DIFFERENT METHODS FOR PARTICLE DIAMETER DETERMINATION OF LOW DENSITY AND HIGH DENSITY LIPOPROTEINS – COMPARISON AND EVALUATION

A Thesis

by

VIDYA VAIDYANATHAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2006

Major Subject: Nutrition

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ABSTRACT

Different Methods for Particle Diameter Determination of Low Density and High Density Lipoproteins – Comparison and Evaluation. (December 2006)

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Predominance of small dense Low Density Lipoprotein (LDL) is associated with a two to threefold increase in risk for Coronary Heart Disease (CVD). Small, dense HDL (High Density Lipoprotein) particles protect small dense LDL from oxidative stress. Technological advancements have introduced an array of techniques for measuring diameters of LDL and HDL as well as estimating overall particle heterogeneity. However, there is lack of comparative studies between these techniques, and, hence, no conclusive evidence to establish the merits of one method relative to others. The primary purpose of this study was to compare Nondenaturing Gradient Gel Electrophoresis (NDGGE) and Dynamic Laser Light Scattering (DLLS) methods in determining LDL and HDL particle diameter. Our comparison entailed: 1) Evaluating the two methods in terms of their reproducibility 2) Correlating the two methods(in future studies method selection would be driven by time and cost considerations if the two methods correlate), and 3) Evaluating the two methods in terms of their ability to identify bi-modal samples. A secondary purpose of this research was to investigate the effect of refrigerated plasma storage on particle diameter. Reproducibility was measured as Coefficient of Variance

(CV). Within and between runs, CV for LDL and HDL for NDGGE were <6% and <15%, respectively and for DLLS, CV within runs were <3% and <5.5%, respectively. No correlation was observed between LDL diameter from the two methods. NDGGE showed two bands for 157 HDL samples of which only 24 samples showed bimodal peaks in DLLS. In order to study the effect of storage, three sample sets of LDL and two sample sets of HDL were used. NDGGE showed a significant difference between mean diameter of fresh and stored LDL and HDL sample for all sets, whereas DLLS showed a significant difference in only one LDL sample set and none for HDL sample sets. We conclude that DLLS may be a better method for measuring LDL diameter because NDGGE overestimated LDL diameter. However, NDGGE was able to resolve subpopulation better in an HDL sample than DLLS. Thus, NDGGE may be a better choice for measuring HDL diameter than DLLS.

DEDICATION

To my family and friends for their love and support

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CHAPTER I

INTRODUCTION

Atherosclerotic Cardiovascular Disease (ASCVD) is characterized by abnormal deposit of lipid and fibrin in the vessels which subsequently results in the thickening of vessel walls (1). Coronary Artery Disease (CAD) refers to a condition in which arteries supplying blood to the heart muscle are affected. Coronary Heart Disease (CHD) encompasses both CAD and the resulting complications from ASCVD like angina and heart attack. Elevated levels of low density cholesterol are known to be associated with increased risk for ASCVD(2). Conversely, high concentrations of high density cholesterol are known to protect against the development of ASCVD(3). More recently, attention has been given to the diameter of lipoproteins and their role in ASCVD. Several lines of evidence suggest that low density lipoprotein (LDL) diameter is an important predictor of cardiovascular events and progression of CHD. Evidence suggests that LDL physical properties (particularly small, dense LDL) as well as quantity may increase cardiovascular risk (4). Austin et al. used mathematical modeling techniques to separate LDL tracings on gradient-gel electrophoresis into individual underlying Gaussian curves of LDL subspecies. From this technique Austin et al identified two subclass patterns of LDL: phenotype A and phenotype B (5). Phenotype B has been found in 40-50% of heart attack victims and individuals with ASCVD (6). Phenotype B is characterized by a relatively small diameter (< 25.5nm) and high density (d = 1.050-

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1.063 g/ml) whereas Phenotype A is characterized by a larger diameter (> 25.5nm) and more buoyant density (d = 1.025-1.033 g/ml) (6).

The National Cholesterol Education Program Adult Treatment Panel III has recognized a predominance of small, dense low-density lipoprotein (LDL) as an emerging risk factor for ASCVD (7). Predominance of small dense LDL is associated with a two to threefold increase in risk for CHD(8). Several epidemiological, perspective and case control studies support this estimate (9, 10). LDL exhibiting small dense phenotype B also possess an increased susceptibility to oxidative modification. This has been demonstrated by in- vitro studies which suggest that particles of a small diameter may directly promote atherosclerosis (6).

Differential precipitation (11, 12)and particle diameter analysis using nondenaturing polyacrylamide gradient gel electrophoresis (NDGGE) have been used to identify subclasses of high density lipoprotein (HDL) (13). Differential precipitation was used to broadly classify HDL populations as a less dense HDL_2, and a more dense HDL_3. Particle diameter analysis has helped to refine population boundaries: HDL_{2a} (8.8 to 9.7 nm in diameter), HDL_{2b} (9.7 to 12 nm), HDL_{3a} (8.2 to 8.8 nm), HDL_{3b} (7.8 to 8.2 nm), and HDL_{3c} (7.2 to 7.8 nm) (14). Dense HDL_3 particles are smaller in diameter than the less dense HDL_2 particles.

The role of smaller, denser LDL phenotype B particles and their major apolipoprotein, apoB-100 in ASCVD (15) is well established. A similar association between ASCVD and HDL particle diameter has not yet been established due to limitations imposed by biological variability, particle instability, and measurement error

with HDL subclasses (14). Miller (1987) showed that antiatherogenic property of HDL was associated with HDL_2 subclass (16), and smallest HDL particles ($HDL_{3b, c}$), have been associated with increased ASCVD risk in several studies (17).

On the contrary, several researchers have shown that small HDL particles possess antiatherogenic property (18). According to the theory put forth by these researchers the reduced free cholesterol and phospholipid (PL) content of small HDL increases the ability of this particle class to efficiently remove cellular cholesterol. HDL expresses its antioxidative property chiefly through its apolipoprotein A-1 (apoA-1). Apo A-I delay or prevent LDL oxidation, or both, by removing the oxidized PLs from LDL, artery wall cells or both. Paraoxonase (PON 1), an antioxidative enzymatic component of HDL also protects LDL against oxidative modification by reducing formation of hydroperoxides in LDL (19).

Technological advancements have introduced an array of techniques capable of measuring the diameters of LDL and HDL lipoproteins as well as estimating overall particle heterogeneity. Of these, NDGGE is most commonly used. Though accurate, this method is laborious and time consuming (20). Nuclear Magnetic Resonance (NMR) spectroscopy, Electron Microscopy (EM) and Dynamic Light Scattering (DLS) techniques are other methods of measuring particle diameter. However there is a lack of comparative studies between these techniques and hence there is no conclusive evidence to establish the merits of one method relative to others.

In the present study we measured particle diameter of LDL and HDL by NDGGE and DLS. The principle underlying particle diameter measurement by DLS is light

scattering arising from Brownian motion of suspended particles. Particles suspended in liquid exhibit random Brownian movement. The rate of motion is inversely proportional to the diameter of the particles. Within light scattering measurement instruments, light emitted by the laser upon incident on the particles is reflected back to the detector. Depending on the motion of the particles the reflected light reaches the detector at different time interval depending upon light wavelength. The signal generated by the detector will resemble a noise signal due to the constantly changing diffraction pattern caused by destructive and constructive interference with incident light as the particles change their position. Analysis of the intensity fluctuations yields a diffusion coefficient (D_T), which is related to particle diameter. In our case we will use a specialized form of DLS which employs an unshifted light control as a reference. This special technique is referred to as Dynamic Laser Light Scattering (DLLS).

In NDGGE particle diameter is determined by the migration distance of the particles in the gel. In this application, gel is a porous media in which pore diameter is of the same order as the diameter of the particle under investigation in order to effect molecular sieving of individual lipoprotein particles.

The primary purpose of this study is to compare NDGGE and DLLS methods in determining LDL and HDL particle diameter. Our goal was to determine whether the methods were comparable. Our comparison entailed:

1) Evaluating the two methods in terms of their reproducibility. Reproducibility is an important characteristic for any technique and it is crucial to know the reproducibility of a method.

2) Method correlation: If the two methods correlate well then in future studies method selection would be driven by time and cost considerations.
3) Evaluating the two methods in terms of their ability to identify bi-modal samples. LDL and HDL diameters have been associated with ASCVD. So it is important for a method to have the capacity to identify the distinct lipoprotein sub-populations, if any, in a sample.

Based on our preliminary analysis we provide suggestions for future comparative studies between these methods.

A secondary purpose of this research is to investigate the effect of refrigerated plasma storage on particle diameter. Our hypothesis is that particle diameter changes with storage. An observation of a change in the stored sample will substantiate this hypothesis.

CHAPTER II

LITERATURE REVIEW

Lipoproteins are spheroid particles that consist of a nonpolar core of triaclyglycerides (TAG) and cholesteryl esters (CE) surrounded by an amphilicic coating of protein, PL, and unesterified cholesterol (UC) (21). The amphipathic nature of these particles makes them ideal lipid transporters in the polar environment of the blood (21). Lipoproteins are divided in five broad classes, based on the relative ratio of lipid to protein in the particle (22). These lipoprotein classes are typically characterized by density, composition, particle diameter, and particle mass and distinct apolipoproteins (**Table 1**). In this section we will limit our discussion to LDL and HDL. Reported effects of monounsaturated fats (MUFA) on the diameter of LDL and HDL will be discussed. Finally, a review of the different methods used for measuring lipoprotein diameter will be presented.

Low density lipoprotein

LDL are spherical particles with diameters ranging from 18-26 nm (23). The particle core is composed of CE and TAG, surrounded by a lipid coat of UC and PL. LDL contains approximately 20-25% protein, 15-20 % PL, 7-10% UC, 35-40% CE and 7-10% TAG. The protein part which primarily comprises of apolipoprotein is embedded in the lipid coat. Apolipoprotein B_{100} (apoB_{100}) is an essential structural protein for LDL. It serves as the ligand for the LDL receptor in peripheral tissues and liver. ApoB_{100} is a component of plasma Chylomicron (CM), Very Low Density Lipoprotein (VLDL),

Intermediate Low Density Lipoprotein (IDL) and LDL (24). LDL sometimes contains other small apolipoproteins, such as apolipoprotein CIII (apoCIII) and apolipoprotein E (apoE) that modulate LDL metabolism (25). The diameter of LDL depends on how much lipid is in the core, and the lipid content determines LDL density. Thus, smaller LDL is more dense, while the larger LDL contain greater fractional core lipid content and therefore are more buoyant (**Table 2**)(26). Early studies using analytical ultracentrifugation determined that distinct LDL subpopulations are present in each individual (5).

Formation of small dense LDL

Very low density lipoproteins (VLDL) are the precursors for LDL particles (27). Plasma TAG concentration has been correlated with the diameter of VLDL and also the relative amount of each VLDL subclasses. The relative amount of large VLDL (VLDL1: S_f 60-400) (27) increases with increasing TAG concentration (28). Schneeman et al (1993) showed that 80% of the increase in lipoprotein particle number was accounted for by VLDL containing apo B_{100} after fat intake (29). This increase in number was confined to VLDL1 particles. Thus in hypertriacylglyceridemia (HTAG), there is an accumulation of VLDL1 in the plasma. Coronary artery disease patients were shown to accumulate large apolipoprotein C-I (apoC-I)-rich VLDL in their plasma after an oral fat- tolerance test. These subjects had an exaggerated postprandial TAG response to the fat-tolerance test (30). Enrichment of VLDL1 with apo C-I was reported to delay particle clearance and cause an accumulation of these VLDL in plasma. In-vitro studies supported this suggestion, and have shown that apo C-1 enrichment of VLDL inhibits apoE- mediated

uptake of VLDL by both the LDL receptor (LDL_R) and the LDL receptor – related protein (LRP) (31). Thus, though VLDL1 have large amounts of apoE, the key ligand mediating VLDL uptake by hepatic cells, the presence of apo C-I in these VLDL inhibits the ability of apoE to bind to its receptors. Possibly apoC-I masks the receptor binding domain of apoE (30) or displaces apoE from the lipoprotein particle (31). In addition, Berbee et al. has shown that apoC-I inhibits lipoprotein lipase (LPL) mediated TAG-lipolysis (32). Thus reduced binding of VLDL1 to its receptor along with decreased lipolysis of VLDL1 creates an environment ideal for the formation of small dense LDL (Figure 1) (27). Triacylglycerol rich VLDL1 remain in circulation for a longer time because of its decreased clearance rate. LDL formed from these VLDL1 have a greater residence time in blood (5 days) compared to LDL formed from normal VLDL (2 days) (27). This is because LDL from VLDL1 have an altered apoB conformation that reduces binding to LDL receptors (33). This increased residence time in the circulation allows LDL to undergo remodeling (27) and reduces oxidative stability (34). One important enzyme involved in the remodeling of LDL is CE transfer protein (CETP). It faciliates the loss of CE from LDL in exchange for TAG from VLDL. In the presence of slowly clearing VLDL1 there is increased transfer of TAG into LDL. In subsequent steps TAG is removed from LDL by Hepatic Lipase (HL). In case of increased hepatic lipase function, rapid removal of TAG from LDL leads to the formation of small dense LDL particles. Thus the key players in this model for generating small dense LDL are HTAG, CETP and HL (27) (**Figure 1**).

Modified function of small dense LDL

Small dense LDL binds less efficiently to the LDL receptors. This is primarily due to structural modification in the $apoB_{100}$ of LDL (33). Since $apoB_{100}$ is the ligand for LDL receptor, its modification decreases the affinity of LDL towards LDL receptors. Thus small LDL remain in circulation for a longer time. Another functional modification of small dense LDL is it reacts more strongly with the arterial wall proteoglycans (35). This interaction leads to accumulation of LDL in the subendothelial space of the artery wall, further modification, and uptake by macrophages thereby promoting foam cell formation. Small dense LDL is also more susceptible to oxidation as it has less vitamin E. Oxidized LDL provokes inflammation that underlies atherosclerosis (36).

Epidemiological studies linking LDL diameter and CAD

Predominance of small dense LDL is associated with a two to threefold increase in risk for CHD (8). Case-control study of myocardial infarction supports this conclusion as risk of myocardial infraction was significantly associated with predominance of small dense LDL. This association was independent of age, sex and relative weight. Subjects with increased small dense LDL also showed decrease in HDL-cholesterol (HDL-C) and increase in TAG and LDL-cholesterol (LDL-C). Small dense LDL was also shown to be associated with angiographically diagnosed CAD (9, 10, 37). Prospective studies involving three population cohorts demonstrated that reduced LDL particle diameter at baseline was a significant predictor for the development of CAD. But in most of the above studies the disease risk associated with small dense LDL was no longer significant after adjusting for TAG or other risk factors (9).

High density lipoprotein

High Density Lipoprotein (HDL) are a class of lipoproteins whose primary function is to carry cholesterol from the body tissues to the liver for disposal. Human HDL contains approximately 40-55% protein, 20-35% PL, 3-4% UC, 12% CE and 3-5% TAG. HDL are the smallest of the lipoproteins (5-12 nm) (38). They are the most dense lipoprotein class because they contain the highest proportion of protein (**Table 3**). In humans, the two major apolipoproteins in HDL are apoprotein A-I (apo A-I) and apoprotein A- II (apo A-II). HDL can also be grouped into subclasses based on their particle diameter (14) (15) (16). The discoidal HDL particles are small, lipid-poor, nascent particles (7.5nm) (39). They are made of apolipoproteins and enveloped by a layer of PL and UC. Spherical HDL are larger because they contain core lipids and are enriched with CE and TAG. These heterogeneous subclasses of HDL perform different physiological functions in the body (38).

Formation of small dense HDL

Spherical HDL particles are generated from lipid-free apoA-I or discoidal HDL (38) which are mainly produced by the liver and intestine or can be released as surface fragments from TAG rich lipoproteins during lipolysis (40) (**Figure 2**)(41). Nascent native HDL particles readily pick up cholesterol and PL at cellular membranes via efflux (40). The HDL cholesterol is subsequently esterified by the enzyme lecithin:cholesterol acyltransferase (LCAT) thus converting the nascent HDL into mature CE rich HDL (42). Cholesteryl ester from the mature HDL may be removed through several pathways. First, the CE in mature HDL maybe exchanged for TAG of apoB containing lipoproteins

via CETP (43). Second, there may be selective uptake of CE by the liver. This process is mediated by a separate class of scavenger receptors (44). Third, apoE-containing HDL, which constitute the minority of HDL, maybe internalized by hepatic apoE receptors (LDL_R and LRP) (45, 46). Cholesteryl ester transfer protein facilitates the exchange of HDL CE for TAG in VLDL or chylomicrons. The subsequent action of HL on the TAG-rich HDL generates small, PL- rich HDL that are the optimal mediators of cellular cholesterol efflux (46).

Function of small dense HDL

Small, dense HDL particles protect small dense LDL from oxidative stress. Apolipoproteins and enzymes (like PON1) associated with HDL possess antioxidative properties which reduce formation of peroxides in LDL (47). This antioxidative property of the apo lipoproteins and enzymes are unevenly distributed across HDL subfractions, being more pronounced in small HDL (19). Consequently, small, dense HDL potently protects LDL against oxidative stress (19). Small HDL probably acquires oxidized lipids more efficiently than large HDL, because of their distinct cholesterol efflux capacity (48).

Epidemiological studies linking HDL diameter and CAD

Several studies have investigated the importance of HDL particle diameter as predictor of future coronary events (48). In the male participants of the Framingham Offspring Study, concentration of large diameter HDL particle was found to be lower in subjects with CAD than in control (49). In vitro studies have documented that small HDL are more potent in removing cellular cholesterol than large HDL (18, 50) and thus

concluded small dense HDL to be a more potent antiatherogenic agent. These studies have shown that in metabolic diseases like Metabolic Syndrome (MetS) and type 2 diabetes, the antiatherogenic property of the small dense HDL is the most affected (51). In insulin resistance small dense HDL showed TAG enrichment, CE depletion and attenuated antioxidative property. Attentuation of the antioxidative function of small HDL may not be the only consequence related to HDL in MetS. Studies show that in MetS the total concentration of HDL-C on the whole may be decreased (52) and subsequently less HDL maybe available to protect LDL against oxidation. Decrease in HDL concentration was measured by level of apoA in circulation and these studies showed that apoA-I concentration was decreased in MetS.

Monounsaturated fat and particle diameter

The effect of dietary fat on LDL -C concentration is well documented (53). The effect of MUFA on LDL diameter has also received much attention. However, literature on the effects of MUFA on HDL particle diameter is limited. A study showed that HDL particle diameter did not differ significantly between Saturated Fat (SFA), MUFA and Polyunsaturated fat (PUFA) in fatty fed individuals. Healthy men and women participated in this randomized multiple crossover design study and were fed diets enriched with stearic acid, oleic acid, and linoleic acid (54). The main source of stearic acid in the stearic acid- rich diet was coco butter (33.5%), oleic acid enrichment was achieved with high oleic acid sunflower oil (47.0%), while linoleic acid enrichment was achieved with safflower oil supplementation (52.0%). The diets provided 38% of total energy requirement from fat, of which 60% was supplied by the experimental fats.

Subjects were provided with a list of fat- containing food from which they could choose foods to meet their remaining 40% of the daily fat intake. Except for the 7% of energy specifically provided as stearic, oleic, or linoleic acid, the nutrients composition of the diets were the same across the three treatments. Each subject consumed one of the three diets for 5 wks followed by a washout period of > 1 wk before crossing over to the next diet. At the end of the experimental period, LDL and HDL particle diameters were measured using NMR spectroscopy. The result showed there was no significant difference of LDL and HDL particle diameter across the three fats.

Compared to HDL, dietary fat has been reported to alter LDL diameter in a number of studies. A study involving apparently healthy individuals showed that both MUFA and PUFA had similar effect on the diameter of LDL particles. Both dietary fats produced very small reduction in the diameter (55). In this parallel design study each individual consumed one of the three test fats – olive oil (rich in MUFA), rapeseed oil (rich in MUFA and PUFA) and sunflower oil (rich in PUFA) for four wks. At baseline each individual were required to consume a diet high in SFA for two wks. Subsequently subjects were randomly assigned to the three test fats. Other than the different fatty acid composition all the three diets were identical. Energy percent from total fat was same at baseline and during the treatment. Energy percent from SFA was similar between the three test diets and was ~ 9% less than baseline. LDL diameter was measured using NDGGE after four wks of feeding experimental fat. It was shown that all endpoint LDL diameter was significantly smaller than baseline LDL diameter for all treatments (olive oil, 26.28 vs 26.36 nm; rapeseed oil, 26.11 vs 26.29 nm; sunflower oil, 25.89 vs

26.18nm), although no significant difference was noted in LDL diameters between the three diets at the end of the experimental phase. The study concluded that dietary MUFA and PUFA similarly reduce LDL diameter relative to SFA.

Another study showed that LDL diameter increased in healthy subjects fed a Mediterranean diet, high in MUFA-fat compared with a low fat, high carbohydrate diet (CHO) (56). Subjects were initialized on a saturated rich diet for four wks (47% CHO, 38% fat, 20% SFA, 12% MUFA, 6% PUFA). At the end of four wks they were randomized into two groups. First group was assigned to a MUFA rich diet (47% CHO, 38% fat, <10%, 6% PUFA, 12% MUFA). At the end of four wks, the group was put on a CHO rich diet (55% carbohydrate, <30% fat, <10% SFA, 6% PUFA, 12% MUFA). The second group was assigned to the high CHO diet first followed by the high MUFA diet. There was no washout period between the two diets in either group. At baseline LDL particle diameter was significantly larger in subjects with the apoE 4/3 genotype compared with those with apoE 3/3 and apoE 3/2 (**Table 4**)(56). There was a decrease in LDL diameter in subjects with apoE 4/3 after changing from a high-CHO diet to a MUFA-rich diet (26.47 vs 26.26), whereas in subjects with apoE 3/3 there was an increase (25.74 vs 25.91). The study concluded that high MUFA diet increases LDL diameter when compared to high CHO diet, but the effect was dependent on apoE genotype. The study did not compare LDL diameter among the two diets independent of genotype. In contrast to the above studies, some studies have documented no effect of MUFA on LDL diameter or have show that though MUFA-rich diets increased LDL

diameter, they caused more plaque in monkeys than PUFA. A few of these studies are described below in brief.

The first study was done in a group of African monkeys (57). The objective of the study was to determine if MUFA was as effective as PUFA in limiting the development of coronary artery atherosclerosis. Monkeys were fed a challenge diet containing SFA (40% of total energy as lard) and cholesterol (0.8mg /kcal) for eleven wks. At the end of the challenge diet period, plasma concentrations of total cholesterol, VLDL-C, IDL-C, LDL-C, HDL-C, apoB, apoA-I, apoE, and LDL particle diameter were measured and monkeys with equivalent means were grouped together in order to create three experimental groups. The challenge diet was followed by a 10-wk washout period in which monkeys were fed monkey chow diet. Subsequently each group was fed for about 5 years with experimental diets containing 35% of kilocalories as fat and 0.8 mg cholesterol/kcal. The diets differed only in the type of fat. Two diet periods with different degree of fatty acid enrichment was used (**Table 5**)(57). The degree of fatty acid enrichment was more exaggerated during the final 42 months (the longest period of the study, period 2). During period 2, the SFA diet, mainly palm oil contained 40% of the fatty acids as palmitic acid. The MUFA diet, mainly oleic acid–enriched safflower oil contained over 70% of the fatty acids as oleic acid. The PUFA fat diet, mainly safflower oil contained over 70% of the fatty acids as linoleic acid. At the end of the study LDL were isolated by size exclusion chromatography and average LDL diameter was measured as LDL molecular weight. The LDL particles were large in the SFA fat group, were of comparable diameter in the MUFA fat group, and were smaller in the PUFA fat

group. The study also showed that at end of the 5 years of diet feeding, the atherosclerosis extent in MUFA-fed group was similar to that in the SFA-fed group, while the PUFA-fed animals had less.

The second study was a clinical trial involving healthy, nonsmoking males 35 to 55 years of age and postmenopausal females 50 to 60 years of age showed that MUFA enriched sunflower oil diet providing 40% to 42% of energy from fat, with 26% to 28% from MUFA and 40% to 45% of energy from CHO caused no significant change in the LDL diameter as compared to a low fat, high CHO diet providing 22% to 25% of energy from total fat, 7% to 8% of energy from MUFA and 55% to 60% of energy from CHO(58) . A group of subjects was randomly assigned to either of the diets for one month with a two-week washout period in between, during which subjects consumed their habitual diet. LDL diameter of the subjects was measured after each experimental period. No difference was observed in LDL diameter between subjects when consuming the high CHO diet (26.15 nm ± 0.23) and MUFA- enriched sunflower oil diet (26.20 nm ± 0.23).

The third study involved one hundred and sixty-two healthy Caucasian men and women within the age group of 30–65 years with normal or moderately increased body weight (59). Subjects consumed an isoenergetic diets, with same amount of total fat, but one diet had a high proportion of SFA while the other had a high proportion of MUFA. Both the diets derived 37% of energy from fat. The high SFA diet provided 17%, 14% and 6% of energy from SFA, MUFA and PUFA respectively. The MUFA diet provided

8%, 23% and 6% of energy from SFA, MUFA and PUFA respectively. LDL diameter did not differ in the two groups.

Thus, we see that while some studies reported an effect of MUFA on the LDL diameter, there are studies which have shown MUFA to have no effect on the particle diameter of LDL.

Methods to measure LDL and HDL diameter

Diameter of LDL and HDL maybe used as risk determinants for ASCVD (8). Therefore, there is need for accurate methods to determine the diameter of these particles. A number of tools are available currently to assess the heterogeneity of LDL and HDL. Of these, NDGGE is the most commonly used method (60). Though accurate, this method is laborious and time consuming (20). Dynamic light scattering (DLS) technique is another particle diameter measurement technique which uses the principle of Photon Correlation Spectroscopy (PCS). Studies have showed good correlation of data obtained from NDGGE and DLS methods (61). Nanotrac®NPA250 (Microtrac, FL) is a particle size analyzer that uses DLLS to determine particle diameter distribution in a sample. Zetasizer® (Malvern Instruments, UK) 3000 is another particle diameter analyzer, which operated on the principle of Photon correlation spectroscopy (PCS). It is a DLS instrument. The basic difference between a DLLS and DLS is the former has a heterodyne configuration and the latter has a homodyne configuration. The two configurations will be described in later section.

Non-denaturing gradient gel electrophoresis

The basic principle of gel electrophoresis is that charged particle migrates through a gel under the influence of an electric field. In this application, gel is a porous media in which pore diameter is of the same order as the diameter of the particle under investigation in order to effect molecular sieving of individual lipoprotein particles. In many applications, the separation is dependent on both charge density and particle diameter (or protein molecular weight) (62, 63, 64) .

Different media are used for electrophoresis. In the present study, we used polyacrylamide gel. Polyacrylamide gel has number of advantages over other media like agarose and starch. Polyacrylamide gel is a synthetic polymer of acrylamide monomer, and is prepared from highly purified reagents in a reproducible manner if the polymerization conditions are standardized. Polyacrylamide gel is chemically inert, stable over a wide range of pH, temperature, and ionic strength, and is transparent. Polyacrylamide is better suited to a size fractionation of particles since the gels it forms can have a wide range of pore sizes. The effective pore size of polyacrylamide gels is determined by the total acrlyamide concentration in the polymerization mixture. Effective pore size decreases as acrlyamide concentration increases. Thus, two particles with widely differing diameters, but identical charge densities will separate on polyacrylamide gel cast to contain gradient of increasing acrylamide content as the molecular sieving effect would slow down the migration rate of the larger particle relative to that of the smaller particle (62). The electrophoretic conditions created by

0.09 M Tris, 0.08M borate, 0.003 M Na_2 EDTA, pH- 8.35, cause all applied lipoprotein particles to adopt a similar charge density. Thus, particles separate according to their diameters. A gradient increases the range of diameter of particles that can be separated, and also causes sharpening of bands. The most extensively used gradient gel for lipoprotein separation are commercially prepared slabs with linear gradients of 2-16% for LDL and 4-30% for HDL (62).

Dynamic light scattering method

Dynamic laser light scattering operates on the principle of PCS or Quasi-Elastic light scattering technique. Macromolecules suspended in liquid exhibit Brownian motion which scatter light causing either a constructive or destructive interference. The interference changes with the movement of the molecules causing a fluctuation in the intensity of the scattered light. The fluctuation ranges between zero and twice the average intensity. The rate of fluctuation depends on the velocity of molecules. The measurements of the change in fluctuation rates are thus used to calculate the transitional D_T. Macromolecules are small fast moving particles, and thus scatter very little light, causing rapid intensity fluctuations. Diffusion coefficient is obtained by counting the individual scattered photons. These photons are counted into a channel or time window and autocorrelation of this data gives the intensity fluctuation constant. Once D_T has been determined, the hydrodynamic radius, $R_{H,}$ can be calculated using the Stokes-Einstein equation:

$$D_T = kT/ 6\pi\eta R_H$$

Where k is Boltzmann's constant, T is the absolute temperature and η is viscosity (65, 66, 67, 68).

Factors affecting particle diameter measurement

Velocity: Particle diameter is derived from the velocity distribution of the particles. Median velocity is inversely proportional to the particle diameter, i.e. small particles have a higher median velocity than large particles (65).

Temperature: It is obvious from Stokes-Einstein equation, that valid calculation of particle diameter depends on the absolute temperature of the fluid molecules in which the particles are suspended. Fluid molecules with high temperature have high average thermal energy and so will impart higher velocities to the particles during collision. Median particle velocity is directly proportional to the absolute temperature of the fluid.

Viscosity: Particle velocity is inversely proportional to fluid viscosity. Viscosity is a complex but predictable function of temperature. If the operating temperature and viscosity at two temperatures spanning the operation interval are known, the viscosity at that temperature can be calculated. Thus, compensation can be made for both temperature, and fluid viscosity. The median particle velocities range from 5 micron per second to 6000 micron per second (65).

Heterodyne and homodyne configurations

Commercially available PCS instruments use two type of configurations, namely heterodyne and homodyne. In heterodyne configuration the frequency shifted light is mixed with stable, unshifted light (**Figure 3**) (65). The unshifted light is called the

reference light (or baseline) for the scattered, shifted light from each particle. The reference light originates from the laser and is reflected in the instrument to the detector. The interaction of the reflected and shifted light allows for removal of the high optical frequency present in the particle-scattered light. This leaves only the lower shifted frequencies, which are the values that are related to the diameter of the particles.

Homodyne detection uses a reference that is not stable and unshifted (Figure 3). The reference light actually denotes the light shifted by other randomly moving, unstable particles in the solution (65).

Advantages/ disadvantages of heterodyne and homodyne configuration

First in heterodyne configuration the power spectrum (frequency distribution) of particles depends upon twice the characteristic frequency of particle i.e. 2γ, while in homodyne configuration, power spectrum depends on γ. This means that a slight change in frequency of particle may result in a greater change in the power spectrum in homodyne configuration. Second, the homodyne power is proportional to the square of scattered light intensity, while the heterodyne power is proportional to the product of the scattered intensity and reference (laser) intensity. This implies that the heterodyne signal level can be made many orders of magnitude larger than the homodyne signal by providing a large reference beam using the reflected laser. This enables measurement of small particles through back scattering and also measurement of very dilute suspensions. In the present study we used Nanotrac®NPA250 (Microtrac, FL) particle size analyzer to determine particle diameter distribution through DLLS technique. The following section describes the operation of Nanotrac®NPA250.

Nanotrac®NPA250 operates on heterodyne configuration. Light from a laser diode passes through an optical beam splitter and strikes the sample. A sapphire window located at the probe tip forms an interface between the sample and the probe. The sapphire window has two functions. First, it reflects the original laser back through the optical beam splitter to a photodetector. This signal acts as a reference signal for detection as it has the same frequency as the original laser. Second, the laser passes through the sapphire window and is scattered by the suspended particles, which are moving in Brownian motion. The frequency of this laser is Doppler shifted relative to the velocity of the particles it encounters. Light is scattered in all directions including 180 degrees backwards. This frequency shifted light is transmitted by the sapphire window through the optical beam splitter to the photodetector. These signals of various frequencies combine with the reference signal to generate a wide spectrum of frequencies. A high speed digital signal processor calculates the power spectrum of the interference signal. A power spectrum describes how the power of a signal or time series is distributed with frequency.The power spectrum is analyzed mathematically and the particle diameter distribution is calculated.

Some of the parameters obtained from Nanotrac®NPA250® NPA250 are:

MV (Mean volume diameter) - Mean diameter in microns, of the volume distribution- represents the center of gravity of the distribution. This parameter is influenced by the presence of large particles in the sample. It maybe considered as a type of average particle diameter.

MI (Mean intensity diameter) - Mean diameter, in microns, of the intensity distribution-is calculated from the intensity distribution. Intensity mean does not take into consideration the effects of refraction and only reflects the relationship of the light signals detected. It is influenced by the intensity of the signals, and the value is displayed only when the intensity distribution is presented.

MA (Mean area diameter) - Mean diameter, in microns, of the Area distribution is calculated from the volume distribution. This parameter is less influenced than MV by the presence of larger particles, and therefore shows smaller particle. MA is a particle surface measurement.

MN (Mean number Diameter) - Mean diameter, in microns, of the number distribution-is calculated from the volume distribution data and is influenced by presence of small particles in a sample. This parameter is related more to the number of small particles in a population.

Microtrac Nanotrac®NPA250 measurement approach

The Microtrac Nanotrac® NPA250 operates on heterodyne detection. **Figure 4** (65) is a diagram of the Nanotrac®NPA250 measurement system. It shows the region of light interaction with the suspended particles. The Nanotrac®NPA250 has an optical beam splitter which ends on a sapphire window. The sapphire window is immersed in the suspension. The sapphire window delivers the laser beam to the sample. The sapphire window and optical beam splitter also collects light reflected back at 180°. The unshifted beam reflected at the interface between the sapphire window and the medium, is mixed with the back-scattered light. This unshifted beam provides the reference for

heterodyne detection. The high refraction index of sapphire produces a reflected reference beam with adequate intensity. There are two important features to this type of configuration. First, the high intensity reference beam allows the heterodyne component to dominate the power spectrum and to provide a signal which is much stronger than the equivalent homodyne signal. The high signal allows for the use of a silicon photo detector and a solid-state laser diode source which are very stable and reliable.

The second important feature of Nanotrac®NPA250® is the scattered light signal travels a very short path length in the suspension. This allows measurement of high particle concentration while preventing effects of multiple scattering.

Comparison between Nanotrac®NPA250 (Model NPA250) and Zetasizer® (Model 3000)

The frequency spectrum is uniquely determined by the particle velocity distribution that in turn is uniquely determined by the particle diameter distribution. The particle diameter distribution is calculated directly from the frequency spectrum obtained from the Doppler-shifted scattered light.

Transparent particles like LDL and HDL that have diameters less than 100nm are transparent and give rise to optical interference effects. Optical interference may be constructive or destructive. The amplitude of the signal received at the photo-detector will be larger if the interference is constructive and smaller if the interference is destructive. If the particles in a sample are of a single uniform diameter, optical interference is not a problem. The shape of the frequency spectrum is unaffected, resulting in the proper computation of particle diameter. Optical interference effects can

be significant if the particle distribution in a sample is bimodal, or if the particle population has a broad distribution of diameters. The computed particle diameter distribution would be distorted and skewed towards those particles that scatter light more efficiently. A true Volume distribution can be obtained when the distorting effects of the scattering efficiency function are properly compensated. If the distorted effects of the scattering efficiency function are not compensated, the resulting computed particle diameter distribution is more properly termed an Intensity weighted distribution.

Instruments based on older technologies were unable to make the necessary[104] compensation and provided only intensity weighted particle diameter distributions. Nanotrac®NPA250 compute true volume distributions in the standard mode of operation and offer the user the alternative of selecting Intensity weighted or Mono-disperse modes of operation. The Nanotrac®NPA250 incorporates a highly accurate temperature sensor in the sample cell. By describing the fluid temperature and viscosity characteristics in the Nanotrac®NPA250 algorithm, these parameters can be included in determining accurate particle diameter distributions. Also, because the laser light needs only to penetrate approximately 100 microns into the sample to generate a power spectrum, the Nanotrac®NPA250 can accurately determine particle diameter distributions at significantly higher concentrations than other methods.

Zetasizer® (Model 3000) produces particle diameter information as Z-average size. The mean size or z-average is a intensity weighted mean. It is calculated from the signal intensity (69). This measurement can only be comparable to other techniques if the sample is monomodal (i.e. only one peak), spherical and monodisperse (i.e. no width

to the distribution), and the sample is prepared in the correct solvent. The instrument gives two values, a mean value for the diameter, and a width parameter known as the Polydispersity, or the Polydispersity Index (PDI). Polydispersity maybe defined as the state of nonuniformity in Molecular Weight (MW) of a material. It indicates whether the sample is composed of molecules with homogenous MW. A homogenous or a monodisperse sample has a polydispersity equal to1. A heterogenous or polydisperse sample is a mixture of species of different MW and has polydispersity that is different from 1.

The Z-average is a D_T, which is converted to a diameter using the dispersant viscosity and some instrumental constants (68).

For Zetasizer the cutoff PDI value for sample is 0.5.Thus the measurements from a Zetasizer® may give a more accurate description of the diameter that is comparable with other methods of analysis only if the samples are spherical and have a reasonably narrow distribution, i.e. with polydispersity below a value of 0.1. If the polydispersity is over 0.5, the Z-average mean may not be a reliable measurement, and a distribution analysis (using instruments like Nanotrac®NPA250) could be expected to provide more accurate peak positions (68). Nanotrac®NPA250 instruments uses advanced calculation to compute the full particle diameter distribution without a priori assumptions about that distribution and thus avoid the ambiguity of distribution approximation methods and assure maximum information about the sample particle distribution. In addition, because the laser light needs only to penetrate approximately 100 µm into the sample to generate

a power spectrum, the Nanotrac®NPA250 can accurately determine particle diameter distributions at significantly higher concentrations than other methods.

CHAPTER III

METHODOLOGY

Blood samples for our study were obtained from an ongoing study (main study) in our laboratory.

Subject selection

Thirty Texas A & M University faculty and students were recruited for the main study. The subjects were normal healthy males between the ages of 30 and 60 yr. Subjects were screened with a battery of blood chemistry tests including plasma glucose, BUN, major minerals, protein, albumin, bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase,TAG, and all lipoprotein cholesterol fractions. All subjects had total serum cholesterol values between 200 and 260 mg/dl, were non smokers and were not on restrictive diets or medication. All subjects were free-living. Participants were advised of study risks and provided informed consent prior to study participation. The study was approved by the Institutional Review Board of Texas A & M University.

Dietary intervention

Preparation of ground beef

Fat trim were collected at Texas A & M Rosenthal Meat Science & Technology Center from carcasses of Angus cattle fed either a corn-based, finishing diet (to 16 mo of age; MUFA: SFA ratio= 1.1), or a hay-based diet (to 20 mo of age; MUFA: SFA ratio=0.8), supplemented to attain a live weight of 500 kg at slaughter. Carcasses of

Angus cattle fed a corn-based diet to 24 mo of age (600 kg live weight) was the source of fat trim with the highest MUFA: SFA ratio (1.4). Cattle were adapted to their diets at weaning (8 mo of age). Fat trim was removed, vacuum –packaged, and stored frozen at -80°C until used in ground beef formulations. Lean trim and fat trim were derived from carcasses available at the Texas A & M University Rosenthal Meat Science & Technology Center at the time of ground beef preparation. Each type of fat trim was ground separately with a Hobart grinder using a coarse (0.64 cm) grinding blade. Fat and lean trims were combined at appropriate ratios to yield 30 % total extractable lipid and reground using a fine (0.32 cm) blade. Ground beef patties (110g: 4 oz) were formed with an automated patty maker, quick frozen to -20°C, individually vacuum- packed and aged in polypropylene film. Final composition of the ground beef patties is given in **Table 6**.

Experimental design

Three groups of 10 men each were assigned randomly to one of the ground beef patties diets for 5-wk durations followed by crossover after habitual diet washout periods. The washout period was for 3wks to allow complete recovery from test diets. The 5-wk dietary periods excluded major holidays. A study phase was defined as the time prior to beginning of a 5-wk dietary period to the end of the 3-wk washout period. The study was conducted in three phases (**Figure 5**).

Sample collection

Fasting blood samples were collected at the start of each phase. (*baseline*), and again at the end of the 5-wk dietary period (*endpoint*). Within each baseline and

endpoint wk two blood samples were obtained. Therefore each subject had six baseline and six endpoint samples available for analysis (**Figure 5**). **Table 7** shows the nomenclature used for labeling the six baseline samples and the six endpoint samples at three study phase.

Diet instruction

Participants were instructed to incorporate the study beef into their diet with as little disruption as possible. No restrictions were placed on how the beef patty is to be prepared, but it was emphasized that all of the beef patty must be consumed within a single meal period. Participants substituted 110 g (4 Oz) of ground beef for the meat they typically consumed. Participants were required to consume one patty per day for a total of five patties in a wk. Participants were frequently contacted by e-mail to provide updates and encourage compliance. On the day of the initial blood sampling each participant received an unlabeled box containing all of the patties necessary for each 5-wk trial.

Laboratory analysis

Blood draw

Plasma was harvested from blood collected into tubes containing EDTA, which prevents blood clotting and preserves plasma lipoproteins prior to their separation by sequential density gradient ultracentrifugation (20). LDL and HDL were isolated by sequential density gradient ultracentrifugation using human density intervals (20). Particle diameter distributions were measured in prepared LDL and HDL using two methods namely, NDGGE and DLLS.

Storage study

To study the effect of storage on plasma lipoprotein diameter, 2ml of plasma from each of endpoint of phase I, baseline of phase II and baseline of phase III were held for four days at 4°C. At the end of 4 days they were prepared and analysized as were fresh plasma samples.

Separation of plasma lipoprotein fractions

Plasma lipoprotein separation was accomplished by density gradient ultracentrifugation. In this method lipoprotein separates into layers based on their density. Particle with the lowest density has the greater non-polar lipid content within its core. As such, it has been shown that when centrifuged, larger TAG- rich lipoproteins rise to the top of the centrifugal field, while particles containing progressively less core distribute in a gradient fashion below. Thus VLDL +IDL with over 50% TAG, has the lowest density and the largest particle diameter when compared to LDL and HDL fractions (Table 1). This implies that lipoprotein density is inversely proportional to its diameter.

Density solutions were constructed for separation of different lipoprotein fractions. At first a large volume of sodium chloride (NaCl) stock solution was made with the same molar background density as plasma (1.0063gm/ml). Distilled water was used for making the solution and the entire preparation was done under sterile conditions. Other, progressively more dense solutions were constructed from this stock solution by adding NaBr. All density solutions were measured with a digital density meter (Mettler/Parr, DMA 46, Graz, Austria). Saline solutions with given densities were

added to plasma samples in order to reach final densities of 1.026g/ml, 1.063g/ml, and 1.216g/mL for VLDL+ IDL, LDL and HDL fractions, respectively. Balanced centrifuge tubes were placed in a Sorvall 45.6 TFT rotor (Sorvall-Dupont, Wilmington, DE), spun in a Beckman L8M Ultracentrifuge at 40K rpm (272,000g), and 14°C for 18 hours. HDL was spun at 14°C for 24 hours. The first ml of each fraction was carefully aspirated and was used for measuring particle diameter.

Analysis of particle diameter

I) NDGGE: NDGGE is an external standard method that was used to indirectly estimate LDL and HDL particle diameters for all the samples. In this method particle diameters are estimated from a standard curve constructed from the migration distance of globular protein standards of know diameter.

We employed the method of Nicholas et al (20). Briefly, 20µl of lipoprotein fractions were mixed with 5 µl of sucrose/dye solution (40% sucrose, 0.01 % bromophenol blue) to facilitate sample application. A vertical electrophoresis apparatus was used for the experiment (Model GE-2/4 LS, Pharmacia, Uppsala, Sweden). Polyacrylamide gradient gels (Alamo Gel San Antonio TX; 2-16% for LDL, 4-30% for HDL) were loaded with the sample and standard proteins. Standard proteins of known hydrated diameter were used as calibrators (High Molecular weight standards, Amersham Pharmacia, Piscataway, NJ)(20). Prior to actual run, the gels were equilibrated in a buffer solution (41.01 gm Trizma base®, 19.17 g boric acid, 3.5 g EDTA and 0.73 g sodium azide, pH 8.3, 4L) for 30 minutes (70 volts, 40 ampere current, 4°C). The samples and the standards were loaded into the wells at the top of

each gel prior to application of 125 volts for 24 hrs at 4°C. Separated lipoprotein bands were stained overnight with 0.05% Coomassie Blue R-250 in methanol:acetic acid:water (45:10:45). The gels were destained with methanol –acetic acid – water (20: 5:75) solution. Individual gels were digitized using an Odyssey (LiCor, Lincoln NB) imaging device and associated software. Each band center was identified manually and the software then calculated the migration distance of the band center from the top of the gel. Particle diameters were calculated in the digitized scans by comparison with migration distances of standard proteins of know hydrated diameter: thyroglobulin (17.00 nm), apoferritin (12.20 nm), catalase (10.40), lactase dehydrogenase (8.16) and bovine serum albumin (7.10). For calculation of the diameter of LDL the three larger standards (thyroglobulin, apoferritin and catalse) were used and for calculating HDL diameter all the five standards were used.

Chicken VLDLy contains apoVLDL-II which is an inhibitor of LPL. So these particles can resist the intermediate metabolism of TAG hydrolysis and remain very stable in composition and diameter (70) regardless of lipoprotein age or turnover rate. This uniformity in diameter produces a narrow or “monodisperse” distribution range. So the diameter of a chicken VLDLy sample was determined using DLLS. Diameter of the same sample was determined by NDGGE using the standard curve of the five standard proteins. This information was used to construct two separate standard curves - one using chicken VLDLy diameter measured by DLLS and the standard proteins and the second using chicken VLDLy diameter measured by NDGGE and the standard proteins. LDL diameter of few samples were measured using the two standard curves with the

chicken VLDLy and also the original standard curve without the chicken VLDLy. Our purpose was to see if there was a significant change in the diameter of LDL if a standard was introduced that was larger than 17nm in diameter. Particle diameter of LDL was also calculated from a standard curve constructed by plotting logarithm of radius against the distance migrated relative to that of apoferritin or R_f. R_f of apoferritin was set at 1.0. II) DLLS: The first milliliter of each isolated lipoprotein fraction was used to measure the particle diameter distribution. Samples were measured in triplicate with each measurement allowing 1 minute for signal collection. Particle refractive index, fluid refractive index and viscosity of the suspending fluid at 20°C and 26°C solution were provided to the software (**Table 8**). Prior to sample analysis, set zero was established by analysis of the suspending fluid free of lipoproteins. The suspending fluid, or the background solution, was composed of the same salt solution and was of the same density as that in which lipoproteins were suspended. This step is necessary to compensate for background solution light scattering. Averages from triplicate measurements were used for statistical analyses.

Statistical analysis of data

Measurement of LDL diameter from DLLS were excluded if they were <16nm or > 28nm. For HDL, DLLS measurements were excluded if they were <7 nm or >14 nm. The Loading Factor (LF) and Concentration Index (CI) of discarded samples were compared to included data to see if the two were significantly different. Particle diameter of LDL obtained from NDGGE which had a chicken VLDLy (measured by DLLS) as a standard was correlated with MV, MN, MA and 50th percentile of MN distribution of

LDL diameter measured by DLLS to see which of the four correlated best with NDGGE measurements. Similar correlation was done for HDL. Chicken VLDLy diameters obtained from NDGGE and DLLS were compared. LDL diameter measured from the standard curve with chicken VLDLy (diameter measured by NDGGE) as a standard was compared to LDL diameter obtained from a standard curve that did not have chicken VLDLy as a standard. LDL diameter obtained from the above two standard curves was also compared to LDL diameter obtained from the standard curve which had a chicken VLDLy as a standard but the value of diameter of the chicken VLDLy was obtained from DLLS. The LDL diameter obtained from all of the above curves were compared to the LDL diameter obtained from the standard curve obtain by plotting log(r) against R_f of standards.

To determine the effect of storage on particle diameter, particle diameters measured using fresh samples were compared with diameters measured using samples stored at 4°C for four days. The LF and CI of stored sample were compared with that of fresh samples to see if there was a significant change. The number of HDL bands resolved on NDGGE was compared with the number of HDL samples for which DLLS found bimodal peaks. Our aim was to determine if DLLS showed bimodal peaks for samples that had double bands in NDGGE. We were also interested to know if the width of the samples identified as bimodal by NDGGE but not by DLLS were greater than the width of samples with single bands in NDGGE and monomodal in DLLS. For this we compared thirty monomodal HDL samples from DLLS which showed two bands in NDGGE to thirty monomodal HDL samples from DLLS which showed one band in

NDGGE. Measurements from NDGGE and DLLS were evaluated for reproducibility by using the coefficient of variation (CV) as calculated from the arithmetic mean ($\overline{X}$) and standard error of mean (SEM) where CV= ($\frac{SEM}{\overline{X}}$) 100%. For NDGGE, CV for within runs and between runs was calculated using the relative migration values for the standards. For DLLS, CV among runs were calculated using the triplicate measurements of a sample. Paired, two-tailed t-tests were done using SPSS software for comparisons and $p<0.05$ were considered as significant.

CHAPTER IV

RESULTS

Subjects

Twenty-eight out of the thirty subjects, who participated in the study, successfully completed it. Two of the participants were unable to complete the study due to illness not related to study.

Data exclusion

For DLLS, data of the 28 subjects were further scanned to remove suspect data. For LDL, a sample run was discarded if its corresponding MN value was < 16nm or > 28nm. Further if all three runs of a single measurement had a MN value less than 16nm, then that particular sample was discarded. Similarly, for HDL a sample run was discarded if its corresponding MN value was < 7nm or > 14nm. Thus if all three runs of a single measurement had a MN value < 7nm or > 14 nm, then that particular sample was discarded. Finally for both LDL and HDL, data were also excluded if they were incomplete i.e. if the subjects missed any of the twelve draws (six baseline draws and six endpoint draws). **Table 9** summarizes the criteria for exclusion of data and also indicates how many of the analysis were excluded at each collection point based on each criterion. Note from the Table that more HDL analyses were discarded than LDL as they did not meet our criteria.

Thirty excluded and included DLLS measurements were randomly selected for both HDL and LDL. The corresponding LF's and CI's were compared (**Table 10**). The

mean LF for included and excluded measurements were 0.1. Loading Factors of both included and excluded HDL analysis were < 0.1 and there was a significant difference between the LF factors for excluded and included analysis of HDL ($p<0.05$). Difference in concentration indices between excluded and included analysis for both LDL and HDL were significantly different (0.8 vs 0.3) ($p<0.05$). For NDGGE none of the LDL and HDL data were discarded.

Comparisons between NDGGE and DLLS

Reproducibility

Reproducibility of the two methods was determined (**Table 11**). For NDGGE, CV of LDL for both within and between runs was < 6% and for HDL, CV for within and between runs was < 15%. CV within runs for DLLS was calculated for MV, MN, and MA of both LDL and HDL. CV of LDL for within runs was < 3% for MV, MN, MA and CV of HDL for within runs was < 5.5% for MV, MN and MA.

Overall Mean Diameter of LDL and HDL

Mean diameters of LDL and HDL were obtained from the two methods. To calculate the mean, measurements of all 12 collections were pooled together. Samples which were suspect were discarded before calculating the mean. For HDL samples which showed two bands on NDGGE, the average diameter of the two bands was calculated and that single value was used for computing the mean. The mean diameter of LDL as measured by NDGGE and DLLS is presented in **Table 12**. Mean diameter of LDL measured by DLLS was smaller than that measured by NDGGE (19.1 vs 26.2 nm).

Mean diameter of HDL measured by DLLS matched the mean diameter of HDL obtained by NDGGE (9.8 nm vs 9.8 nm).

Comparison between baseline pairs and endpoint pairs for each Phase

Means of the two measurements obtained at baseline for each phase were compared to each other to see if there was any significant difference. Similar comparison was done for the two endpoint measurements within each phase (**Table 13**). If there was a significant difference between the baseline pairs and between the endpoint pairs, we were interested to know if both NDGGE and DLLS were able to detect the difference.

Table 12 shows all six pairs of comparison made. For LDL, four of the six comparisons showed no significant difference between the means for both NDGGE and DLLS. For the two pairs of comparisons which were significantly different, the endpoint pair of LDL for phase I (P1E1 & P1E2) were found to be significantly different by DLLS (19.8 nm vs 20.4 nm, $p<0.05$) but not by NDGGE (27.2 nm vs 27.1 nm, $P>0.05$). NDGGE showed a significant difference in endpoint pair of Phase III (P3E1 & P3E2) (27.2 nm vs 26.7 nm, $p< 0.05$) but not DLLS (19.8 nm vs 19.6 nm, $p>0.05$).

For HDL, four of the six comparisons showed no significant difference between the means for both NDGGE and DLLS. Baseline pair of Phase III (P3B1 & P3B2) was found to be significantly different by DLLS (10.6 nm vs 9.0 nm, $p<0.05$) but not NDGGE (9.5 nm vs 9.3 nm, $p> 0.05$). NDGGE showed a significant difference in the baseline pair of phase II (9.5 nm vs 10.0 nm) but DLLS did not show any difference in this pair.

Correlation between NDGGE and DLLS

No significant correlation was obtained between MV diameter of LDL obtained from DLLS and mean diameter obtained from NDGGE (**Figure 6**), between MN diameter of LDL obtained from DLLS and Mean diameter obtained from NDGGE (**Figure 7**), or between MA diameter of LDL obtained from DLLS and mean diameter obtained from NDGGE (**Figure 8**). Further, no correlation was found between the median (50% percentile of MN) diameter of LDL obtained from DLLS and mean diameter of LDL obtained from NDGGE (**Figure 9**). In contrast a good correlation was obtained between MV diameter of HDL obtained from DLLS and mean diameter obtained from NDGGE (**Figure 10**), between MN diameter of HDL obtained from DLLS and mean diameter obtained from NDGGE (**Figure 11**), and between MA diameter of HDL obtained from DLLS and mean diameter obtained from NDGGE (**Figure 12**) and median (50% percentile of MN) diameter of HDL obtained from DLLS and mean diameter of HDL obtained from NDGGE (**Figure 13**).

Estimation of chicken VLDLy diameter by DLLS and NDGGE

DLLS has been used extensively to measure diameter of VLDLy (71, 72, 73) . In our study VLDLy diameter measured by DLLS was ~ 30 nm which agrees with the value reported by Walzem & Perry & Griffin (70). Mean chicken VLDLy diameter obtained on NDGGE was greater than that estimated by DLLS (37.7 nm vs 24.6 nm) (**Table 14**). Note that MV for chicken VLDLy from DLLS was closest to diameter measured by NDGGE.

Diameter of LDL obtained from standard curves with and without chicken VLDLy as standard

Mean LDL diameter measured from standard curves with and without inclusion of chicken VLDLy diameter as a standard is shown in **Table 15**. Mean LDL diameter measured from the standard curve in which chicken VLDLy diameter obtained from DLLS was used as standard, was much smaller (18.4 nm) than the mean LDL diameter obtained from the standard without chicken VLDLy (Table 15).

Bimodal Peaks obtained for HDL in NDGGE and DLLS

NDGGE showed double bands for 157 HDL samples. Of these, 24 samples also showed bimodal peaks in DLLS. There was no significant difference between the width of HDL samples with monomodal peaks in DLLS but two bands in NDGGE and HDL samples with bimodal peaks in DLLS and one band in NDGGE (**Table 16**).

Effect of storage on LDL and HDL diameter

For LDL effect of storage was studied using three different sample sets. NDGGE showed a difference between mean diameter of fresh and stored LDL sample for all three sample sets, while DLLS showed a significant difference in only baseline of phase III (**Table 17**). For NDGGE there was a decrease in mean LDL diameter after storage for endpoint of phase I (26.3 nm vs 25.8 nm) and baseline of phase II (25.7 nm vs 25.1 nm) while an increase in mean LDL diameter after storage was observed for baseline of phase III (25.6 vs 27.6 nm).

DLLS showed a significant difference in the LDL diameter between fresh and stored samples for phase III baseline. Mean diameter of fresh LDL sample was less than

the mean diameter of stored sample(19.0 nm vs 21.0 nm).There was no significant difference between the diameter of fresh and stored LDL samples of phase I endpoint (19.8 nm vs. 19.3 nm) and phase II baseline (19.9 nm vs. 20.0 nm).

For HDL effect of storage was studied using two different sample sets. NDGGE showed significant difference between mean diameter of fresh and stored sample for both the sample sets (**Table 18**). Mean diameter of stored HDL samples were smaller than the diameter of fresh samples in phase I endpoint (8.9 vs 9.4 nm) and phase II baseline (9.1 nm vs 9.4 nm). In DLLS no significant difference was observed between fresh and stored HDL samples at endpoint of phase I (9.9 nm vs 9.4 nm) and baseline of phaseII (9.1 nm vs. 8.6 nm). For DLLS mean diameter of HDL increased in the stored sample for endpoint of phase I. To check if there was a change in LF and CI in the stored samples, CI and LF of thirty of the samples for LDL and HDL were compared to fresh samples. There was no significant difference in the LF (0.1 vs 0.1) and CI (0.8 vs 0.7) for LDL. No significant difference was observed in the LF (0.01 vs 0.01) and CI (0.3 vs 0.7) for HDL (**Table 19**).

Overall CV of LDL and HDL measurements

Overall CV for LDL was less for NDGGE than DLLS (1.8 vs 3.2). Overall CV for HDL was also less for NDGGE than DLLS (2.6 vs 5.4).

CHAPTER V

DISCUSSION

Aim of this study was to compare NDGGE and DLLS methods for measurement of LDL and HDL particle diameter. Our goal was to determine whether the methods were comparable.

Exclusion of LDL and HDL readings

LDL and HDL diameters are an important predictor of CVD. Evidence suggests that small dense LDL are the atherogenic subpopulation of this class of lipoprotein (5). Measurement of LDL diameter has been suggested as an important component of risk assessment for CVD. Nondenaturing gradient gel electrophoresis and DLS has been used to measure has been used to measure the diameter of LDL (60) (61). These two techniques show good correlation with each other (60). Electron microscopy have determined mean diameter of LDL to be about 20 nm (74). LDL diameter by DLS has shown diameter of about 23.1nm, and NDGGE have shown diameter of about 26.1nm (61). Thus we see that depending on the technique used to measure LDL diameter its value could range from anywhere between 19 nm to 26 nm. Based on this information we fixed the range of LDL diameter obtained from DLLS between 16 nm and 28 nm and any measurement out of this range was discarded.

For HDL, mean diameter by DLS have been reported to be around 9.8nm (75). This is in close agreement with values obtained from nuclear magnetic resonance spectroscopy (9.2 nm) (76) and gradient gel electrophoresis (8.4 to 9.6nm) (77). In our

study we fixed the lower limit of HDL as 7nm and values < 7nm were discarded. Values > 14nm obtained from DLS are inconsistent with the mean values obtained for HDL and were also found to be irreproducible (75), therefore any values of HDL diameter > 14nm were also discarded from the sample set.

Loading Factor is a measure of the total signal obtained from the light scattered by particles in motion. Concentration index is a measure of amount of particle in a given amount of solvent. Mictrotrac recommends a LF between 0.1 to 100 for all samples. Within this range the instrument can cover a wide domain of CI. However for LF < 0.1, CI could be a concern for instrument limitations. We hypothesized that LF and CI of LDL and HDL data whose diameter did not meet inclusion criteria may be significantly different from the LDL and HDL data that did meet the inclusion criteria. While the mean LF for LDL was within the range recommended by Microtrac, the mean LF for HDL was much less then 0.1 (Refer Table 10). Thus, LF is not a universal index for measurement quality. It is interesting to note that CI of those samples whose diameter measurements met the inclusion criteria for both LDL and HDL were greater than included data. It is possible that an increase in CI limited the function of DLLS. This may have resulted in exclusion of more HDL data. We conclude from our results that CI is an important factor in measuring a sample.

Comparison between NDGGE and DLLS

Precision and reproducibility of the two methods

Precision of an instrument is defined as a measure of variance in repeated measurements on the same sample. Even when the accuracy of an instrument cannot be

determined, precision can prove to be a useful criterion for assessing the performance of an instrument. It is common to have good precision but poor accuracy (78). Reproducibility may be defined in terms of the variance observed from sample-to-sample, instrument-to-instrument or operator-to-operator, etc (78). Both NDGGE and DLLS measures LDL with better precision than HDL as was observed from their CV within runs.(Refer to Table 11). Note that in NDGGE, HDL standards with smaller diameter were measured more precisely than standards with larger diameter. A similar trend was observed by Perusse et al (79) This may suggest that smaller HDL may be measured with better precision that larger HDL by NDGGE. For DLLS as observed earlier, more HDL data were excluded as compared to LDL data. The increased CV for HDL as compared to LDL confirms further that DLLS is not very precise in reading a HDL sample.

A comparison of CV of NDGGE and DLLS for within runs shows that DLLS is better than NDGGE for measuring LDL .But again the imprecision in NDGGE could be due lack of appropriate standards. For DLLS, CV within runs for HDL was comparable to CV within runs for HDL from NDGGE. However, as the diameter of the standards increased CV of HDL from NDGGE became larger. Though the CV of NDGGE was greater when measuring larger standards, it would still be a better choice than DLLS for measuring HDL because DLLS gave a number of excluded data for HDL as discussed earlier.

Mean diameter of LDL and HDL

Mean LDL diameter obtained from DLLS fell within the ranges reported for LDL diameters obtained by DLS and electron microscopy (61) (74). DLLS measured LDL diameter matches closely with values obtained by electron microscope (Refer to Table 11). Reported mean values of LDL measured by NDGGE matches closely with the values we obtained for LDL diameter by NDGGE (26.2 nm)(61). Our results further corroborate previous study which showed the LDL diameter as measured by NDGGE is greater than that obtained by DLS (61). With the assumption that electron microscope gives the most accurate results for LDL diameter, DLLS could be a better choice for measuring LDL as compared to NDGGE.

For HDL, the average diameter obtained from DLLS was 9.8nm which agrees to value reported by Lima et.al using the same method (75). HDL diameter obtained from NDGGE in this study also agrees closely with values reported in earlier studies (76) (77). On the other hand many of the HDL data from DLLS were unreliable and it is possible that CI of samples greatly influence the way DLLS measures the sample. Therefore, CI of a sample has to be considered when measuring by DLLS. In contrast, CI is not a limitation with NDGGE. Hence NDGGE might be a better choice for measuring diameter of HDL.

Comparison between baseline pairs and endpoint pairs for each Phase

Paired baseline samples were collected with a gap of one day between collection days. This was also true for paired endpoint pair samples. Given the short interval between the two collection days, we predicted that there would be no difference in the

values of the measurements of each individual sample in a pair. Indeed these results show that there was no significant difference between the two collections for majority of the pairs (four out of six). Significant difference was seen between LDL pairs (P1E1 and P1E2) for DLLS (19.8 nm vs 20.4 nm, $p<0.05$) and LDL pairs (P3E1 and P3E2) for NDGGE (27.2 nm vs 26.7 nm, $p<0.05$). For HDL significant difference was seen between P3B1 and P3B2 for DLLS (10.6 nm vs 9.0 nm, $p<0.05$) and P2B1 and P2B2 for NDGGE (9.5 nm vs 10.0 nm) (Refer to Table 13). These differences could be due to technical oversight like incorrect aspiration of LDL fraction from the centrifuge tube or presence of contamination in the sample. Since both NDGGE and DLLS showed no difference between the two collections for majority of the pairs, we may conclude that both DLLS and NDGGE were consistent in their readings.

Correlation between NDGGE and DLLS

We did not find any significant correlation of mean LDL diameter between NDGGE and DLLS. This is in contrast to the findings of O'Neal who obtained good correlation using the two methods. In our study we used standards which have been used in other studies (20),(61). Our standards have a range from 17 to 7.1 nm. Unlike other studies we did not have any standard beyond 17nm. Since LDL diameter is typically beyond 17nm which is outside the standard curve, we extrapolated the diameter from our standard curve. We assume that beyond a certain range of standard curve extrapolation may not produce accurate values for a sample. To eradicate error due to extrapolation we introduced a chicken VLDLy standard to extend the range of the standard curve. We constructed two standard curves- one using chicken VLDLy standard measured by

NDGGE and another using chicken VLDLy standard measured by DLLS. We failed to observe a correlation between mean LDL diameters obtained from the above two standard curves and mean LDL diameter values obtained from DLLS. A possible explanation for this observation could be, chicken VLDLy standard (measured by NDGGE using extrapolation method) in itself may not be accurate. For the standard curve that had chicken VLDLy measured by DLLS, migration of chicken VLDLy cannot be calculated similar to the other five standard proteins as the diameters of these five standard proteins was determined by a different method.

Diameter of Chicken VLDLy measured in NDGGE and DLLS

Diameter of chicken VLDLy obtained from NDGGE was significantly larger then diameter obtained from DLLS (Refer to Table 14). This may suggest that NDGGE may overestimate the diameter of LDL particles. Others have noted discrepancies between LDL diameters estimated by EM and NDGGE (60, 61).

Diameter of LDL obtained from standard curves with and without chicken VLDLy as standard

The values of LDL diameter obtained from standard curve which used the diameter value of chicken VLDLy (obtained by DLLS) as one of the standards, was much smaller when compared to standard curve without chicken VLDLy and was also smaller when compared to standard curve which used the value of chicken VLDLy diameter obtained from NDGGE (Refer to Table 15). LDL diameter obtained from a graph of log(r) vs R_f were greater than that obtained from standard curve with and without chicken VLDLy(Refer to Table 15). We used chicken VLDLy as a standard so

that we could get a wide range on the standard curve. But the diameter of chicken VLDLy was measured by NDGGE which as we discussed earlier overestimates the measurement. Technically, the standard curve did not have a standard protein whose diameter was larger than 17 nm. In studies, which have used standards to cover the range beyond 17nm, the standards either were made in the lab or were purchased from manufacturers who no longer make those standards. NDGGE may give better results for LDL diameter if appropriate standards are used. However, obtaining suitable standards is a challenge.

Bimodal Peaks obtained for HDL in NDGGE and DLLS

NDGGE is more sensitive in resolving HDL subpopulations in a sample than DLLS because NDGGE showed double bands for 157 HDL samples of which DLLS resolved bimodal peaks in only 24 of the samples. Since there was no significant difference in the width of monomodal samples from DLLS which showed two bands in NDGGE and bimodal samples from DLLS with two bands in NDGGE, we concluded that DLLS could not affectively resolve two populations in a sample if the population was bimodal. So NDGGE may be the preferred method for measuring HDL.

Effect of storage on LDL and HDL diameter

Changes in structure and composition of lipoprotein have been reported in stored plasma (80). We hypothesized that a significant difference would be found in comparisons between fresh and stored samples. For NDGGE a change in LDL was obtained in all the three collections but DLLS showed a significant difference only for one collection (Refer to Table 17). For HDL, NDGGE showed a significant difference in

diameter between stored and fresh sample in both the collections but DLLS showed no significant difference in either of the collections (Refer Table 18).To better explain the results of DLLS we noted the CI of stored samples. We assumed that DLLS would have detected a difference if there was variation in CI. We found no significant difference in CI for either LDL or HDL (Refer to Table 19). We assumed that the change in the diameter caused by storage was too small to cause any significant change in the amount of light scattered by these particles. Since DLLS measurement is based on the amount of light scattered by these particles, we did not see any significant change in LDL and HDL diameter when measured by DLLS. NDGGE on the other hand measures particle diameter relative to the particles migration on the gel. A small change in the particle diameter may have resulted in an appreciable change in the migration of particle in the gel and so NDGGE was able to detect the change in particle diameter. We conclude that storage did cause a change in the particle diameter of both LDL and HDL and NDDGE was able to detect the change but not DLLS.

CHAPTER VI

CONCLUSION

LDL and HDL diameter is an important risk factor for CVD. As such to determine an accurate method for determining the diameter of LDL and HDL is very important. From our study we conclude that DLLS gave reliable reading for LDL and may be used for measuring the diameter of LDL. NDGGE on the other hand overestimated the value of LDL diameter. But the primary reason for this was lack of appropriate standards. This problem of overestimation could be solved with use of suitable standard. But given the not so ready availability of such standards, this process may be tedious and expensive. NDGGE may be a better choice for measuring HDL than DLLS. DLLS gave a large number of excluded HDL samples. This may be because DLLS depends on CI of samples and any technical error or contamination of sample that may cause a change in CI of sample may affect the results obtained from DLLS. This is not the case with NDGGE as this method is CI independent. NDDGE was able to resolve the subpopulation in a HDL samples better than DLLS. NDDGE detected a refrigerated storage effect in LDL and HDL while DLLS did not.

Another important factor that may influence our choice of equipment is time and cost. Compared to NDGGE, DLLS is very fast and results can be obtained within very short time. It is very convenient to use. Though the price of equipment is high, the equipment incurs minimal maintenance cost. NDGGE on the other hand is very laborious and time consuming process. It takes more than 24 hours to obtain results from

this technique. Though the equipment itself is less expensive, the gels and standards are costly. For studies like our which require use of large number of gels and standards this may be a costly methods. Thus both NDGGE and DLLS have their own merits and demerits in measuring particle diameter and cost and time may be an important factor influencing the choice of equipment.

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APPENDIX A

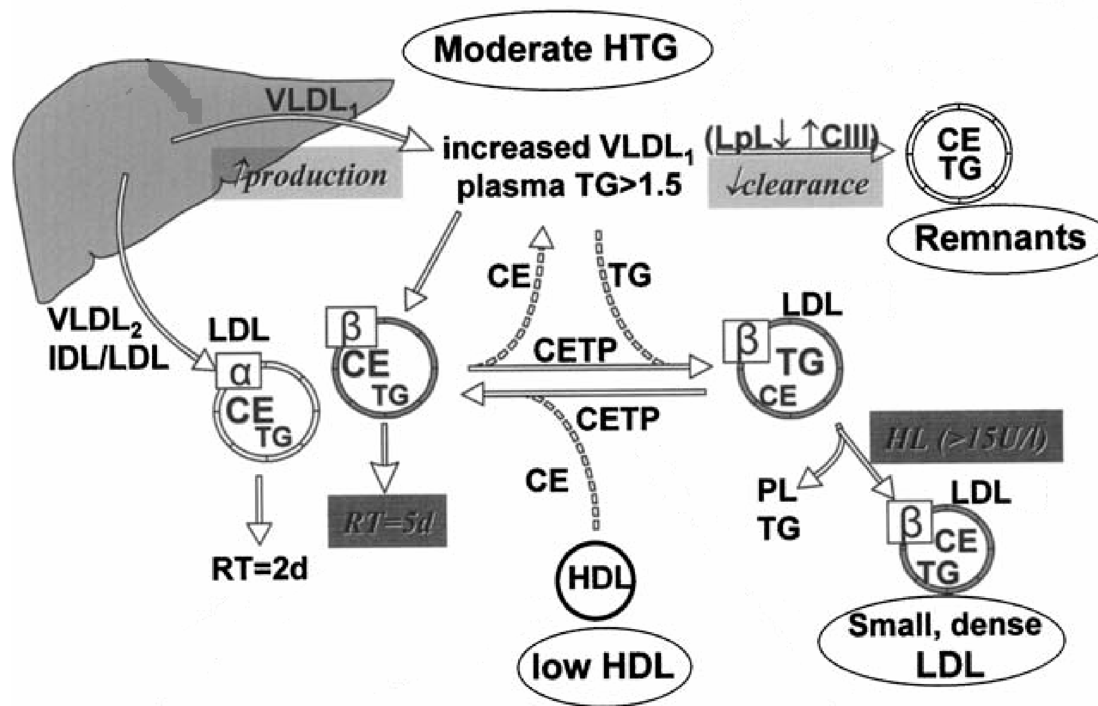

FIGURE 1. Formation of small dense LDL. Mild to moderate hypertriacyglyceridemia (HTG) leads to accumulation of large triacylglycerol rich VLDL (VLDL1) due to ovrproducton by liver or reduced clearance from circulation. Low Lipoprotein Lipase (LpL) activity or an excess of apoCIII (CIII; inhibitor of LpL) can impede the efficient lipolysis of $VLDL_1$. $VLDL_1$ when lipolysed produce a population of Low density lipoprotein (LDL) particles (denoted β) which have an altered apoB100 configuration.These particles do not bind well with LDL receptors and so habe prolonged residence time (Rt) in the circulation(d=day).Pool prolonged Retention (RT) in the circulation (d=days). Pool β LDL has therefore increased likelihood of undergoing remodelling. CETP removes cholesteryl ester (CE) and replaces it with triacylglycerol as the protein shuttle between VLDL, LDL and HDL particles. Triacylglycerol-enriched LDL is a good substrate for HL, an enzyme that removes triacylglycerol from smaller lipoprotein particles. Small, dense LDL is generated in this final lipolytic step.

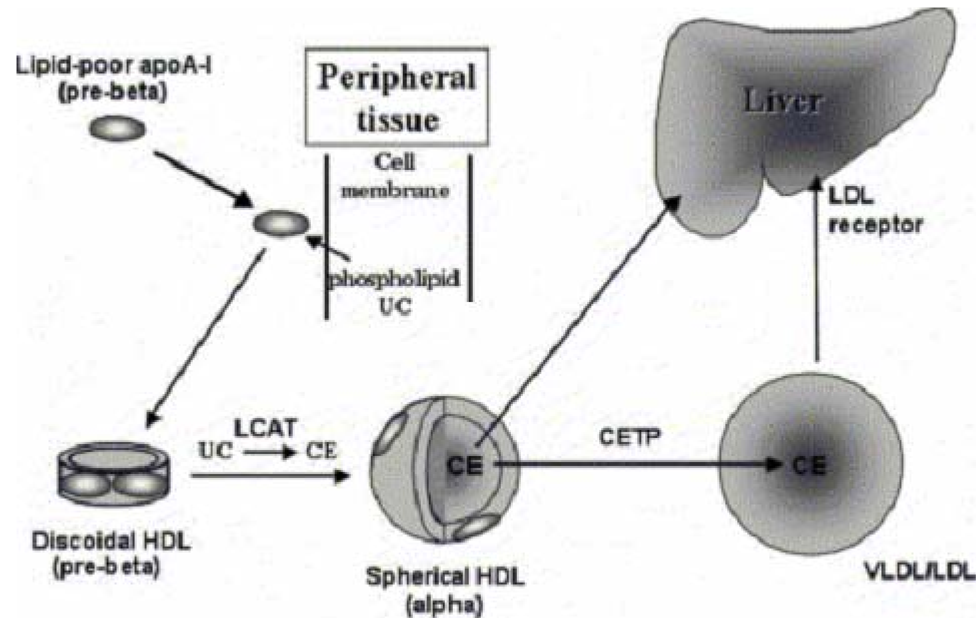

FIGURE 2. Formation of small dense HDL. Lipid poor pre-beta high density lipoprotein acquires phospholipids and unesterified cholesterol (UC) from peripheral tissues to form discidal HDL (pre-beta). Lecitin:cholesterol acyltransferase(LCAT) facilitates exchange of UC for cholesterol ester (CE) in discoidal HDL to form spherical HDL. Cholesterol ester partition into center of thesespherical HDL which increases in size to form large HDL. Cholesterol ester transfer protein transfers CE from large HDL to Very Low Density Lipoprotein (VLDL) and Intermediate Density Lipoprotein (IDL).

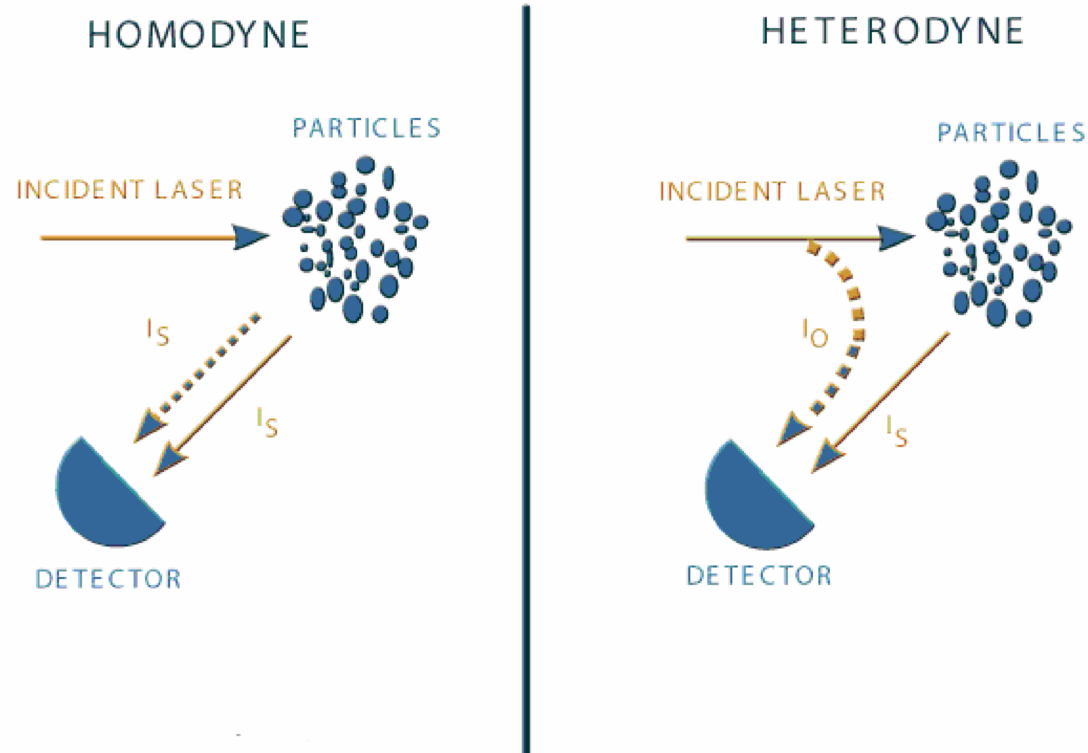

FIGURE 3: Comparison of homodyne and heterodyne configuration used in Dynamic Laser Light Scattering. I_o represents the reference beam which is reflected back from the sapphire window. I_s represents the scattered light from the suspended particles.

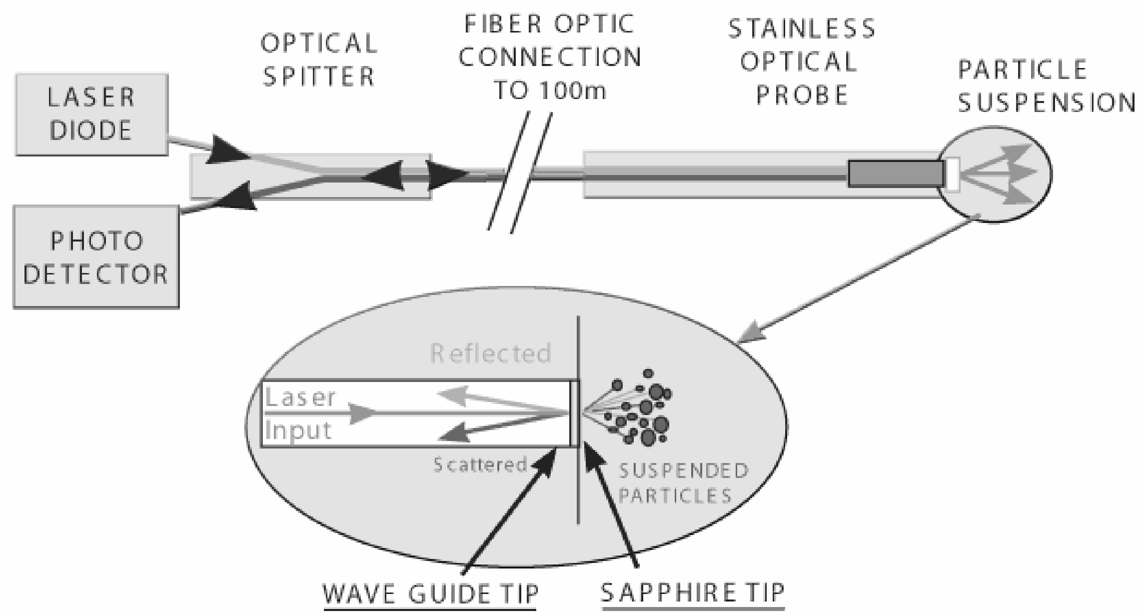

FIGURE 4. Controlled Reference Method used in Nanotrac. The wave guide includes the optical splitter, fiber optic connector and the enclosed stainless probe. A part of the laser beam does not penetrate into the suspended particle and is reflected back from the sapphire window. Another part strikes the suspended particle and are scattered.

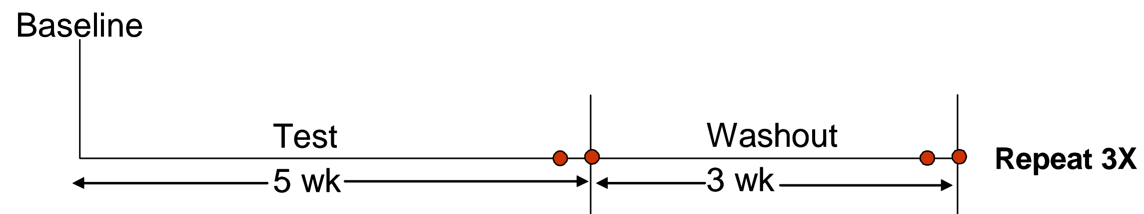

FIGURE 5. Experimental design of the study. Study was done in crossover design and was conducted in 3 phases. Each group consumed each of the three different diets at different phases. Each baseline and endpoint consisted of 2 collections.

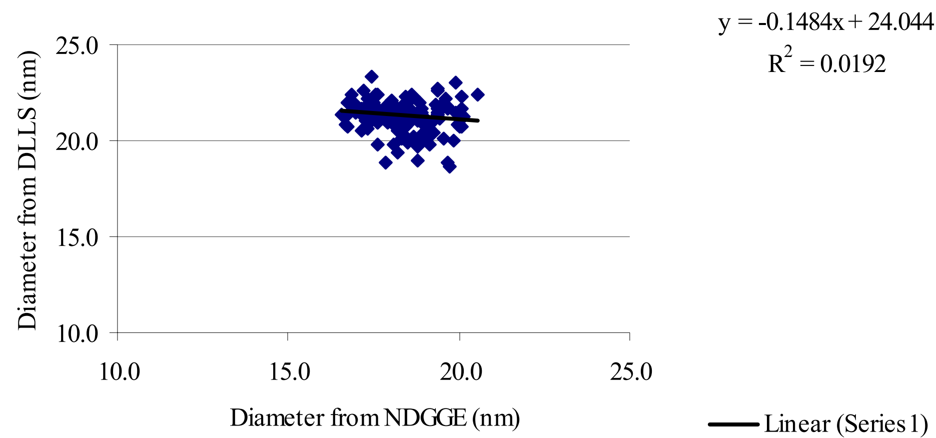

FIGURE 6. Correlation between Mean Volume LDL diameter from DLLS and Mean diameter from NDGGE. Chicken VLDLy measured by DLLS was used as a standard for obtaining LDL diameter by NDGGE.

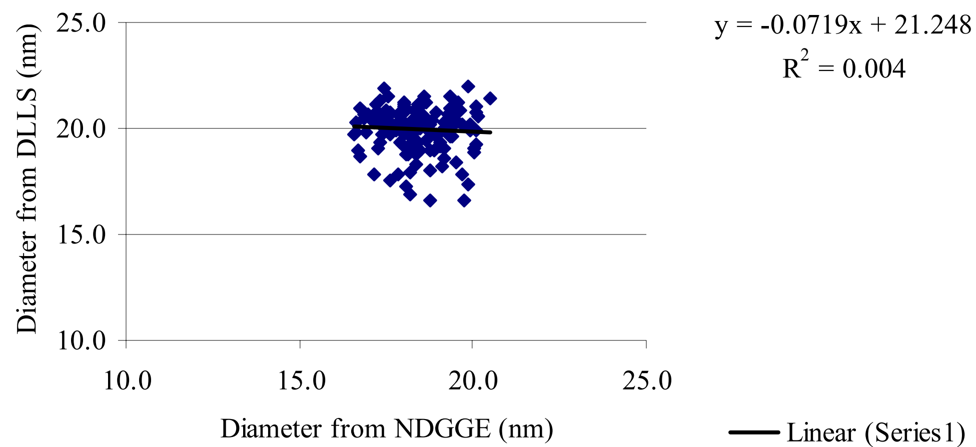

FIGURE 7. Correlation between Mean Number LDL diameter from DLLS and Mean diameter from NDGGE. Chicken VLDLy measured by DLLS was used as a standard for obtaining LDL diameter by NDGGE.

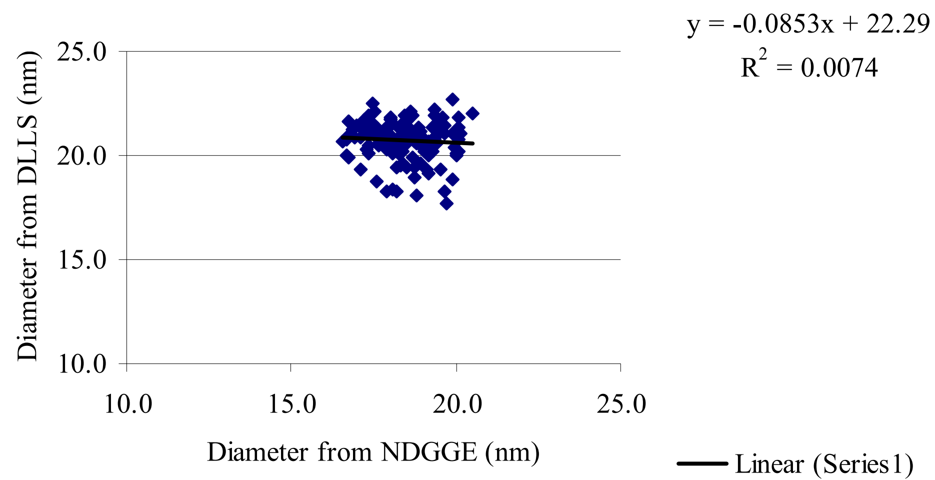

FIGURE 8. Correlation between Mean Area LDL diameter from DLLS and Mean diameter from NDGGE. Chicken VLDLy measured by DLLS was used as a standard for obtaining LDL diameter by NDGGE.

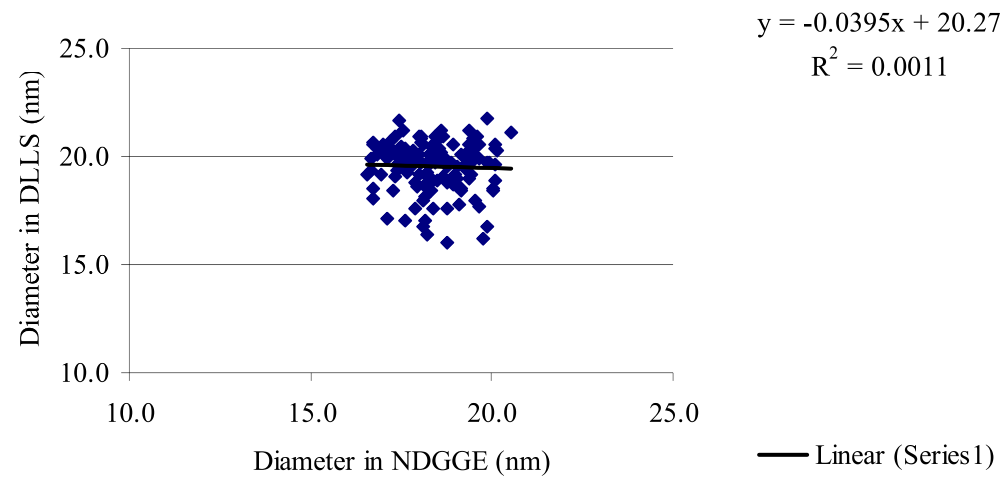

FIGURE 9. Correlation between Median Number LDL diameter from DLLS and Mean diameter from NDGGE. Chicken VLDLy measured by DLLS was used as a standard for obtaining LDL diameter by NDGGE.

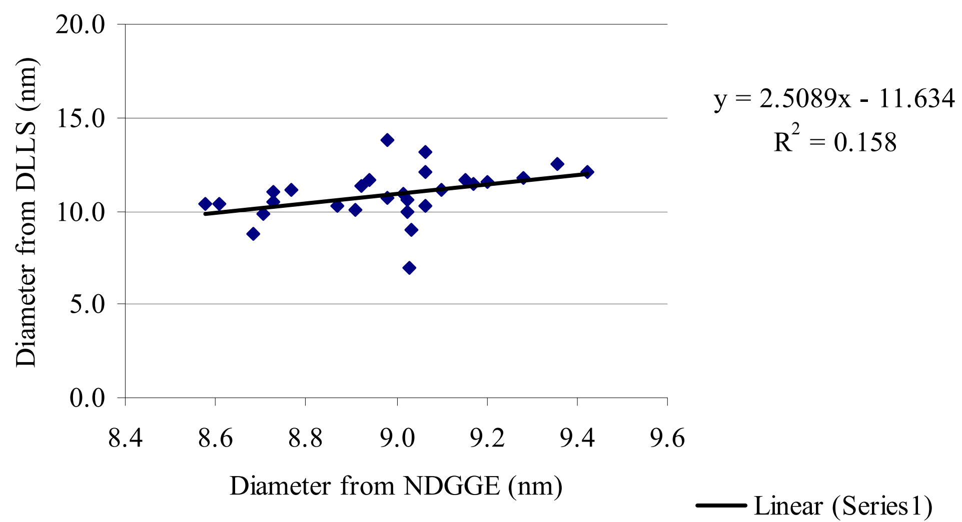

FIGURE 10. Correlation between Mean volume HDL diameter from DLLS and Mean HDL diameter from NDGGE.

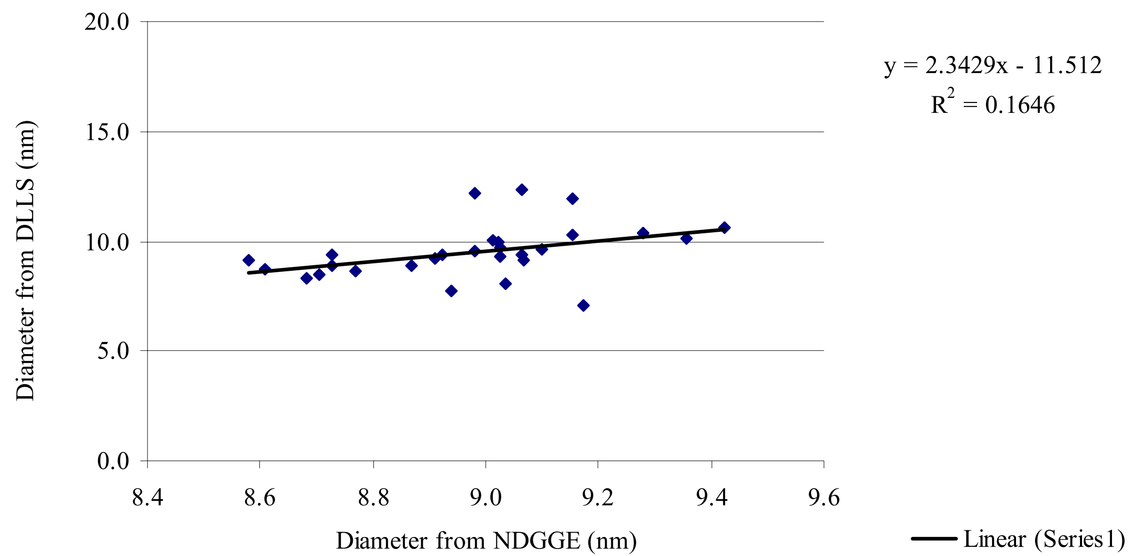

FIGURE 11. Correlation between Mean number HDL diameter from DLLS and Mean HDL diameter from NDGGE.

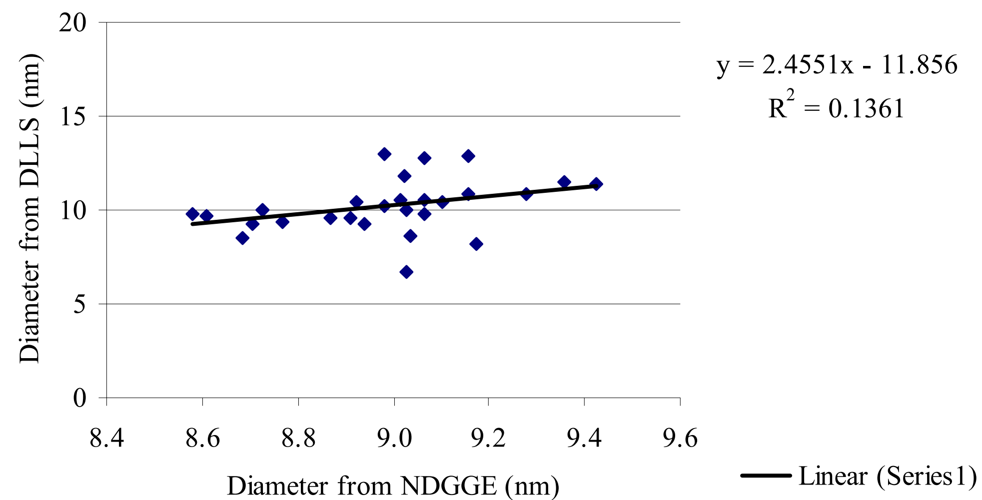

FIGURE 12. Correlation between Mean area HDL diameter from DLLS and Mean HDL diameter from NDGGE.

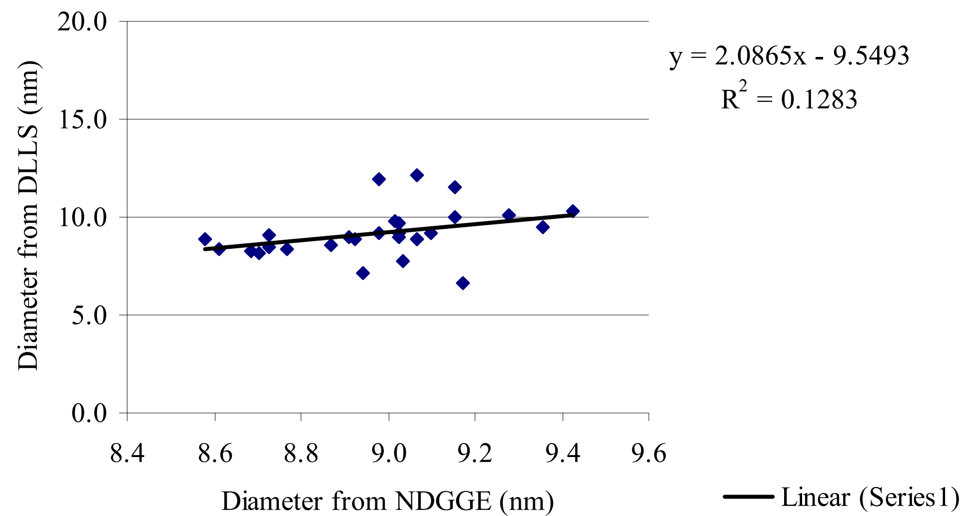

FIGURE 13. Correlation between Median number HDL diameter from DLLS and Mean HDL diameter from NDGGE.

APPENDIX B

TABLE 1. Lipoprotein characteristics found in human plasma

	Chylomicron	VLDL	IDL	LDL	HDL
Density (g/ml)	<0.95	<1.006	1.006-1.019	1.019-1.063	1.063-1.210
Particlediameter (nm)	1200-75	80-30	35-25	25-18	12-5
Particle Mass (kD)	400,000	10,000-80,000	5,000-10,000	2,300	175-300
% Protein[a]	1.5-2.5	5-10	15-20	20-25	40-55
% Phospholipid[a]	7-9	15-20	22	15-20	20-35
% Free Cholestrol[a]	1-3	5-10	8	7-10	3-4
% Triaclyglyceride[b]	84 - 89	50-65	22	7-10	3-5
% Cholesterol Ester[b]	3-5	10-15	30	35-40	12
Major Apolipproteins	AI, AII, B48, CI CII, CIII, E	B100, CI, CII, CIII, E	B100, CIII, E	B100	AI, AII, CI, CII, CIII, D, E

VLDL= Very Low Density Lipoprotein
IDL= Intermediate Density Lipoprotein
LDL= Low Density Lipoprotein
HDL= High Density Lipoprotein
[a]Surface Component
[b]Core Lipids

TABLE 2. Low Density Lipoprotein characteristics found in human plasma

	LDL-I	LDL-II	LDL-III	LDL-IV
Peak Sf	12-Jul	07-May	05-Mar	0-3
Density(gm/ml)	1.019-1.023	1.023-1.028	1.034-1.041	1.044-1.051
		1.028-1.034	1.041-1.044	1.051-1.06
Diameter (nm)	27.2-28.5	26.5-27.2	24.7-25.6	23.3-24.2
		25.6-26.5	24.2-24.7	22.0-23.3
%Protein	18	19	22	26
		21	24	29
% Cholestrol ester	43	45	46	42
		45	44	40
%Unesterified cholestrol	9	10	8	7
		9	7	7
%Triacylglycerol	7	4	3	5
		3	3	6
%Phospholipid	22	23	21	19
		22	21	18

LDL= Low Density Lipoprotein

TABLE 3. High Density Lipoprotein characteristics found in human plasma

	HDL2b	HDL3	
		HDL3L	HDL3D
Density (g/ml)	1.063-1.125	1.1.3-1.14	1.15-1.16
Particlediameter (nm)	9.5-12	5.5-9.5	5.5-9.5
Particle Mass (kD)	358,000	182,000	152,000
% Protein	37	50	59
% Phospholipid	30	27	23
% Free Cholestrol	5	3	2
% Triaclyglyceride	8	5	3
% Cholesterol Ester	20	15	13
Major Apolipproteins	A-I, A-II	A-I, A-II	A-I, A-II
Minor Apolipoproteins	C-I, C-II.C-III,E	C-I, C-II.C-III,E	C-I,C-II, C-III, E

HDL_{2b} = Large high density lipoprotein
HDL_{3} = Small High Density Lipoprotein
HDL_{3L}, HDL_{3D} = Subpopulation of small high density lipoprotein
[a]Surface components
[b]Core lipids

TABLE 4. Baseline characteristics of plasma lipids and apolipoproteins according to the apoE genotypes

	ApoE 4/3	ApoE 3/3	ApoE 3/2
LDL size(nm)	26.3 ± 0.2[a]	25.8 ± 0.1[b]	25.7 ± 0.2[b]

Values are mean ± SD

Means in a row with superscripts without a common letter differ, $P < 0.05$

TABLE 5. Fat composition of the experimental diet

[a]Fat for Each Experimental Group and Diet Period (g/100 g)						
Period	Saturated		Monounsaturated		Polyunsaturated	
1	Lard	15.6	Oleinate[b]	9.8	Safflower oil	9.8
1	Safflower oil	0.8	Lard	6.6	Lard	6.6
2	Palm oil	15.4	Oleinate	16.4	Safflower oil	16.4
2	Safflower oil	1				

[a]All diets contained a calorie distribution of 35% fat, 48% carbohydrate, and 17% protein.
[b]Oleinate is oleic acid-rich safflower oil.

TABLE 6. Composition of ground patties.

Ground patties	MUFA:SFA
1	0.8
2	1.1
3	1.4

MUFA = Monounsaturated Fatty Acid
SFA = Saturated Fatty Acid

TABLE 7. Nomenclature for labeling six baseline samples and six endpoint samples at three study phase.

	Draw	Phase I	Draw	Phase II	Draw	Phase III
Baseline 1	1	P1B1	5	P2B1	9	P3B1
Baseline 2	2	P1B2	6	P2B2	10	P3B2
Endpoint 1	3	P1E1	7	P2E1	11	P3E1
Endpoint 2	4	P1E2	8	P2E2	12	P3E2

TABLE 8. Particle and fluid information fed into Nanotrac®UPA 250

	Particle Information		Fluid information		
	RI	Density	RI	Viscosity	
				26°C	20°C
VLDL+IDL	1.46	1	1.337	1.0197	1.0224
LDL	1.46	1.02	1.344	0.9087	1.0462
HDL	1.46	1.15	1.3685	1.065	1.226

VLDL= Very Low Density Lipoprotein
IDL = Intermediate Density Lipoprotein
LDL = Low Density Lipoprotein
HDL = High Density Lipoprotein
RI = Refractive Index

TABLE 9. Number of data that were excluded from the study

	Exclusion criteria	P1B1	P1B2	P1E1	P1E2	P2B1	P2B2	P2E1	P2E2	P3B1	P3B2	P3E1	P3E2
LDL	Incomplete	1	1	0	1	1	4	1	1	1	1	2	2
	MN < 16nm	1	1	1	0	0	0	0	0	0	0	0	0
HDL	Incomplete	1	1	0	1	1	4	1	1	1	1	2	2
	MN < 7nm or MN > 14nm	0	4	1	1	2	8	2	5	2	5	2	4

LDL= Low Density Lipoprotein
HDL = High Density Lipoprotein
MV = Mean Volume Diameter
MN = Mean Number Diameter
MA = Mean Ares Diameter

TABLE 10. Loading Factor and Concentration Index of included and excluded data

	HDL (n=30)			LDL (n=30)		
	Included Data	Excluded Data	p value	Included Data	Excluded Data	p value
LF	0.009±0.003	0.01±0.01	<.05	0.1±0.02	0.1±0.1	>0.05
CI	0.3±0.1	0.8±0.4	<.05	0.7±0.3	1.0±0.5	<0.05

LF= Loading Index
CI= Concentration Index
Included Data = MN between 7nm and 14 nm for HDL
MN greater than 16 nm for LDL
Excluded Data = MN less than 7nm or greater than 14 nm for HDL
MN less than 16 nm for LDL

TABLE 11. Covariance within and in between runs for NDGGE and within runs for DLLS

NDGGE

	LDL		HDL	
Std	Within runs	Between runs	Within runs	Between runs
T	5.7	4.3	14.1	11.1
F	4.7	3.9	8.9	7.8
C	4.3	4.4	6.4	5.0
LDH			5.3	4.5
BSA			4.5	4.0

DLLS (within runs)

LDL			HDL		
MV	MN	MA	MV	MN	MA
2.6	2.7	2.5	4.3	5.3	4.9

NDGGE = Non Denaturing Gradient Gel
DLLS = Dynamic Light Scattering
LDL= Low Density Lipoprotein
HDL = High Density Lipoprotein

TABLE 12. Mean diameter of LDL and HDL as measured by NDGGE and DLLS

LDL				HDL		
DLLS	p	NDGGE	p	DLLS	NDGGE	p
19.1±1.4		26.2±1.4	<0.0001	9.8±1.6	9.8±0.7	>0.05

[a]value is mean ± SD
NDGGE = Non Denaturing Gradient Gel
DLLS = Dynamic Light Laser Scattering
LDL= Low Density Lipoprotein
HDL = High Density Lipoprotein

TABLE 13. Comparison between baseline pairs and between endpoint pairs

				Baseline					**LDL**					Endpoint				
Method	**1** [*]	**2**	**p**	**3**	**4**	**p**	**5**	**6**	**p**	**7**	**8**	**p**	**9**	**10**	**p**	**11**	**12**	**p**
DLLS	21.2 ±1.2	20.5 ±1.8	>0.05	19.9 ±1.0	19.9 ±1.0	>0.05	20 ±1.1	20.5 ±0.8	>0.05	19.8 ±1.0	20.4 ±0.8	<0.05	20 ±0.9	19.8 ±1.2	>0.05	19.8 ±1.0	19.6 ±1.0	>0.05
NDGGE	25. 9 ±0.7	25.7 ±0.8	>0.05	26.6 ±1.9	26.3 ±1.2	>0.05	25.1 ±1.8	25.8 ±1.2	>0.05	27. 2 ±1.3	27.1 ±1.6	>0.05	26. 0 ±1.0	26.4 ±1.5	>0.05	27. 2 ±1.3	26.7 ±1.2	<0.05

				Baseline					**HDL**					Endpoint				
Method	**1**	**2**	**p**	**3**	**4**	**p**	**5**	**6**	**p**	**7**	**8**	**p**	**9**	**10**	**p**	**11**	**12**	**p**
DLLS	9.6 ±1.4	8.7 ±1.1	>0.05	10.1 ±1.6	8.8 ±3.1	>0.05	10.6 ±1.6	9 ±1.1	<0.05	9.9 ±1.8	9.6 ±1.1	>0.05	9.4 ±1.3	9.4 ±1.2	>0.05	11.8 ±1.5	11.8 ±1.6	>0.05
NDGGE	9. 9 ± 0.6	9. 9 ±.0.7	>0.05	9.5 ±.0.6	10. 0 ± 1.1	<0.05	9.5 ± 0.6	9.3 ± 0.6	>0.05	9.3 ± 0.6	9.4 ± 0.7	>0.05	10.3 ± 0.5	10.2 ± 0.6	>0.05	9. 9 ± 0.7	9.9 ±0.7	>0.05

[*] Draw Number

NDGGE = Non Denaturing Gradient Gel

DLLS = Dynamic Light Laser Scattering

LDL= Low Density Lipoprotein

HDL = High Density Lipoprotein

n = Sample size

TABLE 14. Chicken VLDLy diameter as measured by DLLS and NDGGE

	DLS (nm)		
NDGGE (nm)	MV	MN	MA
34.9	28.5	23.7	26.1
34.1	30.7	23.5	26.6
41.7	30.7	23.5	26.6
37.7	31.3	26.1	28.7
40.3	31.3	26.1	28.7

NDGGE = Non Denaturing Gradient Gel
DLLS = Dynamic Light Laser Scattering
MV = Mean Volume Diameter
MN = Mean Number Diameter
MA = Mean Ares Diameter

TABLE 15. Mean LDL diameter obtained from different standard curves

Without VLDLy Std	With VLDLy Std measured by NDGGE	With VLDLy Std measured by DLLS	R_f/Log(r)
26.0±1.3	25.8± 1.1	18.4± 1.0	27.1±1.5

VLDLy= Chicken Very Low Density Lipoprotein
NDGGE= Non Denaturing Gel Electrophoresis
DLLS= Dynamic Laser Light Scattering

TABLE 16. Peak width comparison between DLLS samples which showed one or two bands in NDGGE

	1 bands in NDGGE	2 bands in NDGGE
Peak width	2 nm±.0005[a]	2 nm±.0006

[a]value is mean ± SD

TABLE 17. Effect of storage on LDL diameter for 3 collections

Method	DLLS			NDGGE		
Days stored prior to analysis	0	4	p	0	4	p
Phase I endpoint	19.8	19.3	>0.05	26.3	25.8	0.05
Phase II baseline	19.9	20.0	>0.05	25.7	25. 1	0.05
Phase III baseline	19.0	21.0	<0.05	25.4	26.8	<0.05

NDGGE = Non Denaturing Gradient Gel
DLLS = Dynamic Laser Light Scattering
LDL= Low Density Lipoprotein
HDL = High Density Lipoprotein

TABLE 18. Effect of storage on HDL diameter for 2 collections

Method	DLLS			NDGGE		
Days stored prior to analysis	0	4	p	0	4	p
Phase I endpoint	8.2	9.8	0.05	9.4	8.9	<0.05
Phase II baseline	9.4	8.3	>0.05	9.4	9.1	<0.05

NDGGE = Non Denaturing Gradient Gel
DLLS = Dynamic Laser Light Scattering

TABLE 19. Effect of storage on LF and CI in DLLS

	HDL (n=30)			LDL (n=30)		
Days stored prior to analysis	0	4	p	0	4	p
LF	0.01±0.0	0.01±0.0	>0.05	0.1±0.2	0.1±0.0	>0.05
CI	0.3±0.1	0.7±1.3	>0.05	0.7±0.3	0.8±0.3	>0.05

LF= Loading Factor
CI= Concentration Index
LDL= Low Density Lipoprotein
HDL = High Density Lipoprotein

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