patients with type III or type I hyperlipoproteinaemia (Rifai, N., Warnick, G. R., McNamara, J. R., Belcher, J. D., Grinstead, G. F., & Frantz, I. D. 1982). The use of this formula is also not recommended for Type II diabetes mellitus, nephrotic syndrome and chronic alcoholic patients, because, in these conditions too, the triglyceride to cholesterol ratio in VLDL is altered (Matas et al., 1994 ; Rubies-Prat et al., 1993).

Despite the widespread belief that the calculation or measurement of LDL cholesterol is standardized and reproducible, data indicates that results can vary significantly with methods from different manufacturers, and the calculated LDL cholesterol may not agree with measured LDL cholesterol (Contois, Warnick, & Sniderman, 2011).

Cholesterol measurement is challenging because clinically important differences in the lipoprotein concentrations are minor and even small analytical errors can contribute to misclassification of dyslipidemic patients (Kimberly et al., 1999).

Direct methods for HDLC and LDLC are easier to perform, faster but more expensive, while precipitation/calculated methods is more time consuming but less expensive and not suitable for use in busy clinical establishments.

### **1.3 Justification**

Adoption of automated direct assays for HDL-cholesterol (HDL-C) is increasing, driven by the need of clinical laboratories to cope up with increasing workloads. However, performance characteristics of direct assays often differ in important aspects from those of the conventional precipitation.

The findings generated from the study will aid clinical laboratories choose analytical methods for lipid profile analysis based on Laboratory setting including quantity of workload, availability of analytical machines and skilled technologists

The study will enable diagnosis and monitoring using non-fasting and postprandial samples enhancing management of cardiovascular conditions without compromising



the health of the patients. The findings will improve overall management and reduce risks of heart diseases associated with cholesterol

#### **1.4 Research Questions**

1. What are the concentrations of serum HDL-C and LDL-C by use of direct analytical methods?
2. What is the concentration of HDL-C and LDL-C using precipitation method and Friedwald equation?
3. Are concentrations of serum lipoprotein determined by precipitation/ Friedwald estimation comparable with those of direct assays?

#### **1.5 Hypothesis**

The mean concentrations of major serum lipoprotein estimated by direct method does not differ with that of precipitation method.

#### **1.6 Objectives**

##### **1.6.1 General Objective**

To compare precipitation and direct analytical methods of major serum lipoproteins

##### **1.6.2 Specific Objectives**

1. To determine HDL-C and LDL-C human serum concentration by direct analytical methods
2. To determine HDL-C and LDL-C serum concentration by precipitation method and Friedewald equation.
3. To compare direct and precipitation/calculated lipoprotein analytical methods

## **CHAPTER TWO**

### **LITERATURE REVIEW**

#### **2.1 Lipids**

Lipids consist of the diverse groups of molecules, which are nearly insoluble in water, but soluble in organic solvents (Fielding & Frayn, 2003). The most important lipids in the human body are cholesterol and cholesterol esters, fatty acids, triglycerides, glycerophospholipids, sphingolipids, bile acids, steroid hormones, and fat-soluble vitamins (Libby, Ridker, & Maseri, 2002). Lipids are structural components in biomembranes (glycerophospholipids, sphingolipids, and cholesterol); they function as hormones, precursors of hormones, signaling molecules, metabolic fuel (fatty acids), or energy storage (triglycerides), and aid in digestion of fat (bile acids) (Vance & Vance, 2002).

Cholesterol is an essential molecule for maintenance of membrane fluidity and permeability, and it serves as a precursor of bile acids, steroid hormones and vitamin D (Caserly & Topol 2004). Cholesterol homeostasis in the cells is maintained by the dietary absorption, de novo synthesis, metabolism to bile acids and sterol hormones, and excretion as bile acids (Hu et al., 2010). All human cells are capable of synthesizing cholesterol from acetate, which originate from the metabolism of carbohydrates or fats, but the liver and intestine are the main organs for de novo synthesis of cholesterol (Vance & Vance 2002). Dietary lipids and endogenous lipids synthesized by the liver and extra hepatic tissues are transported in the circulation with Apo lipoproteins as lipoprotein complexes (Grundy, 2011).

Lipids are either yield fatty acids when hydrolyzed or complex alcohols that can combine with fatty acids to form esters, for example, cholesterol ester forms from cholesterol and fatty acid (Unger, 2003)

Lipids are not soluble in the plasma water, thus they travel in micelle-like complexes composed of phospholipids and protein on the outside with cholesterol, cholesterol esters, and triglycerides on the inside (Arneson, & Brickell, 2007).

## 2.2 lipoproteins

The classification of lipoproteins has traditionally been based on the different density of lipoprotein particles separated by ultracentrifugation. The main classes of lipoproteins are chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), lipoprotein(a) [Lp(a)], and high-density lipoproteins (HDL) (Mahley, R. W., Innerarity, T. L., Rall, S. C., & Weisgraber, K. H. (1984). In spite of possible contaminations with adjacent lipoprotein fractions, ultracentrifugation has remained Review of the literature as a gold standard for lipoprotein research. According to their electrophoretic mobility, lipoproteins are classified to  $\alpha$ ,  $\beta$ , and  $\beta$  lipoproteins that are HDL, VLDL, and LDL classes, respectively (Larossa, 2004). Also chromatographic methods have been used for separation of different lipoprotein subclasses based on the size of lipoprotein particles or their immunoaffinity. High-resolution nuclear magnetic resonance (NMR) spectroscopy is a new technique to separate lipoprotein subclasses according to their different sizes (Langlois and Blaton 2006). Some of the small-size HDL and LDL particles are particularly atherogenic, therefore the possibility to separate these subclasses and estimate their proportion in the whole particle population could improve the risk assessment of CVD (Rizzo & Berneis 2006; Movva & Rader 2008).

Lipoproteins are a heterogeneous group of lipid populations differing in density, size, and lipid compositions, therefore classified according to their major apolipoproteins (Mahley, Innerarity, Rall, & Weisgraber, 1984).). All other lipoproteins except HDL contain apoB-48 (chylomicrons) or apoB-100 (VLDL, IDL, LDL, Lp [a]), which makes it possible to assess the amount of atherogenic lipoprotein particles in the circulation by the apoB measurement (Hattori et al., 1998). The major apolipoproteins of HDL particles are apoA-I and apoA-II. Apo lipoproteins A-I, A-II, C-I, C-II, C-III, and E can exchange between lipoproteins during the metabolism whereas apoB-48 and apoB-100 are non-exchangeable (Walldius, G., Jungner, I., Holme, I., Aastveit, A. H., Kolar, W., & Steiner, E. 2001). All lipoproteins share a similar particle structure: phospholipids, unesterified cholesterol, and apolipoproteins are on the surface and triacylglycerols, cholesterol esters and other neutral lipids are situated in the core of the particle (Planella et al., 1997). Apolipoproteins determine

the metabolism of lipoproteins: they direct lipoprotein transport and redistribution between tissues, function as cofactors for a variety of enzymes, and maintain the structure of lipoproteins (Mahley et al., 1984).

### **2.3 Metabolism of lipoproteins**

Lipoproteins transport lipids from the intestine and liver to the peripheral tissues (DeLong et al., 1986). During their metabolism lipoproteins exchange apolipoproteins with each other and modify their lipid composition with the exception of apoB-48 and apoB-100 (Vance & Vance 2002). The role of VLDL and LDL particles is to deliver cholesterol, triglycerides, and other lipids to peripheral tissues in a process called forward cholesterol transport (Estrada et al., 1990). Instead, HDLs transport excess cholesterol from the peripheral cells back to the liver in a process called reverse cholesterol transport (RCT) (Mattock et al., 1982). Finally, all lipoproteins are catabolized in the liver, kidney or peripheral tissues via apolipoprotein-receptor-interactions or other mechanisms (Lewis & Rader 2005)

Chylomicrons are secreted from the intestine after a fat-containing meal with apolipoprotein B-48 as a major protein component (Joven et al., 1990). They deliver fat as an energy source for muscles or to adipose tissue for storage. The liver uses free fatty acids from the circulation and excess carbohydrates, fat, and proteins from the meal to synthesize triacylglycerides, which form very low-density lipoproteins (VLDL) together with apolipoprotein B-100, cholesterol and other lipids (Ahmadi et al., 2008). ApoB synthesis is highly regulated by the availability of core lipids, the type of dietary fatty acids, and by the hormonal balance (Ginsberg & Fisher, 2009).

High-density lipoproteins (HDL) are secreted from the liver (70 %) or intestine (30%) as lipid-poor nascent particles that contain only apolipoprotein A-I and phospholipids (Harris et al., 1996). The maturation of HDL from nascent discoidal particle to larger, spherical HDL demands the action of lecithin-cholesterol acyltransferase (LCAT), which converts free cholesterol to cholesterol esters (Scanu & Edelstein 2008). These cholesterol esters are partly exchanged with triglycerides from apoB containing lipoproteins via cholesterol ester transfer protein (CETP)

before transport to the liver for catabolism as a part of reverse cholesterol transport (Lewis & Rader 2005). HDLs are further divided into larger, cholesterol ester-rich HDL particles and smaller HDL particles (Movva & Rader, 2008).

Excess carbohydrates and sugar in the diet may cause endogenous hypertriglyceridemia, which activates CETP and hepatic lipase (HL) leading to formation of small-size HDL particles (Gou et al. 2005). While LDL-Cholesterol is considered harmful when in excess, the elevation of HDL-Cholesterol is viewed as a positive cardiovascular biomarker for a patient. Elevated HDL-C has a beneficial effect for the vascular system, due to the role that HDL plays in the body (Albers & Marcovina, 1989). HDL removes excess cholesterol from tissues and routes it to the liver for reprocessing and/or removal. HDL-C are heterogeneous and polydisperse population of particles that are densest and generally smallest in size among the lipoproteins. Fourteen subclasses of HDL-C, each with varying proportions of lipids and proteins may be found depending on the analytical technique used for separation (Miller W.G. 2012).

#### **2.4 Low density lipoprotein cholesterol**

About 70% of plasma cholesterol occurs as LDL-C (Grundy et al., 2004). LDLs are chiefly involved in the transport of the cholesterol manufactured in the liver to the tissues, where it is used. Uptake of cholesterol into cells occurs when lipoprotein binds to LDL receptors on the cell surface. LDL is then taken into the cell and broken down into free cholesterol and amino acids (Grundy, et al., 2004).

Disorders involving a defect in or lack of LDL receptors are usually characterized by high plasma cholesterol levels (Ellerbe et al., 1990). The cholesterol cannot be cleared efficiently from the blood and therefore accumulates. This is the case in the inherited disorder familial hypercholesterolemia. High levels of LDL in the blood are associated with an increased risk of coronary heart disease (National Heart Lung and Blood Institute. 2002).

By definition, LDL comprises the population of particles with hydrated density between 1.006 and 1.063 kg/L (Gylling et al., 1995). This definition referring to LDL separated by sequential density ultra centrifugation, or the so-called beta quantification method combining ultra centrifugation and chemical precipitation, has seen the basis for measurement in most epidemiological studies (Rifai et al., 1992). The LDL population is subdivided into large, medium, small, and very small particles (Rizzo & Berneis 2007). The prevalence of small particles varies with age, sex, genetic factors, and diet (Rizzo & Berneis 2006). LDL can be separated into phenotype A with large, buoyant particles and to phenotype B with small, dense particles (Criqui, & Golomb, (1998). The proportion of phenotype B increases with increasing triglyceride levels and decreased levels of HDL-C. This lipid combination has been defined as atherogenic lipoprotein phenotype (Austin, King, Vranizan, & Krauss, 1990). These small LDL particles are more easily oxidized; one possible reason could be the increased content of polyunsaturated fatty acids (Rizzo & Berneis 2006b).

## **2.5 High density lipoprotein cholesterol (HDL-C)**

Previously, HDL-C was defined as the particles with a density between 1.063 and 1.21 kg/L, i.e., the bottom fraction after ultracentrifugation at a density of 1.063 kg/L was readjusted to a density of 1.21 kg/L and again subjected to ultracentrifugation (Bayer et al., 2005). Cholesterol recovered in this top fraction was taken as a measure of HDL-C. Because there is virtually no cholesterol in particles with a density .1.21 kg/L, in practice cholesterol was often simply measured in the 1.063 kg/L bottom fractions (Warnick, Kimberly, Waymack, Leary, & Myers 2008).

Ultracentrifugation as a separation technique is not only tedious and time-consuming, but the labile lipoproteins can be substantially altered by the high salt concentrations and centrifugal forces used (Warnick *et al.*, 2008). Furthermore, there is a plethora of different types of equipment, making conditions extremely difficult to reproduce from one laboratory to another, and separations are highly dependent on the skills of the technician. Achieving complete and reproducible recovery is difficult, even for experienced technologists, and fractions may be cross-contaminated (Contois et al.,

2012). Although ultracentrifugation has been highly useful in research and as a comparison method for validation of other methods, this approach is not considered practical for routine analytical measurements (Baruch et al., 2010). Therefore, in this study we compared both direct and precipitation methods for estimation of major serum lipoproteins

## **2.6 Precipitation methods for HDLC estimation**

Burstein Scholnick and Morfin (1970) of France pioneered the precipitation methods to separate lipoproteins rapidly on a preparative scale. They and other groups later adapted the preparative reagents as a convenient means for research and routine analytical separations of serum lipoproteins. One of the methods developed Chemical precipitation utilizes polyanions such as heparin and dextran, sometimes combined with divalent cations such as lead( $Mn^{2+}$ ) and manganese( $Mg^{2+}$ ), to selectively aggregate and render insoluble the lower density lipoproteins, leaving HDL-C in solution. The insoluble lipoproteins can then be sedimented by centrifugation at 3000 rpm. The resulting supernatant solution can be recovered by pipetting or decanting for cholesterol analysis as a measure of HDL-C (Miller et al., 2010).

Heparin with manganese chloride ( $MnCl_2$ ) was previously a popular early combination used in pioneering lipoprotein research at national institutes of health (Fredrickson, Levy, & Lindgren, 1968). Because commercial heparin preparations were somewhat inconsistent in properties and manganese ions ( $Mn^{2+}$ ) was observed to interfere with the early enzymatic assays, this combination was generally replaced for routine use by dextran sulfate or phosphotungstic acid, both used together with magnesium ions ( $Mg^{2+}$ ). Use of phosphotungstic acid or polyethylene glycol became more common in Europe (Warnick benerson & Albers., 1982). A major problem with the precipitation methods has been interference from increased triglycerides (TGs) which make aggregated lipoproteins lighter, preclude the sedimentation of the insoluble aggregates, and yield turbid supernatant. The resulting cloudy supernatant is contaminated with lipoproteins other than HDL-C, leading to an over assessment of HDL-C (Arneson, & Brickell, 2007).

Each of the precipitation reagents evolved with modifications reported to change the selectivity or performance (Warnick, Kimberly, Waymack, Leary, & Myers, 1981). For example, the heparin-Manganese chloride method was first described with 1.0 mol/L MnCl<sub>2</sub>. Subsequent studies concluded that this concentration was appropriate for serum specimens but that a higher concentration, 2.0 mol/L, improved specificity in Ethylene diamine tetra acetic acid (EDTA) plasma specimens, presumably to compensate for chelation of some of the divalent cation by EDTA and to avoid incomplete precipitation of Apo B-containing lipoproteins (Benlian, et al., 2000). Scharnagl et al., (2001) found that the Friedewald equation was inaccurate at lower LDL-C concentrations compared with ultracentrifugation reference method. A recent study reported poor agreement between results calculated with the Friedewald equation and the direct LDL-C assay from Siemens despite a good correlation, more than 25% of results differed by more than 30 mg/ dl (Scharnagl et al., 2001). The provision of a reliable standardization program was a factor in improving accuracy in HDL-C measurements, but imprecision remained a problem with the conventional precipitation methods.

## **2.7 Direct assays for HDLC estimation**

A major breakthrough was reported in 1994, with the first of a series of so-called “direct” methods capable of full automation and considered third generation (Baruch et al., 2010).). The new-generation homogeneous assays do not require off-line pretreatment and separation, eliminating the manual pipetting, mixing, and centrifugation steps (Iwasaki Y, *et al.*, 2006). The first reagent contained polyethylene glycol, which caused aggregation of the apo B-containing chylomicrons, VLDL-C and LDL-C (Nauck et al., 2002). The second reagent protected or blocked the aggregated lipoproteins with antibodies to apo B and apo C ((Hirano et al., 2009)). The third reagent included the cholesterol reaction enzymes (cholesterol esterase, cholesterol oxidase, and peroxidase), which reacted only with the unprotected HDL-C (Warnick et al., 2008). A fourth and final reagent stopped the enzymatic color reaction and cleared the reaction mixture with guanidine salts, which solubilized the aggregates (Warnick et al., 2008). HDL-C was quantified based on



the final reaction absorbance, monitored at 600 and 700 nm(Arneson, & Brickell, 2007).

The direct methods for the quantification of HDL-C seem to be increasingly accepted by clinical laboratories, steadily replacing the conventional precipitation methods (Hirano *et al.*, 2009). Data gleaned from the proficiency testing reports of the College of American Pathologists indicate that the homogeneous assays first appeared as an identified class in 1997, with 550 laboratories reporting their use. One year later, the number of laboratories reporting use of homogeneous assays had increased to 853, and by the year 2000, the number had increased dramatically to 2578 (Warnick *et al.*, 2008).

The dextran sulfate (50 000Da with  $Mg^{2+}$ ) method peaked in 1995 at 1479 laboratories, and by year 2000 had decreased to 510. The PTA assay, which was most common in 1994 with; 1300 laboratories, decreased steadily to 281 by the end of the year 2000.. A second-generation direct method with dextran sulfate linked to magnetic beads increased sharply from 117 laboratories in 1994 to 530 in 1996, declined moderately through 1999, and then increased slightly to 591 by the end of year 2000(Warnick *et al.*, 2008). In the last survey of year 2000, these patterns clearly indicate the preference for fully automated, convenient, and labor-saving methods. The data also suggest that the homogeneous assays enabled more laboratories to perform the HDL-C assay with an overall increase in the number of laboratories measuring HDL-C in the proficiency surveys (Warnick *et al.*, 2008).

## **2.8 Friedwald's equation for LDLC estimation**

LDL-C is accurately measured by ultra centrifugation as recommended by Lipid Research Clinic Beta quantification (LRC-BQ) (Marniemi *et al.*, 1995) However, its use in routine clinical laboratories is limited., because it is costly, labor intensive, requires expensive ultracentrifuges, rotors, and tubes, is time consuming and can be performed only on a few samples a day. Until recently, LDL-C has been estimated from Friedewald's equation for clinical purposes, based on three independent measurements: HDL-Cholesterol (HDL-C), Triglycerides (TGs) and total Cholesterol (TC) (Friedewald *et al.*, 1972). The

Friedewald equation assumes that dividing the blood TG concentration by a factor of 5 can approximate the amount of cholesterol in VLDL. Studies have shown that the use of the Friedewald's formula (FF) has shortcomings: firstly combining three measurements increases analytical variability; secondly, the formula has been shown to be invalid in samples with high triglyceride concentrations and can therefore only be used in fasting blood samples. Thirdly the assumption that the relationship between cholesterol and triglyceride in VLDL is constant has been shown to be inaccurate in some hyperlipidemias because samples contain chylomicrons (CM), chylomicron remnants and VLDL remnants all of which predict higher TG/total cholesterol ratio than in normal VLDL concentrations (McNamara et al., 1990).

## **2.9 Direct method for LDL-C estimation**

Direct assays for LDL-C have been developed to be used especially when the triglyceride concentration is higher than 4.5 mmol/l (Warnick et al., 2008). Currently, there are several different Small, dense low-density lipoprotein cholesterol direct LDL-C assays based on selective detergents or other elimination methods to separate chylomicrons, VLDL, and HDL from LDL (Contois et al., 2011). A precipitation assay for Small, dense low-density lipoprotein (sd-LDL) or Small, dense low-density lipoprotein cholesterol (sd-LDL-C) has also been developed (Hirano *et al.*, 2009). According to the latest evidence, homogeneous LDL-C assays have limited specificity against unusual specimens, which may cause misclassification of patients (Miller et al., 2010). Different LDL-C assay kits may react partially also to VLDL, IDL, Lp(a), or apoE-rich HDL particles producing variations in LDL-C concentrations (Nauck et al., 2002). LDL-C has been accepted as a novel cardiovascular risk factor, but the measurement of LDL particle size has not been transformed into clinical practice (Warnick et al., 2008). However, the amount of all apoB-containing particles is possible to estimate with apoB measurements instead of LDL-C (Davidson 2008).

## **CHAPTER THREE**

### **MATERIALS AND METHODS**

#### **3.1 Introduction**

This chapter gives in detail all the aspects of the study design. It describes how the study subjects were recruited, the reaction principles of the parameters studied and the equipment used to carry out the analytical work. It also describes the quality control aspects of the study and finally the statistical methods used for data analyses

#### **3.2 Study Site**

The study was conducted in Kenyatta National Hospital (KNH). The hospital serves all regions of the country and is the largest referral hospital in Eastern and Central Africa region. The analysis was conducted in Clinical Chemistry Laboratory. Kenyatta National Hospital is situated along Ngong Road; it is about 5 Kilometers from Nairobi Central business district, opposite Nairobi Hospital and next to the Nairobi area traffic police station, Nairobi County. KNH is the largest referral and teaching hospital in Kenya.

#### **3.3 Study design**

The study was a descriptive cross sectional study Conducted between the months of May and August 2014

#### **3.4 Study Population**

The study population comprised of 384 Patients (192 males and 192 females) sent to the clinical chemistry laboratory for blood collection for lipid profile analysis. The patients were recruited from medical outpatient clinic (MOPC)

#### **3.5 Inclusion and Exclusion Criteria**

##### **3.5.1 Inclusion Criteria**

1. Participants who gave informed consent

2. Participants who fasted for 8-12 hours.c
3. Patients with valid laboratory request form for lipid profile

### 3.5.2 Exclusion criteria

1. Participants Previously diagnosed with type II diabetes mellitus, type III or type I hyperlipoproteinemia and nephrotic syndrome
2. Participants who are chronic alcoholics

### 3.6 Sample size

Sample size was calculated using Step by step Sample size determination by Bartlett, *et al.*, (2001) method: -

$$n = Z^2 P (1-P)/d^2$$

Where,

$n$  = expected sample size

$Z$  = Z statistic for a level of confidence, 0.05=1.96

$P$  = expected prevalence or proportion of hypertensive patients if prevalence is not known, =50%,  $P = 0.5$ , and

$d$  = precision = 5%,  $d = 0.05$ ).

$$n = (1.96)^2 0.5(1-0.5) / (0.05)^2$$

$$=384$$

Therefore, sample size was 384.

### 3.6 Sampling Method

Purposive sampling method was used to select study samples in which all patients who met the inclusion criteria were prospectively recruited to the study

### **3.7 Sample Collection and Processing**

The study participants were instructed to fast for 8-12 hours before collection of the blood sample. Using a 5 ml syringe, 4ml of venous blood was collected and put into plain vacutainers. All the sample vacutainers were labeled correctly with the subject's name, study number and gender of the patient and age. Serum was separated by centrifugation after blood clotting using a speed of 3000 r.p.m. for five minutes at room temperature (18<sup>0</sup>-22<sup>0</sup> C). Serum was separated using a Pasteur pipette for each sample and transferred into specific vials labelled with patient details. The vials were stored at -20<sup>0</sup>C and analyzed in batches for total cholesterol (TC), triglycerides (TG), high density lipoproteins cholesterol (HDL-C) and low density lipoproteins (LDL-C) by direct assay. High density lipoprotein cholesterol HDL-C was estimated by precipitation method and low density lipoprotein cholesterol (LDLC) calculated by Friedewald's formula.

All the lipoproteins in the serum were analyzed using Mindray BS 800(Scheenzhen,china) a closed automated Clinical Chemistry analyser. All lipid analyses were performed within three hours of sample collection.

#### **3.7.1 Cholesterol oxidase/peroxidase enzymatic (CHO-PAP) method for blood total cholesterol (TC) measurement**

Total cholesterol was measured enzymatically with the appropriate reagents using Mindray BS 800 Clinical chemistry analyser. Cholesterol ester was catabolized by cholesteryl ester hydrolase (CHE) and cholesterol oxidase (CHO) to yield H<sub>2</sub>O<sub>2</sub>, which oxidated 4- Aminoantipyrine with phenol to form a colored dye of quinoneimine. The absorbency increase at 600nm was directly proportional to the concentration of cholesterol.

### **3.7.2 Glycerol-3-phosphate/ peroxidase enzymatic method for serum Triglycerides (TG) measurement**

Through a sequence of enzymatic catalysis steps by lipase, GK and GPD, triglycerides was catalyzed to yield H<sub>2</sub>O<sub>2</sub>, which oxidized 4-aminoantipyrine to yield a colored dye of quinoneimine. The increase in absorbance at 600nm was directly proportional to the concentration of triglycerides in the sample.

### **3.7.2 Direct assay methods for major serum lipoproteins**

#### **3.7.2.1 High Density Lipoprotein Cholesterol (HDL-C)**

HDL reagent kit from mindray Ltd. (Schenzhen, china) was used. The HDL kit has two liquid reagents that directly measures the HDL-C concentration by a direct homogenous assay method Anti-human lipoprotein antibody binds and inactivates chylomicrons, LDL and VLDL. Cholesterol esterase and oxidase then react with HDL cholesterol resulting in the production of hydrogen peroxide. This then reacts with chromogens to form a coloured dye, which absorb at a given wavelength. Analysis was done on the Mindray BS 800 in accordance to manufacturer's protocol.

#### **3.7.2.2 Low Density Lipoprotein Cholesterol (LDL-C)**

The assay uses two reagents. First Reagent solubilizes only the non LDL particles. The cholesterol released is consumed by cholesterol esterase and cholesterol oxidase in a non colour forming reaction. Reagent 2 solubilises the remaining LDL particles and a chromogenic coupler allows for colour development. Analysis was done on the mindray BS 800 according to the manufacturer's protocol.

### **3.7.3 Precipitation method**

#### **3.7.3.1 High Density Lipoprotein –Cholesterol (HDL-C) determination by phosphotungstic acid and magnesium chloride precipitation**

The precipitating reagent consisted of 0.55 mmol/l phosphotungstic acid and 25 mmol/l magnesium chloride. Plasma (0.2 ml) was mixed in a tube containing the precipitation reagent (0.5 ml) and, after 10 min at room temperature, was centrifuged for 10 min at 2,000g. The clear

Supernatant was separated and stored in capped glass tubes for a maximum of 2 days at 4°C before the cholesterol content was determined by the cholesterol oxidase-peroxidase

### **3.7.3.2 Low Density Lipoprotein-Cholesterol Estimated Using the Friedewald Formula**

Calculated LDL cholesterol was derived from the following formulae;  $LDL-C = TC - (TG/2.2 + HDL-C)$  mmol/L. Samples with fasting TG concentrations  $>4.5$  mmol/L were excluded from the FF calculations because the equation has been clearly shown to be invalid in hypertriglyceridemic samples

### **3.8 Quality management**

Internal quality control was adhered to throughout all the stages of testing process. Only fasting patients participated in the study. All samples were given a unique identification number which was traceable throughout the testing process. Quality control material were analyzed before any study on participants specimens to ensure accuracy and precision

### **3.9 Data management**

All study data was entered into MS-Excel (Microsoft corp.) and analyzed using SPSS version 21. Paired t-test was used to test significance of results at  $p=0.05$ . Data was presented in the form of tables and graphs

### **3.10 Ethical consideration**

Permission to carry out the research study was granted by the Department of Medical Laboratory Sciences Jomo Kenyatta University of Agriculture and Technology (JKUA, Nairobi). Ethical clearance was given by the Ethics and Research Committee of Kenyatta National Hospital and University of Nairobi (approval no P497/08/2014). Informed consent was sought from all participants

**CHAPTER FOUR**  
**RESULTS**

**4.1 Quality control of the analytical work**

An internal quality control serum for specific parameter was included in each analytical session throughout the study period. There were 32 analytical sessions since all the parameters were analysed at the same time. Quality control results for the analyzed parameters were within the specific assigned QC range as shown in the table 4.1 below

**Table 4.1: Internal Quality Control (IQC) report for the studied analytes**

Analyte	Assigned IQC		Study IQC.	
	Mean	Range	Mean	Range
TC	2.29	2.2 - 5.0	3.63	2.2 - 5.0
TG'S	1.22	0.36 - 2.04	1.22	0.36 - 2.04
HDLC Precipitation Assay	1.93	0.80 - 3.29	1.93	0.80 - 3.29
HDLC – Direct assay	1.93	0.80 - 3.29	1.93	0.80- 3.29
LDLC-Direct Assay	2.05	0.52 - 2.16	2.05	0.52 2.16



#### 4.2 HDL-C and LDL-C serum concentration by direct analytical methods

HDLC and LDLC Concentration by direct analytical methods was  $1.52 \pm 0.47$  and  $2.88 \pm 1.01$  mmol/L respectively. The minimum and maximum concentration for the studied samples was 0.38 -2.90 and 0.23-5.90mmol/l as shown in table 4.3 below.

**Table 4.2: mean concentration of Serum HDLC and LDLC concentration by direct analytical methods (n=384)**

Lipid profile	Range(mmol/L)	Mean $\pm$ S.D
Direct HDLC	0.38 – 2.90	$1.52 \pm 0.47$
Direct LDLC	0.23 - 5.90	$2.88 \pm 1.01$

#### 4.3 HDL-C concentration by precipitation method and LDL-C concentration by friedewald's formulae

HDLC and LDLC Concentration by precipitation method was  $1.51 \pm 0.47$  and  $2.89 \pm 0.47$  mmol/L respectively with a range of 0.35-2.86 mmol/L and 0.18-5.80mmol/l respectively as shown in figure 4.3 below

**Table 4.3 median HDLC and LDLC concentrations by precipitation / friedewald formulae (n=384)**

Lipid Profile	Range(mmol/l)	Mean $\pm$ S. D(mmol/L)
Precipitation HDLC	0.35-2.86	$1.51 \pm 0.47$
Friedewald LDLC	0.18-5.80	$2.89 \pm 0.47$

## 4.7 Comparison of direct and precipitation/calculated lipoprotein analytical methods

### 4.7.1 Comparison between precipitation and direct analytical methods for estimation of serum HDLC

Serum HDLC estimated by direct analytical method was higher than those estimated by precipitation method at different total cholesterol concentration ranges. There was no significant difference in serum HDLC estimated by Friedewald's formulae and direct method regardless of total cholesterol concentration range as shown in figure 4.6 below.

**Table 4.4: Comparison of mean and S.D of HDL-C by direct and precipitation method at different total cholesterol (TC) ranges**

TC range (mmol/L)	N	Mean± S.D HDLC (Direct assay) in mmol/L	Mean± S.D HDLC (Precipitation) in mmol/L	p-value
< 3	14	0.90 ± 0.38	0.86 ± 0.41	0.79
3 - 6	285	1.52 ± 0.44	1.51 ± 0.45	0.78
6 - 9	85	1.62 ± 0.50	1.59 ± 0.49	0.69

### 4.7.2 Comparison of serum LDLC by direct and Friedewald's formulae

The serum LDLC estimated by direct method analytical method and Friedewald's formulae were highest at Triglyceride levels of 3.43 to 4.56 mmol/L and lowest at triglycerides levels of 2.29 to 3.39 mmol/L. LDLC estimated by Friedewald's formulae was slightly higher than that estimated by direct method at all ranges of triglycerides.

**Table 4.5: mean and SD (in mmol/L) of serum LDLC by direct and Friedewald's formulae at different TG ranges.**

Triglyceride range	n	LDLC by direct assay	LDLC by friedewald's	p-value
<1.14	88	2.86 ± 0.94	2.88 ± 0.96	0.89
1.15-2.28	215	2.88 ± 0.99	2.90 ± 0.01	0.77
2.29 -3.42	71	2.81 ± 0.07	2.82 ± 1.06	0.93
3.43-4.56	10	<b>3.48 ± 1.47</b>	3.50 ± 1.48	0.97

#### **4.7.3 Comparison of mean differences for direct analytical assays and precipitation /Calculated methods for HDLC and LDLC**

The paired t test showed no significance at p=0.093 and p=0.91 for serum HDLC and serum LDLC respectively hence there is no difference between precipitation/calculated assays and direct assays as shown in the table 6 below.

**Table 4.5: Paired differences in mean and standard deviation for HDLC and LDLC for the studied samples**

Lipid profile	Paired Differences		t	Df	Significance (2-tailed)
	Mean(mmol/L)	S.D(mmol/L)			
HDLC	0.02	0.19	1.69	383	0.093
LDLC	0.01	0.18	1.70	383	0.091

## **CHAPTER FIVE**

### **DISCUSSION**

#### **5.1 Introduction**

The study population comprised of male (192) and female (192) population of the ages 16 to 74 years of age. The choice of study population was comparable with that of comparative and correlative study on friedewald's and direct assay in rural Kolar population (Chatterjee, & Mendez, 2011)

#### **5.2 HDL-C and LDL-C human serum concentration by direct analytical methods**

The current study the mean for LDLC measured directly was  $2.88 \pm 1.01$ mmol/L differing slightly with a similar study by Anwar, M., Khan, D. A., & Khan, F. A. (2014) in Pakistanis population whose mean concentration for directly measured LDLC was  $2.93 \pm 0.81$ mmol/L. The mean directly measured HDLC concentration in our study was  $1.52 \pm 0.47$  mmol/L also differing with Anwar (2014) whose Mean HDLC Concentration was  $1.06 \pm 0.26$  mmol/L Differences in the results of different studies may be attributed to diversity in population, pathologies and kits used. These differences not only arise from imprecision within laboratories, but also from lot to lot variation and assay.

#### **5.3 HDL-C and LDL-C Concentration by precipitation and friedewald formulae**

The study had a mean HDLC and LDLC Concentration by precipitation and friedewald formulae of  $1.51 \pm 0.47$  mmol/L and  $2.89 \pm 0.47$ mmol/L respectively differing with a study Jabbar, J., Siddiqui, I., & Raza, Q. (2006) also carried out in pakistanis population whose mean concentration were  $1.11 \pm 0.23$ mmol/L and  $2.89 \pm 0.23$ mmol/L respectively. The difference is may be attributed to diversity in populations studied as well as the Quality and accuracy of the method which depends upon the centrifugation speed and time of centrifugation. This aspects may vary from laboratory to laboratory and with different technologists.

#### **5.4 comparison of direct and precipitation/calculated lipoprotein analytical methods**

The current study compared direct assays with precipitation /friedewald's formulae for the estimation of major serum lipoproteins. In the current study, HDLC determined by direct assay was slightly higher than that estimated by precipitation method in each study group as shown in table 4.4. This was in agreement with a similar study by Jensen et al., (2002) who found directly measured HDLC concentrations higher than HDLC Measured after precipitation by 1.0 mmol/L in all study groups based on total cholesterol. Despite the small difference in cholesterol measurement by the two methods, the clinical implications needs to be considered. This is because LDLC/HDLC ratio increase by 1 unit increases the risk of myocardial infarction by 53% (Stampfer, Sacks, Salvini, Willett, & Hennekens, 1991). In kenya, most clinical laboratories are performing precipitation (indirect) method for the estimation of HDL-C. Quality and accuracy of the results depends upon the centrifugation speed, time of centrifugation, accurate pipetting and the test has to be performed by skilled medical technicians. The indirect method is slightly cumbersome since it is a twostep method, more time consuming but less expensive.

There was non-significant difference in mean and standard deviation of HDLC measured directly and by precipitation method at different total cholesterol levels  $p=0.79, p=0.78, p=0.69$  respectively as shown in table 4.4. Similar findings have been reported by Martin, et al. (2013 in their study found non-significant difference at varied cholesterol levels ( $p>0.05$ ). Similar findings were reported by a recent study by (Burstein, Scholnick, & Morfin, 1970)). No significant difference in the mean levels of HDLC was observed between both methods irrespective of serum total cholesterol levels  $P=0.94, P=0.77$  and  $P=0.62$ . This implies that total cholesterol did not affect measurement of LDLC by the two techniques.

The current study shows no significant difference in the mean concentration of HDLC assayed directly and by precipitation methods with a mean difference of 0.02mmol/L and  $p=0.093$  as shown in table 4.6.

The study findings were in agreement with a similar study by Jabbar, (2006) carried out in Pakistanis population with mean difference of 0.74mg/dl (0.02mmol/L) and  $p=0.301$ .

There was no significant difference in LDLC estimated directly and friedewald's formulae at different triglyceride levels  $p=0.89$ ,  $p=0.77$ ,  $p=0.93$ ,  $p=0.97$  at triglyceride levels  $>1.14$ mmol/L, 1.15 -2.28mmol/L, 2.29-3.42, 3.43-4.56mmol/L respectively as shown in table 4.5. The findings differed with those reported by Suchanda., Rajinder and Bharti., (2005) .in the earlier study, there was significant difference between the two methods at lower TG levels 1-100mg/dl( $>1.14$ mmol/L) and 101-200 mg/dl(1.14-2.28mmol/L )  $p<0.02$ ,  $<0.01$  respectively. There was no. significant difference at TG levels above 200 mg/dl (2.28mmol/L). The study also differed with study by Anwar et al.,(2014) whose mean value between calculated and direct LDLC differed significantly  $5.5\pm 11.68$  mg/dL( $0.14 \pm 0.31$ mmol/L)  $P <0.0001$ .in this case the difference was within acceptable limit and did not create any discrepancy regarding clinical aspects.

. Direct and precipitation assays were found to be reliable, this is consistent with study by jabbar et al., (2006) who found both precipitation method and direct method precise and accurate in estimation of HDLC and LDLC. However direct assays are time saving and are less labour intensive. Use of direct assays can improve reliability of the results of lipoprotein testing because it avoids the precipitation and centrifugation steps which depend on technical skills and experience of the laboratory technologist.

LDLC analysed by direct method and Friedewald's formulae were consistent with previous findings by Smets et al., (2001) who found agreement between direct and Friedewald's formulae for triglycerides levels up to 4.52mmol/L. This was in contrast with our current study in which the mean for LDLC measured by friedewald's formulae was higher than that of direct method at all triglyceride ranges The NCEP Working Group on Lipoprotein Measurement recommended the development of direct methods for LDL-C measurement (Bachorik & Ross 1995). In earlier studies, three out of five homogenous methods were shown to give results

comparable to the Friedewald's calculation and appeared to meet NCEP performance criteria and therefore despite the technical disadvantages, Friedewald's method was found to be firmly entrenched in routine practice and only likely to be displaced when the homogenous methods can demonstrate clear advantages in reference to cost and overall performance

## **CHAPTER SIX**

### **CONCLUSION AND RECOMMENDATIONS**

#### **6.1 Conclusion**

There is no significant difference in mean serum concentrations of low density lipoprotein cholesterol (LDL-C) estimated by direct assay and the precipitation/Friedwald's formulae in all triglyceride levels. There is no significant difference in mean Concentration of high density lipoprotein cholesterol (HDL-C) estimated by direct and precipitation method at all total cholesterol ranges

#### **6.2 Recommendations**

The following recommendations will provide further information to improve analysis of serum lipoproteins;-

1. Selection of procedures for analysis of serum lipoproteins be based on individual laboratory requirements.
2. More comparison need to be done with different analytical reagents for direct assays and precipitation assays based on different principles to ensure 'generalization' of findings



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## APPENDICES

### **Appendix 1: Informed consent**

**Topic:** Comparison of direct and precipitation methods for the estimation of major serum lipoproteins

**Consent explanation:** My name is Mbithi Mulinge (Cellphone; 0722365468) a Masters student in the Department of Medical Laboratory Sciences, Jomo Kenyatta University of Agriculture and Technology (JKUAT). PO Box 20732 Nairobi, Kenya; Phone: +254-6-752711. I am conducting a study to compare direct and precipitation methods of lipoprotein estimation. The information in this form will help you make an informed decision whether or not to participate in this study. Please read through carefully and feel free to ask any question about the study. I will read it out to those who are not able to read.

**Description:** Previous studies have indicated possibility of variations in serum lipoprotein measurements estimated by direct and precipitation methods

**Purpose:** This study is interested in finding out the magnitude of variation between the two methods routinely used for estimation of serum lipoproteins

**Benefits:** There will be no tokens or direct benefit to the participants. However the final findings can ultimately improve diagnosis of lipid related disorders

**Risks:** One potential risk of being in the study is the loss of privacy. However, the samples will only be used for the intended purpose of this study and any personal information collected will be handled with high confidentiality.

**Procedure:** If you accept to participate in this study, blood samples will be collected for analysis. The procedure of sample collection has no side effects but might cause a slight discomfort.

**Voluntarism:** Enrolment to the study is at free will.

**Subject's rights:** As a voluntary study participant, you have the right to withdraw your consent or discontinue participating at any time without penalty. Your

individual privacy will be maintained in all published and written data emanating from the study.

If you have questions about your rights as a study participant, or are dissatisfied at any time with any aspect of this study, you may contact - anonymously, if you wish – KNH/UoN ERC (Chairperson of the Scientific Steering Committee, PO Box 20732 Nairobi, Kenya; Phone: 02-7263000 Ext 44102.

I have read this form or had it read to me in a language that I understand. I have discussed the Information with study staff. My questions have been answered. My decision whether or not to take part in the study is voluntary. If I decide to join the study I may withdraw at any time.

By signing this form I do not give up any rights that I have as a study participant.

\_\_\_\_\_  
\_\_\_\_\_

Participant Name  
Date

\_\_\_\_\_

Participant Signature/ Thumbprint

\_\_\_\_\_  
\_\_\_\_\_

Study Staff Conducting Study  
Date

Staff Signature

## Appendix 2: Ethical approval letter



**UNIVERSITY OF NAIROBI**  
COLLEGE OF HEALTH SCIENCES  
P O BOX 19676 Code 00202  
Telegrams: varsity  
(254-020) 2726300 Ext 44355

Ref: KNH-ERC/A/14

Mbithi Mulinge  
Reg. No. TM300-1176/2012  
JKUAT

Dear Mr. Mulinge

**RESEARCH PROPOSAL: COMPARISON OF DIRECT AND PRECIPITATION METHODS FOR  
THE ESTIMATION OF MAJOR SERUM LIPOPROTEINS (P497/08/2014)**

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above proposal. The approval periods are 14<sup>th</sup> January 2015 to 13<sup>th</sup> January 2016.

This approval is subject to compliance with the following requirements:

- a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
- b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
- c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
- d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
- e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
- f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
- g) Submission of an *executive summary* report within 90 days upon completion of the study  
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website [www.erc.uonbi.ac.ke](http://www.erc.uonbi.ac.ke)



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14<sup>th</sup> January 2015

Protect to discover

Yours sincerely



**PROF. M. L. CHINDIA**  
**SECRETARY, KNH/UON-ERC**

c.c.     The Principal, College of Health Sciences, UoN  
          The Deputy Director CS, KNH  
          The Assistant Director, Health Information, KNH  
          The Chairperson, KNH/UON-ERC  
          Supervisors: Dr. Sarah Kaggia, Dr. Stanley Kinge Waitthaka

## Appendix 3; Publication Article

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### COMPARISON OF DIRECT AND PRECIPITATION METHODS FOR THE ESTIMATION OF MAJOR SERUM LIPOPROTEINS

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### COMPARISON OF DIRECT AND PRECIPITATION METHODS FOR THE ESTIMATION OF MAJOR SERUM LIPOPROTEINS

J. M. MULINGE, S. K. WAITHAKA and S. N. KAGGIA

#### ABSTRACT

**Background:** There is increase in use of direct assays for analysis of high and low density lipoprotein cholesterol by clinical laboratories despite differences in performance characteristics with conventional precipitation methods. Calculation of low density lipoprotein cholesterol in precipitation methods is based on total cholesterol, triglycerides and high density lipoproteins, thus may cumulatively carry errors of individual methods. Adoption of direct assays is expected to decrease turnaround time and save on cost.

**Objectives:** To compare direct and precipitation methods for estimation of major serum lipoproteins.

**Design:** Cross sectional study.

**Setting:** Clinical Chemistry Laboratory, Kenyatta National Hospital, Nairobi, Kenya.

**Subjects:** Three hundred and eighty four (384) participants were recruited for the study.

**Results:** There was no significant difference in high density lipoprotein cholesterol estimated by direct and precipitation methods  $p=0.091$  as well as low density lipoprotein cholesterol estimated by direct method and Friedwald's formulae  $p=0.093$ .

**Conclusion:** Both direct and precipitation methods give similar results. Selection should be based solely on workload, availability and technical expertise.

#### INTRODUCTION

High density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) are major lipoproteins of cholesterol in human plasma(1) and major transporters of cholesterol in human plasma, therefore measurements of these markers have been proposed as primary tools for risk assessment and monitoring of patients with risk of developing cardiovascular disease(2).

Total cholesterol in humans is distributed primarily among three major lipoprotein classes: very low density lipoproteins cholesterol (VLDL-C), low density lipoproteins cholesterol (LDL-C), and high density lipoproteins cholesterol (HDL-C). Smaller amounts of cholesterol are also contained in two minor lipoprotein classes: intermediate density lipoprotein (IDL) and Lipoprotein (a)(3). In normal individuals, the minor lipoprotein classes can be expected to contribute on average about 0.0621mmol/L to the

total cholesterol measurement. Several studies have indicated that there is an inverse relationship between the risk for coronary heart disease and the plasma concentration of high density lipoprotein (HDL-C) cholesterol(4). Possible mechanisms by which HDL-C might play a direct protective role have also been studied as well as conditions associated with elevated or depressed HDL-cholesterol levels. These investigations have led to an increased interest in the routine determination of HDL-cholesterol levels to aid in the assessment of risk for ischemic heart disease, as well as interest in further studies of the putative protective effect of HDL-C, and have stimulated efforts to understand better the capabilities and limitations of quantitative HDL-C methods (5). There is increase in use of direct assays for analysis of high and low density lipoprotein cholesterol by clinical laboratories despite differences in performance characteristics with conventional precipitation methods. Calculation of low density lipoprotein cholesterol in precipitation

thus may cumulatively carry errors of individual methods. Adoption of direct assays is expected to decrease turnaround time and save on cost(6).

Precipitation method involves precipitation of Apo B lipoproteins followed by centrifugation at 3000g, for 10 minutes, the supernatant is used for estimation of HDL-C using a method similar to that of total cholesterol(6). LDL-C is estimated by use of Friedwald's formulae which is based on total cholesterol (TC), Triglycerides (TG's) and High density lipoproteins cholesterol (HDL-C).

Measurements of HDL-C and LDL-C by direct methods offer the potential to improve both analytical and biological variability, since precision of HDL-C and LDL-C measurement does not depend upon the analytical variability in measurement of total cholesterol and low levels cholesterol in supernatants after precipitation(7). Capabilities and limitations of quantitative HDL-C methods is not known, the study intended to compare quantitative analytical methods used for estimation of the major serum lipoproteins.

In clinical practice, LDL-C is either estimated by the Friedewald's formula or directly measured with a homogeneous assay. Since the calculation is based on serum TG, TC, and HDL-C, it necessarily includes the accumulated errors in all the three measurements(8). Despite the widespread belief that the calculation or measurement of LDL or HDL cholesterol is standardized and reproducible, data indicates that results can vary significantly with methods from different manufacturers, and calculated LDL cholesterol may not agree with measured LDL cholesterol(9). Limitations of the Friedewalds equation were recognised early including the fact that calculation is not valid for specimens having triglycerides >4.52mmol/l for patients with Type III hyperlipoproteinemia or chylomicronemia, or with non-fasting specimens. In fact, the equation is increasingly inaccurate with TG from 2.28 to 4.56 mmol/l(9). Despite the continued efforts to standardize LDL-C and HDL-C analytical methods results have shown method to method variation. Calculated LDL-C may not agree with measured LDL-C cholesterol while direct HDL-C assays have also had reliability issues which relate to ambiguity in definition and heterogeneity of LDL-C and HDL-C particles(9).

Precipitation methods for HDL-C involves multiple pipetting and centrifugation steps which are dependent on experience of the technologist. Automated assays are bound to improve variability of results of serum lipoprotein measurements as it avoids the need to centrifuge and multiple pipetting. The aim of the study was to compare direct assays and precipitation methods used for estimation of serum lipoproteins.

## MATERIALS AND METHODS

*Samples:* Serum samples from 384 adults comprising of 192 males and 192 females received at Clinic Chemistry Laboratory, Kenyatta national Hospital and Nairobi were used. Only those where a fasting lipid profile was requested were considered. The specimen was collected from study participant after (8-12 hours) fasting and placed in plain vial. The serum was separated by centrifugation and was used to estimate various parameters using Mindray BS 800 Clinical Chemistry analyzer (Shenzhen Mindray Biomedical and Electronics Company Limited China).

*Total cholesterol and Triglycerides assay:* Total cholesterol was estimated by Cholesterol Oxidase/Peroxidase Enzymatic Method (10). Usir recommended procedure by Mindray-Shenzhen, China. Cholesterol ester was catalyzed by cholesterol ester hydrolase (CHE) and cholesterol oxidase (CHO) to yield H<sub>2</sub>O<sub>2</sub>, which oxidized 4-Aminoantipyrine with phenol to form a colored dye of quinoneimine.

Triglycerides were estimated using Glycerol 3-Phosphate/ Peroxidase Enzymatic Method(11,12) using recommended procedures and reagents Mindray-Shenzhen, China. Through a sequence of enzymatic catalysis steps by lipase, Glycerol kinase and Glycerol phosphate dehydrogenase, triglyceride was catalyzed to yield hydrogen peroxide which oxidized 4-Aminoantipyrine to yield a colored dye of quinoneimine.

*HDL-C assays:* HDL-C was estimated by precipitation method(13,14) using cholesterol liquid colour test kit manufactured by human diagnostics, Gesellschaft für Biochemie und Diagnostik mbH, Wielsbaden-Germany and distributed in Kenya by Chemlabs (E.A) Limited. The method is based on selective precipitation of Very low density lipoproteins (VLDL), low density lipoproteins (LDL) and Lipoprotein(a) (LP(a)) with phosphotungstic acid/magnesium chloride (MgCl<sub>2</sub>). Sedimentation of precipitant by centrifugation and subsequent enzymatic analysis of high density lipoproteins (HDL) as residual cholesterol remaining in clear supernatant by CHO-PAP Method for total cholesterol.

HDL-C was measured directly in serum (1) using manufacturer recommendations by Mindray Shenzhen- China. HDL-C particles were protected by surfactant as LDL-C, VLDL-C and chylomicrons were removed by cholesterol esterase (CE), and cholesterol oxidase (CO). HDL-C particles in the presence of second surfactant and peroxidase generated hydrogen peroxide which reacts with 4-amino-antipyrine and HSDA to form a purple blue dye. The color intensity of the dye was directly proportional to concentration of HDL-C and was measured photometrically at 585nm.

**LDL-C assays:** LDL-C estimated directly (16) using recommendations by Mindray Diagnostics, Shenzhen-China of protecting agent that protected LDL-C from enzymatic reactions. LDL-C was then catalyzed by cholesterylesterase (CE) and cholesteryl oxidase (CHO) to cholestenone and hydrogen peroxide which reacted of 4-aminoantipyrin in presence of peroxidase to form a coloured dye quinoemine.

Low density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald formulae (18), which states that  $LDL-C = TC - (TG/2.2 + HDL-C)$ .

**Quality control:** An internal quality control for each parameter was included in each analytical session throughout the study period. Quality control results for the analysed parameters were within the specific assigned QC range.

**Statistical approach:** Paired t test was used for comparison of mean difference at an alpha value of  $p=0.05$  to assess significant difference using SPSS version 20. Any  $p$ -value  $\geq 0.05$  was considered not statistically significant while  $p$ -value  $\leq 0.05$  was

considered to be statistically significant.

**Ethical approval:** The study was cleared by Kenyatta National Hospital and University of Nairobi ethics and research committee (approval no P497/08/2014)

## RESULTS

Result of the 384 samples analysed by precipitation methods of HDL-C ranged from 0.35 to 2.86 mmol/l (mean 1.51) and LDL-C from 0.18 to 5.80 mmol/l (mean 2.89).

In direct method, the HDL-C ranged from 0.38 to 2.90 mmol/l (mean 1.52 mmol/l) and LDL-C from 0.1 to 5.80 mmol/l (mean 2.89).

The paired t test showed there was no statistical significant difference between precipitation and direct methods of HDL-C  $p=0.76$ , there was no significant difference between precipitation (Friedewald estimation) and direct methods LDL-C  $p=0.89$  as shown in table 1 below.

**Table 1**  
Results of HDL-C and LDL-C by Precipitation and Direct methods

	Precipitation	Direct method	p-value
Total samples	384	384	384
HDL-C	1.52 ± 0.47	1.51 ± 0.47	0.76
LDL-C	2.89 ± 1.02	2.88 ± 1.01	0.89

There was no significant difference in Low density lipoproteins estimated by direct and Friedewald's formulae at different triglyceride ranges with  $p=0.89$  at triglyceride (TG) levels  $<1.14$  and  $p=0.97$  at TG levels  $>3.42$  mmol/l as shown in table 2 below.

**Table 2**  
Comparison of serum LDL-C by direct and Friedewald's formulae

TG* (mmol/L)	N*	LDL-C(D)* (mmol/L)	LDL-C(FF)* (mmol/L)	p-value
<1.14	88	2.86 ± 0.94	2.88 ± 0.96	0.89
1.15-2.28	215	2.88 ± 0.99	2.90 ± 0.01	0.77
2.29 -3.42	71	2.81 ± 0.07	2.82 ± 1.06	0.93
>3.42	10	3.48 ± 1.47	3.50 ± 1.48	0.97

TG\*-Triglycerides, N\*-Study population, LDL-C (D)\*-Low density lipoprotein cholesterol by direct method, LDL-C (FF)\*-Low density lipoprotein cholesterol by Friedewald's formulae.

The paired t test for comparison of mean differences was not significant at  $p=0.093$  for HDL-C and  $p=0.091$  for LDL-C as shown in table 3 below.

**Table 3**  
Paired Samples Test for comparison of HDL-C and LDL-C by direct assay and precipitation/friedewald's formulae

Analyte	Paired Differences		S.E* of Mean	95% C.I of the Difference		t	df	Sig. (2-tailed)
	Mean(mmol/L)	SD*(mmol/L)		Lower	Upper			
HDLC	0.016	0.187	0.010	-.003	.035	1.685	383	0.093
LDLC	0.015	0.179	0.009	-.034	.002	-1.697	383	0.091

SD\*-standard deviation, S.E\*-Standard error, C.I\*- Confidence interval

## DISCUSSION

The study compared direct assays with precipitation /friedewald's formulae for the estimation of major serum lipoproteins. LDL-C/HDL-C ratio increase by 1 unit increases the risk of myocardial infarction by 53(16).

The paired t-test on comparison of LDL-C by direct method and friedewald's formulae showed no statistically significant difference at  $p=0.091$ , The results differed with findings of a study by Chartejee, C. *et al.*, (2011) whose results were statistically significant at  $p<0.001$ , hence depicting that there was a significant difference in LDL-C measurements by direct and Friedewald's equation at  $p=0.01(17)$ . Friedewald's formula was first developed in 1972(18), to estimate LDL-C as an alternative to tedious ultracentrifugation. Because VLDL carries most of the circulating TGs, VLDL-C can be estimated reasonably well from measured TGs divided by 2.2 for mmol/l units. LDL-C was then calculated as Total Cholesterol minus HDL-C minus estimated VLDL-C.

The mean difference in HDL-C concentration by direct assay and precipitation method was not statistically significant  $P=0.093$ , in agreement with other studies. Arranz-Pena *et al.*, in their study showed close correlation of direct assay and several precipitation methods(19), Nauck, M. *et al.*, also showed that homogeneous assays produce precise and accurate HDL cholesterol concentration even for hypertriglyceridemia samples up to 10.26 mmol/l, the use for patients with higher triglycerides provides an advantage for use of direct techniques in non-fasting samples(20). In the current study direct and

precipitation assays were found to be reliable, this is consistent with study by Jabar *et al.*,(2006) who found both Precipitation method and direct method precise and accurate in estimation of HDL-C and LDL-C, however direct assays have an advantage of time saving and are less labour intensive(21). Use of direct assays can improve reliability of the results of lipoprotein testing because it avoids the precipitation and centrifugation steps which depend on technical skills and experience of the laboratory technologist. The precipitation method for HDL-C testing is highly dependent on pipetting skills of the technologist, the centrifuge speed and hence can lead to variabilities related to personnel hence direct method can provide a better alternative.

In conclusion, results obtained by direct and precipitation/calculated methods of serum lipoprotein measurement are comparable and the methods can be used alternately. The choice of the technique should depend on amount of workflow, technical competence. Despite this direct assays have the capacity to improve precision in laboratory analysis of HDLC and reduce errors due to inaccurate pipetting when performing precipitation assays.

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