COMPARISON OF ULTRACENTRIFUGATION, NMR, AND FPLC METHODS TO ANALYZE LIPOPROTEINS IN CATS

by

ERIN ELIZABETH TITTLE

(Under the Direction of Margarethe Hoenig)

ABSTRACT

Obesity is not only a problem for humans, but the growing trend is also now seen in domestic pets such as cats. Obese cats develop diabetes mellitus like humans; however, atherosclerosis which is often seen in obese humans does not occur in cats. Obesity often leads to the development of dyslipidemia which may lead to the development of disease. The purpose of this study was to determine the effects of diet and obesity on lipoprotein classes and to compare results obtained with the standard method of ultracentrifugation to those obtained with FPLC and to NMR. Twenty-four cats were used for this study; 12 were lean and 12 were considered obese. These results show that all three methods can be used to measure feline lipids and lipoproteins, cats do develop significant changes in lipoproteins with obesity, and dietary components had little effect on the majority of concentrations of lipids and lipoproteins.

INDEX WORDS: Obesity, Lipoproteins, Cats, Nuclear magnetic resonance, Ultracentrifugation, Fast phase liquid chromatography

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CHAPTER 1

INTRODUCTION

Obesity occurs in humans and animals when caloric intake is increased and/or energy output is decreased. In humans, obesity is often seen in combination with or preceding diseases such as diabetes mellitus and cardiovascular disease. The growing trend in obesity is also now seen in veterinary medicine, especially in domestic pets such as dogs and cats. Obesity in cats has been shown to lead to the development of other diseases, among them diabetes mellitus; however, atherosclerosis, a common co-morbidity in people, does not occur in cats.

Obesity is associated with many unfavorable metabolic alterations, including, dyslipidemia, which can be characterized by increased levels of total triglycerides and low density lipoproteins and decreased levels of high density lipoproteins. Lipoproteins are the particles formed to transport both dietary and endogenous lipids throughout the body for metabolism, storage, or excretion. There are five classes of lipoproteins which can be separated by their differing densities; including, chylomicrons, very low density, intermediate density, low density, and high density lipoproteins. Each of the classes of lipoproteins is known to be composed of various concentrations of subclasses. The increased caloric intake which occurs with obesity leads to increased intake and accumulation of lipids in adipose and non-adipose tissues. Increased accumulation of lipids in tissues leads to altered lipoprotein profiles. These alterations include changes in both particle concentration and particle size of the various subclasses of lipoproteins, which may be associated with disease or lead to the development of disease.

Nuclear magnetic resonance spectroscopy allows for the quantification of the complete lipoprotein profile of an individual in only a few minutes. Nuclear magnetic resonance technology allows for the analysis of both particle size and particle concentration of the lipoprotein subclasses which yields the complete lipoprotein profile. Thus, there are many advantages of nuclear magnetic resonance over using standard assays which are only capable of measuring one type of lipid carried by the lipoprotein.

Fast phase liquid chromatography (FPLC) is another method which allows for the separation and quantification of lipoproteins. This method starts with concentrating the plasma by ultracentrifugation to isolate the density < 1.21 gm/mL. Due to the spin step, only 80 – 90% of the lipids in the plasma can be recovered using this method. The pooled plasma is concentrated between 8 to 16 fold the concentration to obtain the lipoprotein-rich fraction from each of the classes. This concentrated solution is then applied to the pressure-packed Superose 6B™ columns which allows for the separation of VLDL, LDL, and HDL by size. The VLDL for this method is calculated as Total – HDL – LDL.

It was our hypothesis that lipoproteins change with different diets, and that changes can be detected equally with different methods. The objectives of this research were twofold:

1. To determine the effects of diet and obesity on lipoprotein classes and their concentration of cholesterol, triglyceride, phospholipid, non-esterified fatty acid, and protein.
2. To compare results obtained with ultracentrifugation with those obtained with FPLC, and nuclear magnetic resonance.

CHAPTER 2

LITERATURE REVIEW

Lipid Metabolism

Lipids are a vital macronutrient required by all organisms. Triglycerides, cholesterol, and phospholipids are consumed and ingested in the diet. Some lipids such as cholesterol and phospholipids are also synthesized in the body from bile (Rhoades et al., 2003). Lipids are stored in adipose tissue as triacylglycerols and provide an excellent source for energy because they contain 9 kcal/g, whereas carbohydrates and protein only provide approximately 4 kcal/g (Stipanuk, 2000). Triacylglycerols may account for up to 100 grams or more of the lipid content in the human diet with the fatty acyl groups varying in length from C2 to C24 containing both saturated and unsaturated fatty acids (Mu et al., 2005). Phospholipids and cholesterol are important structural components in plasma membranes as well as intracellular membranes. Glucocorticoids, mineralocorticoids, estrogens, progestins, and Vitamin D are also derived from cholesterol. Thus, lack of cholesterol or phospholipids might cause intracellular and extracellular signaling impairment as well as Vitamin D deficiencies (Stipanuk, 2000).

In order for the body to use lipids they must be broken down and absorbed from the small intestine. Triglycerides are hydrolyzed into a 2-monoglyceride and 2 fatty acid molecules by pancreatic lipase (Rhoades et al., 2003). Phospholipids are hydrolyzed into lysophospholipids and fatty acids by phospholipase A_2 (Rhoades et al., 2003). Cholesterol esters are hydrolyzed to free cholesterol and fatty acid by cholesterol esterase (Rhoades et al., 2003). The hypothesis that different types of fatty acids may affect lipoproteins has been tested in humans when diets with

saturated fatty acids, monounsaturated fatty acids, and omega-6 unsaturated fatty acids were consumed in moderation (Thijssen and Mensink, 2005). This study found that there were no significant differences between LDL cholesterol or in VLDL, LDL, or HDL particle size or subclass concentration for diets between these three types of fatty acids (Thijssen and Mensink, 2005). Previous reports in humans have shown that a diet high in unsaturated fatty acids will actually lead to a reduction in LDL cholesterol (Mattson and Grundy, 1985). Once these lipids are broken down, bile salts form mixed micelles which can diffuse across the layer of water surrounding the enterocytes (Rhoades et al., 2003). Fatty acids and monoglyceride are absorbed by the enterocyte and travel to the smooth endoplasmic reticulum where triglyceride molecules are formed (Rhoades et al., 2003). Cholesterol can leave the endoplasmic reticulum in the free or esterified form (Rhoades et al., 2003).

Lipoproteins are the particles formed from the combination of lipid with protein inside the endoplasmic reticulum of the enterocyte. There are five major classes of lipoproteins which are classified according to their density including chylomicrons, very low density (VLDL), intermediate density (IDL), low density (LDL), and high density (HDL). The liver and the small intestine account for the rate of synthesis of plasma lipoproteins, and, when combined with peripheral catabolism, account for the total amount of lipoproteins in the circulation (Field et al., 1995). The two main pathways which the body uses to metabolize lipids are the exogenous pathway which refers to the absorption of lipids consumed in the diet from the small intestine and the endogenous pathway which refers to the body's synthesis of lipids in the liver.

The body uses the exogenous pathway to metabolize lipids that are consumed in the diet (Barter, 2005). The lipids consumed in the diet are assembled in the intestine into chylomicron particles which range from 100-500 nm in size, making them the largest lipoprotein particle

(Field et al., 1995). Their main function is to transport dietary triglycerides to peripheral tissues which can account for up to 90% of their total composition (Field et al., 1995). The intestine-derived chylomicrons, which are formed after a meal containing fat is consumed, begin assembly within the enterocytes when nascent apolipoprotein B together with phospholipids are released from the endoplasmic reticulum membrane (Hussain, 2000). Apolipoprotein B48 is the major structural protein in these particles (Stipanuk, 2000). The triglyceride-rich particles which are reformed after absorption from the intestine then fuse with the phospholipid and protein particles to form chylomicrons (Hussain, 2000). Chylomicrons exit the intestine and enter into the circulation picking up additional apolipoprotein C and apolipoprotein E from HDL (Bauer, 2004). These particles travel to muscle and adipose tissue where lipoprotein lipase (LPL) hydrolyzes the triglycerides to produce free fatty acids which can then be transported into tissues and stored (Goldberg, 1996). The chylomicron remnants are finally returned to the liver through the high affinity LDL receptor which recognizes apolipoprotein E (Cooper, 1997).

The model proposed for the assembly of VLDL is slightly different than the model for chylomicron assembly. The assembly of VLDL is thought to occur in two stages. The first step in VLDL assembly occurs within the enterocytes and hepatocytes when microsomal triglyceride transfer protein (MTP) transfers triacylglycerol from the endoplasmic reticulum membrane to the VLDL precursor particle located in the endoplasmic reticulum lumen (Gibbons et al., 2004). Next, apolipoprotein B is released from the endoplasmic reticulum membrane and fuses with the VLDL precursor particle located in the endoplasmic reticulum lumen (Gibbons et al., 2004). Phospholipids are added to the precursor particle when phospholipase D is activated by the protein ADP-ribosylation factor 1 (ARF1) to form phosphatidic acid (Gibbons et al., 2004).

Finally, phosphatidic acid fuses with the triacylglycerol rich particle in addition to the apolipoprotein B to form the mature VLDL particle (Gibbons et al., 2004).

Lipids which are synthesized de novo move through the endogenous route for metabolism. VLDLs are formed in the liver and intestine from endogenous triglycerides. The molecular weight of VLDL is approximately 100 times less than that of chylomicrons (Stipanuk, 2000). The function of these particles is to transport triacylglycerols to adipose and muscle tissue where lipoprotein lipase binds and hydrolyzes the particle. It has also been suggested that in cats hepatic lipase may be involved in the hydrolysis of VLDL triglycerides (Demacker et al., 1988). VLDLs contain apolipoprotein B100, apolipoprotein C, and apolipoprotein E. Loss of apolipoprotein C to HDL in the plasma coupled with triacylglycerol loss to tissues via lipoprotein lipase activity will result in conversion of a small number of particles to IDL and most to LDL (Stipanuk, 2000).

All IDL and LDL in the plasma originate from VLDL (Stipanuk, 2000). IDLs are either removed from the circulation through hepatic LDL receptor where they lose their apolipoprotein E or they are re-released back into the circulation as LDL. Hepatic lipase is responsible for the conversion of IDL to LDL (Connelly, 1999). LDL functions as the major transporter of cholesterol from the liver to the tissues. Apolipoprotein B100 is the only apolipoprotein on the LDL particle. LDL is returned to the liver through the LDL receptor which recognizes the apolipoprotein B100 (Stipanuk, 2000). The LDL particles are endocytosed in vesicles which fuse with lysosomes where protein is broken down and cholesterol ester is hydrolyzed to yield free cholesterol and free fatty acids (Rhoades et al., 2003).

HDL particles are synthesized in the liver and intestine, and are not assembled from the catabolism of any other lipoprotein. The main physiological role of HDL is to return cholesterol

from the periphery to the liver. HDLs are synthesized in the liver and small intestine as protein-rich particles which lack cholesterol. These particles leave the plasma compartment where they pick up free cholesterol in the extracellular space (Fielding, 1995). Cholesterol-rich particles return to the plasma where the free cholesterol is esterified by lecithin cholesterol acyltransferase (LCAT). These cholesterol-rich particles then return to the liver via the HDL receptor or the cholesterol esters may be transferred to VLDL and LDL through cholesterol ester transfer protein (CETP) (Fielding, 1995).

Obesity and Lipids

Obesity is now a major health concern for veterinary medicine due to the increasing trend seen in domestic cats and dogs. It has been estimated that approximately 35% out of 8000 cats examined were considered overweight or obese in United States veterinary practices (Lund et al., 2005). Obesity occurs when energy intake exceeds energy output. Sedentary lifestyles combined with the consumption of high-fat and high-calorie foods lead to weight gain and to the development of obesity. Individuals consuming a high-fat diet will tend to overeat because fat is not as fulfilling as a diet high in protein (Lawton et al., 1993). The prevalence of obesity in humans has doubled in the last 10 years in Western countries and is now recognized as a growing epidemic health problem (James, 2004). Diseases such as diabetes mellitus and cardiovascular disease (CVD) are known to be closely associated with obesity (Despres, 1993). It has been shown that even modest adult weight gain greatly increases the risk for developing type II diabetes (Colditz et al., 1995). The coexistence of diabetes, coronary artery disease, and hypertension is now known as the metabolic syndrome (Eckel et al., 2005). Metabolic diseases

associated with obesity are known to effect over 50% of the adult population (Wellen et al., 2003).

The problem which occurs with obesity and with the metabolic syndrome in humans is that there is increased accumulation and storage of lipids in both adipose and non-adipose tissues (Soufi et al., 2006). In normal individuals insulin inhibits lipolysis in adipose tissue. Hormone sensitive lipase (HSL) is the enzyme responsible for the release of fatty acids from adipocytes (Eckel, 1995) and individuals with insulin resistance in combination with obesity or metabolic syndrome have continued release of fatty acids from adipose tissue because the enzyme is not inhibited (Barter, 2005). These individuals also have impaired esterification and re-esterification of fatty acids in adipose tissue (Lewis et al., 2002). Abnormal esterification and re-esterification of fatty acids leads to increased flux of fatty acids from the adipose tissue (Lewis et al., 2002). Lipoprotein lipase mediated release of free fatty acid from triglyceride in VLDL and chylomicron particles is inhibited during insulin resistance because this enzyme is stimulated by insulin and these particles are not taken up by adipose tissue and are diverted away to other organs (Lewis et al., 2002).

The increased amount of fatty acids being shuttled to the liver results in increased amounts of fatty acids being stored in the liver (Lewis et al., 2002). Insulin resistance and hyperinsulinemia also affect the liver by increasing the esterification of fatty acids, rather than oxidation. This promotes the storage of triglycerides or increased production of VLDL (Lewis et al., 2002). The high rate of VLDL synthesis thus increases the amount of VLDL circulating in the plasma which in turn raises the concentration of chylomicrons because they are both competing for lipoprotein lipase for hydrolysis (Lewis et al., 2002).

Obesity or the metabolic syndrome in humans may also lead to increased concentrations of triglycerides in the plasma and cause cholesterol ester transfer protein-mediated transfer of cholesterol ester and triglyceride between VLDL and HDL to be increased which results in VLDL having high levels of cholesterol ester and HDL having high levels of triglyceride (Barter, 2005). The addition of triglyceride to the HDL particle leads to the formation of small dense HDL which leads to increased clearance of HDL from the circulation (Murakami et al., 1995). This study also demonstrated that the transfer of cholesterol ester out of HDL was higher in hypertriglyceridemic individuals than in normal individuals or in those with hypercholesterolemia (Murakami et al., 1995). Increased clearance of HDL from the circulation leads to decreased reverse cholesterol transport from the periphery back to the liver and higher risk for developing cardiovascular disease.

Obesity or the metabolic syndrome in humans may also lead to the alteration of LDL particles which occurs when cholesterol ester transfer protein shifts cholesterol esters from LDL to VLDL and triglycerides from VLDL to LDL (Barter, 2005). The addition of triglyceride leads to a small dense particle which it is more harmful to the epithelium than larger particles. LDL from hypertriglyceridemic subjects has been shown to be more susceptible to oxidation than in normal subjects (deGraaf et al., 1993). The problem that occurs with LDL oxidation is that now LDL is not able to be recognized by the LDL receptor, but instead is recognized by the scavenger receptor (Sparrow et al., 1989). This results in large amounts of cholesterol being taken up by the macrophages in the arterial wall, which are referred to as foam cells which lead to atherosclerosis (Berliner et al., 1995). This phenomenon is not observed in the cat.

Obesity in cats increases the risk of developing several other diseases, including hepatic lipidosis and diabetes. The metabolic syndrome does not exist in cats because they are not prone

to develop atherosclerosis. It has been estimated that between 0.5-1% of cats are diabetic, whereas it was seen in less than 0.2% of cats approximately 30 years ago (Schaer, 1973). Currently there are no studies which show that obesity in dogs leads to the development of diabetes mellitus; however, obesity can lead to insulin resistance and probably complicates diabetes management. The occurrence of diabetes mellitus in veterinary teaching hospitals, however, has also been increasing in dogs because in 1970 there were approximately 19 cases per 10,000 cases and in 1999 there were 58 cases per 10,000 cases (Guptill et al., 2003).

Very little research has been done to investigate lipid metabolism in cats and dogs. Feline lipoproteins can be separated into five classes similar to human lipoproteins, including chylomicrons, VLDL, LDL, HDL_2, and HDL_3 (Bauer, 2004). In addition to having these five fractions dogs also exhibit a HDL_1 fraction which is not seen in humans or in cats (Bauer, 2004). The two distinct HDL sub-fractions that cats exhibit are also seen in human density profiles which makes them excellent models for studying lipoprotein metabolism (Terpstra et al., 1982). Cats have 5 times more HDL than LDL which serves to increase the amount of cholesterol returned from the periphery (Demacker et al., 1987). HDL is also the main carrier of cholesterol in lean and obese dogs (Jeusette et al., 2005). HDL phospholipids have been found to be significantly higher in obese dogs whereas other HDL lipids were not significantly different between lean and obese (Chikamune et al., 1995). It has been shown that newly obese cats also have more HDL cholesterol than lean cats, which might offer protection against developing atherosclerosis (Hoenig et al., 2003). Interestingly, decreases in HDL cholesterol have been reported in obese insulin-resistant dogs which is similar to what occurs in humans (Bailhache et al., 2003). The fact that cats do not develop atherosclerosis or coronary artery disease with obesity is thought to be due to their lack of cholesterol ester transfer protein (Watson et al.,

1995). Obese cats have higher VLDL concentrations than lean cats and show increases in all components (Hoenig et al., 2003). In obese dogs, VLDL phospholipids were significantly higher than in lean, but other VLDL lipids measured were not found to be different by one investigator (Chikamune et al., 1995), whereas Bailhache et al. (2003) found VLDL triglycerides to be higher in obese dogs. LDL cholesterol has been shown to be higher in obese dogs (Jeusette et al., 2005). This is different from cats, where no change in LDL components was seen (Hoenig et al., 2003).

Measurement of Lipid Particles

Each of the VLDL, LDL, and HDL classes are made up of different sized lipoprotein particles, often referred to as a subclass, which when summed together account for the total class concentration and individually as the particle concentration of each subclass. The VLDLs are composed of large VLDL/chylomicrons, medium, and small VLDL (NMR LipoProfile-II® Research Report, 2004). The LDLs are composed of large, medium-small, and very-small LDL (NMR LipoProfile-II® Research Report, 2004). IDL is also included with the LDL group (NMR LipoProfile-II® Research Report, 2004). The HDLs are composed of large, medium, and small HDL (NMR LipoProfile-II® Research Report, 2004). Accurate measurement of the concentration of the different subclasses of lipoproteins is essential for patients at risk for disease because different subclass particle concentrations can have either negative or positive impacts on the health of humans. The diameter of each of the lipoprotein particles is referred to as the particle size. This is an important measurement because it has been shown that small LDL particles enter the artery walls more easily than large LDL particles, thus having a high

concentration of small LDL would lead to the development of atherosclerosis (Lamarche et al., 1997).

The gold standard method for determining the concentration of lipoproteins in plasma has been by density gradient ultracentrifugation (Bauer, 1991). This method is too time-consuming and too few samples can be analyzed at one time for use in the clinical setting. In addition, analysis of particles by ultracentrifugation does not yield information on the size of the particles. Nuclear magnetic resonance spectroscopy (NMR) is a new method that allows the determination of both size and concentration of lipoproteins in whole plasma in only a few minutes (Otvos et al., 1991). Unlike ultracentrifugation, physical separation of the lipoprotein classes is not required. This method is based on characteristic methyl signals which are transmitted from all of the lipids in the particle to produce a bulk particle signal (Otvos et al., 1991). Thus, the amplitude of each lipoprotein particle determines the concentration of that particular lipoprotein (Otvos et al., 1991). This is the first method capable of directly and efficiently measuring both the concentration and size of lipoprotein particles in an individual. Because disease risk factors are correlated with the concentration of each lipoprotein class and its particle size rather than the lipid carried in them, this information is used for the identification of individual patients who are at a high risk for developing disease. The importance of the nuclear magnetic resonance lipid profile becomes obvious when examining the difference between measuring LDL particle concentration and LDL-cholesterol concentration, as an example. LDL-cholesterol is often measured in the clinical setting for those patients at risk for cardiovascular disease as an indicator of the concentration of LDL particles if it is not possible to determine particles. However, measuring LDL-cholesterol is not a direct measurement of LDL particle concentration because it only measures the amount of cholesterol that the particle is carrying. Thus, the

amount of cholesterol carried per particle varies between individuals and LDL-cholesterol assays underestimate particle concentration by about 10-25% (Otvos et al., 2002).

The correlation between various lipoprotein particle concentrations and particle sizes in relation to cardiovascular and type 2 diabetes have been shown in several studies. Increases in large VLDL have been shown to be associated with coronary artery disease (Freedman et al., 1998) and inversely associated with insulin sensitivity (Goff et al., 2005). Increases in total VLDL particle concentration were found in patients with insulin resistance (Garvey et al., 2003), in patients with increased glucose intolerance in all subclasses except the small VLDL particle (Goff et al., 2005), and found to be inversely correlated to insulin sensitivity (Goff et al., 2005). Increases in VLDL particle size were associated with insulin resistance (Garvey et al., 2003), with increasing glucose intolerance (Goff et al., 2005), and inversely associated with insulin sensitivity (Goff et al., 2005). Regarding LDL particle concentrations, it has been shown that increases in small LDL particle concentration and total LDL particle concentrations were seen in women with coronary heart disease (Kuller et al., 2002), in women with increased coronary artery calcification (Mackey et al., 2002), with insulin resistance (Garvey et al., 2003), and with increased glucose intolerance due to decreases in large LDL particle concentrations (Goff et al., 2005). Others have also shown in humans that significantly increased levels of LDL cholesterol were more associated with intermediate and small LDL particles than in those with large or very small LDL particles (Campos et al., 1992). Diets high in unsaturated fatty acids have been shown in humans to reduce LDL particle size when compared to diets high in saturated fatty acids (Kratz et al., 2002). It has also been shown that the difference between those with coronary artery disease and controls in LDL particle size was that plasma triglyceride levels were increased and HDL-cholesterol levels were decreased (Campos et al., 1992). Small LDL particle

size was associated with coronary heart disease (Kuller et al., 2002), with coronary artery calcification (Mackey et al., 2002), with insulin resistance (Garvey et al., 2003), and with increased glucose intolerance (Goff et al., 2005). Regarding HDL particle concentration, increases in small HDL particle concentrations were found to be associated with increased coronary artery calcification in women (Mackey et al., 2002), with severity of coronary artery disease in men (Freedman et al., 1998), and with insulin resistance (Garvey et al., 2003). Large HDL particle concentrations were inversely associated with coronary artery calcification in women (Mackey et al., 2002), were seen to decrease with insulin resistance (Garvey et al., 2003), and decrease with increasing glucose intolerance (Goff et al., 2005). Total HDL particle concentrations were also seen to decrease with increasing glucose intolerance (Goff et al., 2005). Insulin sensitivity was positively correlated with HDL-cholesterol concentration, HDL particle concentration, large HDL particle concentration, and inversely correlated with adiposity (Goff et al., 2005). HDL particle size has been shown to be decreased in insulin resistant individuals (Garvey et al., 2003) and with increasing glucose intolerance (Goff et al., 2005). Intermediate sized HDL particle size has been shown to be inversely associated with coronary heart disease (Freedman et al., 1998). Insulin sensitivity was positively correlated with HDL size and inversely correlated with adiposity (Goff et al., 2005).

CHAPTER 3

MATERIALS AND METHODS

Animals and diets. Twelve lean (L) neutered adult cats aged 7 ± 2 years and 12 obese (OB) neutered adult cats aged 9 ± 1 years of equal gender distribution were used for this study. Obese cats had been obese for over 1 year prior to the beginning of the study. Cats were maintained at the University Of Georgia College Of Veterinary Medicine Animal Care Facility under standard colony conditions. They were housed in individual cages and given free access to water. Animal studies were approved by the University of Georgia Animal Care and Use Committee and conducted in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Cats were determined to be healthy on the basis of results of physical examination and clinical laboratory data. All cats were accustomed to daily handling.

The cats were fed one of three diets (Table 1) once daily for two months. They were randomly and evenly allocated to 3 diets in a Latin Square rotation. Food intake was recorded at each feeding, and the cats were weighed once weekly. Food intake was adjusted to maintain each cat's weight within 5% of the weight at the beginning of the study. The composition of the diets was only known to one investigator (MW) who was not involved in experimental execution or data analyses.

Postprandial lipoprotein analysis. Postprandial analysis was performed on 12 samples for the lean cats (4 for diet A, 4 for diet B, and 4 for diet C). The average weight of these cats was 3.5 ±

0.5 and there was no significant difference of weights between diets. The weight of lean males was significantly higher than females (3.9 $\pm$ 0.2 and 3.1 $\pm$ 0.2, respectively; $p < 0.0001$).

Experimental protocol. Baseline blood samples were collected from the jugular vein and immediately placed into EDTA coated tubes for plasma collection. In addition to baseline samples, postprandial samples were also taken from lean cats at the first diet rotation at 3, 6, and 12 hours post feeding. Lean cats ate their food within 15 minutes. After centrifugation of the blood, the plasma was harvested and either used immediately for ultracentrifugation and NMR analysis, or it was stored at -20ºC until assays and FPLC analyses were performed. Plasma was used for separating lipoprotein fractions with a single isopycnic density gradient ultracentrifugation method as previously described (Hoenig et al., 2003). Briefly, the plasma was prestained with Sudan black and ethylene glycol solution to enhance observation of the lipoprotein fractions. A swinging bucket rotor (Beckman S 28.1, Fullerton, CA) was used to centrifuge samples at 150,000 *x g* for 31 hours at 20ºC. The fractions were collected from the meniscus downward. The density of each fraction was determined by weighing each fraction and measuring the volume of each fraction. In plasma and also in each fraction, cholesterol (Cholesterol LiquiColor; Stanbio, Boerne, TX), triglycerides (Serum Triglyceride; Sigma, Saint Louis, MO), and phospholipids (Phospholipids B; Wako Chemicals, Richmond, VA) were measured. Protein content was determined by the Bradford method (Bradford, 1976). Plasma nonesterified fatty acids (NEFAs) were measured by use of an enzymatic test kit (NEFA-C; Wako Chemicals, Richmond, VA). Plasma glucose measurements were performed using a colorimetric glucose oxidase method (Glucose Assay; DCL, Oxford, CT). Plasma insulin concentrations were measured as previously described (Hoenig and Ferguson, 1989).

Measurement of body mass index (BMI; expressed in kg/m^2) was performed. The BMI was calculated by use of the following formula:

$$BMI = \frac{\text{body weight (kg)}}{\text{body length (m) x height (m)}}$$

where length was the distance from the point of the shoulder to the tuber ischium, and height was the distance from the point of the shoulder through the point of the elbow to the proximal boundary of the central metacarpal pad. All measurements were performed by the same person to minimize variability (SK).

Nuclear magnetic resonance spectroscopy analysis. Lipoprotein analysis by nuclear magnetic resonance was performed by Liposcience in Raleigh, NC for (Otvos et al., 1992).

Fast phase liquid chromatography (FPLC). FPLC analysis was performed on 22 samples from lean cats (8 for diet A, 6 for diet B, and 8 for diet C) and on 12 samples from obese cats (3 for diet A, 5 for diet B, and 4 for diet C) in the Emory Lipid Research Laboratory (Emory University, Atlanta, GA) as described (as described (Innis-Whitehouse et al., 1998).

Statistical analysis. Data were analyzed by use of a software program (Prism software, GraphPad Software Inc, San Diego, CA). Data are expressed as means ($\pm$SD) unless stated otherwise. The significance of differences of means between groups was evaluated by an ANOVA and differences within a group by the Student t test for paired analysis. Values of $p < 0.05$ were considered significant.

CHAPTER 4

RESULTS

The weight, BMI, girth, and intake of the cats are shown in Table 2. Lean cats consumed significantly more kilocalories per body weight than the OB ($p < 0.0001$) but there was no diet or gender difference. Weight, BMI, and girth were significantly lower in L than in OB ($p < 0.0001$). Weight for L males was significantly higher than L females ($p < 0.0001$). Lean males also had a significantly higher BMI than L females ($p = 0.01$). Girth for OB males was significantly higher than OB females ($p = 0.03$).

Plasma baseline lipid concentrations. Cholesterol concentrations (mg/dL) were significantly higher in L than in OB (156 $\pm$ 28.8 and 140 $\pm$ 22.9, respectively; $p = 0.01$) (Table 3) when results from diets were combined. The only diet difference that was seen in the cholesterol concentrations was between diet A and B in the OB group (A: 151 $\pm$ 19.5 and B: 131 $\pm$ 22.7; $p = 0.02$). Baseline cholesterol concentrations ranged from 106 to 224 in L, and from 90 to 213 in OB. There was no difference in triglyceride concentrations among diets; however, for all 3 diets, triglyceride concentrations were significantly lower in L than in OB ($p < 0.0001$) (Table 3). The baseline triglyceride concentrations (mg/dL) ranged from 12 to 56 in the L and from 21 to 86 in the OB. There was no difference between phospholipid concentrations within L and OB fed different diets; however, L had significantly higher phospholipid concentrations than OB ($p = 0.0004$) (Table 3). The baseline phospholipid concentrations (mg/dL) ranged from 194 to 351 in L and from 109 to 326 in OB. Protein concentrations (mg/dL) were significantly lower in L than

OB (5819 $\pm$ 734.1 and 6301 $\pm$ 867.3, respectively; $p = 0.01$) (Table 3). NEFA concentrations were not different among diets. NEFA concentrations (mEq/L) were, however, significantly lower in L than OB (0.40 $\pm$ 0.19 and 0.56 $\pm$ 0.12, respectively; $p = 0.0002$) (Table 3); they were also higher in obese males than females; however, this was not significant.

Determination of lipoproteins with ultracentrifugation.

Concentration of lipid and protein in plasma lipoprotein fractions: VLDL, LDL, HDL_2 and HDL_3 fractions were collected (Figure 1). Diet had no effect on VLDL concentrations. However, VLDL concentrations were significantly lower in L than OB for cholesterol ($p = 0.01$), triglycerides ($p < 0.0001$), phospholipids ($p < 0.0001$), protein (respectively; $p < 0.0001$), and total lipoprotein mass ($p < 0.0001$) (Table 3). There were no significant gender differences.

LDL concentrations (Table 3) (mg/dL) were not different between lean and obese cats. However, there were diet effects (Table 4). Lean cats fed diet C had significantly higher values of cholesterol, triglycerides, phospholipids, and protein than cats fed either diet B, or A, whereas there was no difference between cats fed diet A and B. Lean males had significantly higher triglycerides in diet A and B than females ($p = 0.01$, and $p = 0.04$, respectively; not shown).

HDL_2 concentrations were significantly higher in the L than the OB for cholesterol ($p = 0.01$), phospholipids ($p = 0.001$), and total lipoprotein mass ($p = 0.05$) when results from all diets were combined (Table 3). There were no diet effects in L in the HDL_2 fraction; however, OB fed diet A had significantly higher cholesterol (58 $\pm$ 9.3 and 48 $\pm$ 15.2, respectively; $p = 0.05$), phospholipids (78 $\pm$ 15.9 and 64 $\pm$ 18.1, respectively; $p = 0.05$), and total lipoprotein mass (250 $\pm$ 48.6 and 205 $\pm$ 64.02, respectively; $p = 0.04$) than OB fed diet C. OB males compared to OB females fed diet A had higher concentrations of cholesterol (63 $\pm$ 4.8 and 52 $\pm$ 10.2, respectively; $p = 0.04$), phospholipids (87 $\pm$ 12.95 and 68 $\pm$ 12.8, respectively;

p = 0.04), and protein (128.1 $\pm$ 12.02 and 88.89 $\pm$ 23.58, respectively; p = 0.01).

HDL_3 concentrations (mg/dL) were not significantly different between L and OB groups (Table 3).

Composition of lipid and protein in plasma lipoprotein fractions: The percent weight of cholesterol, triglyceride, phospholipid, and protein is shown in Table 5 for each lipoprotein fraction. There were no effects of diet on the percent weight in any of the measurements; however, in the VLDL fraction, cholesterol and phospholipids were significantly higher in L compared to OB (p = 0.002 and 0.01, respectively), whereas triglycerides and protein were significantly lower in L than OB (p = 0.02 and < 0.0001, respectively).

Correlation between results from basal lipid measurements with ultracentrifugation, NMR, and FPLC. There was a significant correlation between triglyceride measurements using in-house assays and FPLC (Figure 2a) (r^2 = 0.5599, p < 0.0001) and with NMR (Figure 2b) (r^2 = 0.3162, p < 0.0001). There was also a good correlation between HDL cholesterol measured after ultracentrifugation and measured with FPLC (Figure 3a) (r^2 = 0.4198, p < 0.0001), or NMR (Figure 3b) (r^2 = 0.3141, p < 0.0001). Plasma cholesterol also correlated well using in-house assays and FPLC (Figure 4) (r^2 = 0.4462, p < 0.0001).

Particle size determined by NMR. VLDL average particle size (nm) was too small to be measured in L cats. In OB, VLDL particles had the largest size, followed by LDL, and HDL particles (p < 0.0001 between all 3 fractions). In L, LDL particles were also larger than HDL particles (p = 0.0004, and p < 0.0001, respectively), and both were significantly larger in L than OB. In OB, VLDL particle size was significantly different among diets (all p < 0.04). Diet B had the highest particle size (64.3 $\pm$ 6.9) and diet C had the lowest particle size (47.6 $\pm$ 5.7). Diet affected LDL particle size (nm) in OB with a similar pattern to that seen in the VLDL fraction:

the highest particle size was seen in OB fed diet B (22.1 $\pm$ 1.0), and the smallest size was seen in cats fed diet C (20.8 $\pm$ 1.5; all $p < 0.05$). There were no significant diet differences of the HDL average particle size (nm) within the L or OB group. Table 8 shows the particle size in L and OB after combining the diets.

Particle concentration determined by NMR. VLDL particle concentrations were not able to be measured in the L cats. In OB, the medium VLDL particle concentration (nmol/L) was significantly higher than the large VLDL particle concentration (5.6 $\pm$ 5.3 and 2.3 $\pm$ 2.4, respectively; $p = 0.01$). Concentrations of large LDL particles (nmol/L) were not significantly different between L and OB cats. Concentrations of medium small and very small particles in OB were significantly different (115.4 $\pm$ 64.7 and 440.1 $\pm$ 273.4, respectively; $p < 0.0001$). In L cats these particles could not be determined. Concentrations of large HDL particles (µmol/L) were not significantly different between L and OB cats (Figure 5). However, medium HDL particle concentrations were significantly higher in the L than the OB, whereas concentrations of small HDL particles were significantly lower in L than in OB. (Figure 5).

Postprandial lipoprotein and NEFA concentrations measured with ultracentrifugation. For the postprandial analysis, results from different diets were combined (Table 9). When comparing plasma concentrations in L, there was no significant change in plasma cholesterol or phospholipid concentrations postprandially at any time point. However, triglyceride, and total-protein concentrations increased significantly 3 hours after feeding and returned to baseline at 12 hours. There was no change in NEFA concentrations after feeding. Analysis of lipoprotein fractions showed that the concentrations of cholesterol, triglycerides, and total lipoprotein mass increased in all fractions and were still elevated after 12 hours (Table 10).

Plasma glucose and insulin concentrations. Diet had no effect on baseline glucose concentrations (mg/dL) in L or OB cats. There was no significant difference between glucose concentrations in L (83 $\pm$ 10) and OB (85 $\pm$ 9) cats (data not shown) or in postprandial cats (Table 9). Diet also had no effect on baseline insulin concentrations (data not shown). However, baseline insulin concentrations (pmol/L) were significantly lower in L than OB (131 $\pm$ 68 and 270 $\pm$ 100, respectively; $p < 0.0001$) and increased significantly in L at 3, 6, and 12 hours postfeeding regardless of diet ($p < 0.0001$) (data not shown).

Table 1. Diet Compositions for diets A, B, and C.

Composition	Diet A	Diet B	Diet C
Protein %	44.20	33.80	35.10
Fat %	14.7	16.9	16.6
CHO (by subtraction) %	25.27	32.07	32.82
Fiber (Crude) %	1.44	1.70	1.45
Ash %	8.94	8.19	7.22
Moisture %	5.45	7.34	6.81
Kcal/g (by calculation)	4.1	4.16	4.21
Fatty Acid analysis	**% fat**	**% fat**	**% fat**
14:0	1.6	2.01	2.61
14:1	0.35	0.44	0.31
16:0	21.3	22.3	21.4
16:1n-7	3.63	3.67	4.42
18:0	10	11.8	10.2
18:1n-9	32.9	34.2	30.3
18:1n-7	1.54	1.53	1.77
18:1n-9 T	2.76	3.37	3.37
18:2n-6	18.5	13.5	12.8
20:0	0.19	0.17	0.19
18:3n-3	1.00	0.80	0.93
20:2n-6	< 0.1	< 0.1	0.15
20:3n-6	0.11	0.10	0.12
20:4n-6	0.45	0.36	0.51
24:0	0.12	< 0.1	< 0.1
20:5n-3	< 0.1	< 0.1	1.86
22:5n-3	< 0.1	< 0.1	0.41
22:6n-3	< 0.1	< 0.1	1.49
Total	92.5	91.8	89.92

Table 2. Mean (± SD) values for body weight (kg), body mass index (kg/m^2), girth (cm), and food intake (kcal/kg) in lean (n=12, 6 male and 6 female) and obese (n=12, 6 male and 6 female) cats fed 3 different diets. The results were combined because there were no differences among diets.

Measurements	Weight (kg)	BMI (kg/m^2)	Girth (cm)	Food Intake (kcal/kg)
Lean	3.5 ± 0.5 *	32.3 ± 3.9 *	35 ± 2.8 *	53.8 ± 9.4 *
Males	3.9 ± 0.2 * ŧ	34 ± 3.1 * ŧ	35.8 ± 2.5 *	56.4 ± 11.5 *
Females	3.1 ± 0.2 * ŧ	30.7 ± 4 * ŧ	34.2 ± 2.9 *	51.3 ± 6.2 *
Obese	7.14 ± 1.1 *	62.3 ± 8.6 *	54.6 ± 4.6 *	39.1 ± 7.1 *
Males	7.5 ± 1.1 *	65 ± 9.2 *	56.3 ± 4.4 * ŧ	40 ± 9.2 *
Females	6.8 ± 1.1 *	59.7 ± 7.5 *	53 ± 4.2 * ŧ	38.1 ± 4.1 *

* Significant ($P < 0.05$) difference in measurements within a column between lean and obese cats.

ŧ Significant ($P < 0.05$) difference in measurements within a column between male and female cats.

Table 3. Mean (± SD) concentration of lipids, protein, and NEFA in baseline plasma and plasma lipoprotein fractions in lean (n=12, 6 male and 6 female) and obese (n=12, 6 male and 6 female) cats fed 3 different diets. The results were combined. Diet differences were noted in the LDL fraction (see Table 4).

Measurements	Plasma	VLDL	LDL	HDL_2	HDL_3
Lean Cats					
Cholesterol (mg/dL)	156 ± 28.8 *	3 ± 2.5 *	28 ± 11.7	63 ± 15.7 *	49 ± 11.3
Triglyceride (mg/dL)	21 ± 8.1 *	7 ± 6.6 *	5 ± 3.3	3 ± 2.8	3 ± 1.8
Phospholipids (mg/dL)	261 ± 40.4 *	3 ± 3.4 *	26 ± 12.5	89 ± 22.6 *	82 ± 20.8
Protein (mg/dL)	5819 ± 734.1 *	1.039 ± 1.2 *	13.52 ± 5.04	112.5 ± 33.01	116.4 ± 36.4
Total LP Mass (mg/dL)	---------------	13 ± 6.8 *	72 ± 23.9	265 ± 45.8 *	248 ± 45.4
NEFA (mEq/L)	0.40 ± 0.19 *	---------------	---------------	---------------	---------------
Obese Cats					
Cholesterol (mg/dL)	140 ± 22.9 *	5 ± 2.9 *	24 ± 9.1	53 ± 13.2 *	45 ± 10.7
Triglyceride (mg/dL)	48 ± 18.7 *	33 ± 20.9 *	4 ± 5.1	3 ± 1.8	3 ± 2.4
Phospholipids (mg/dL)	222 ± 46.2 *	10 ± 5 *	23 ± 9.8	71 ± 16.9 *	73 ± 18.7
Protein (mg/dL)	6301 ± 867.3 *	4.8 ± 2.5 *	12.98 ± 4.26	98.18 ± 27.47	110.1 ± 31.1
Total LP Mass (mg/dL)	---------------	52 ± 20.5 *	63 ± 21.8	227 ± 41.5 *	229 ± 43.9
NEFA (mEq/L)	0.56 ± 0.12 *	---------------	---------------	---------------	---------------

* Significant ($P < 0.05$) difference in measurements within a column between lean and obese cats.

Table 4. Mean (± SD) concentration of lipids and protein in the baseline LDL fraction in lean (n=12, 6 male and 6 female) cats fed 3 different diets.

Measurements	Diet A	Diet B	Diet C
Lean Cats	**LDL**	**LDL**	**LDL**
Cholesterol (mg/dL)	25 ± 8 †	24 ± 11.9 ‡	33 ± 13.2 ‡ †
Triglyceride (mg/dL)	4 ± 1.6 †	5 ± 1.7 ‡	8 ± 4.3 ‡ †
Phospholipids (mg/dL)	23 ± 5.9 †	21 ± 10.3 ‡	34 ± 16 ‡ †
Protein (mg/dL)	12.64 ± 3.1 †	11.41 ± 5.1 ‡	16.34 ± 5.6 ‡ †
Total LP Mass (mg/dL)	63 ± 19.3 †	61 ± 27.2 ‡	91 ± 34.6 ‡ †

‡ Significant ($P < 0.05$) difference in measurements within a row between diets B & C.
† Significant ($P < 0.05$) difference in measurements within a row between diets A & C.

Table 5. Mean (± SD) composition of baseline lipids and protein in plasma lipoprotein fractions in lean (n=12, 6 males and 6 females) and obese (n=12, 6 male and 6 female) cats fed 3 different diets. The results were combined because there was no difference among the diets.

Measurements	VLDL	LDL	HDL_2	HDL_3
Lean Cats				
Cholesterol (% weight)	17.3 ± 15.1 *	38.6 ± 4.7	23.9 ± 2.5	20.1 ± 3.5
Triglyceride (% weight)	52 ± 19 *	8.1 + 5	1.1 ± 1.03	1.14 ± 0.9
Phospholipid (% weight)	29.2 ± 19.2 *	35.1 ± 3.9	33.5 ± 2.2 *	33.3 ± 2.14 *
Protein (% weight)	5.2 ± 4.1 *	19.3 ± 4.5	42.4 ± 5.6	46.4 ± 5.82
Obese Cats				
Cholesterol (% weight)	9.6 ± 5.8 *	37.3 ± 4.6	23.5 ± 2.3	19.96 ± 3
Triglyceride (% weight)	61.6 ± 12.5 *	6.6 ± 5.5	1.5 ± 0.9	1.4 ± 1.1
Phospholipid (% weight)	20.2 ± 7.4 *	35.1 ± 4.7	31.9 ± 3 *	31.6 ± 2.3 *
Protein (% weight)	9.7 ± 3.3 *	21.2 ± 3.4	43.22 ± 3.4	47.95 ± 4.5

* Significant ($P < 0.05$) difference in measurements within a column between lean and obese cats.

Table 6. Mean (± SD) concentration of baseline ultracentrifugation (n=24, 12 L and 12 OB), NMR (n=24, 12 L and 12 OB), and FPLC (n=24, 12 L and 12 OB) total triglycerides and HDL-cholesterol.

Measurements	Combined Diets	Combined Diets
	Lean	**Obese**
In-house assayed Triglycerides (mg/dL)	21 ± 8.1 *	48 ± 18.7 *
NMR Triglycerides (mg/dL)	28 ± 7.1 *	43 ± 18.1 *
FPLC Triglycerides (mg/dL)	24 ± 7.5 *	39 ± 10.6 *
Ultracentrifugation HDL-cholesterol (mg/dL)	111 ± 22.4 *	96 ± 17.2 *
NMR HDL-cholesterol (mg/dL)	94 ± 8.7 *	85 ± 6.1 *
FPLC HDL-cholesterol (mg/dL)	134 ± 37.8	124 ± 35

* Significant ($P < 0.05$) difference in measurements within a row between lean and obese cats.

Table 7. Baseline correlations of ultracentrifugation (n=24, 12 L and 12 OB), NMR (n=24, 12 L and 12 OB), and FPLC (n=24, 12 L and 12 OB) measured triglyceride and cholesterol concentrations in cats.

Measurements	All Diets Ultracentrifugation vs. FPLC	All Diets Ultracentrifugation vs. NMR
Lean and Obese Cats		
Total plasma triglycerides	$p < 0.0001$ $r^2 = 0.5599$	$p < 0.0001$ $r^2 = 0.315$
HDL-cholesterol	$p < 0.0001$ $r^2 = 0.4198$	$p < 0.0001$ $r^2 = 0.3141$
Plasma cholesterol	$p < 0.0001$ $r^2 = 0.4462$	N/A N/A

Table 8. Mean (± SD) size of baseline NMR (n=24, 12 L and 12 OB) measured lipoprotein particles in cats.

Measurements	Combined Diets	Combined Diets
	Lean	**Obese**
VLDL Particle Size (nm)	N/A	58.2 ± 9.5
LDL Particle Size (nm)	22.4 ± 0.7 *	21.5 ± 1.3 *
HDL Particle Size (nm)	9.7 ± 0.2 *	9.4 ± 0.2 *

* Significant ($P < 0.05$) difference in measurements within a row between lean and obese cats.

Table 9. Mean (± SD) concentration of lipids, protein, NEFA, and glucose in baseline and postprandial plasma samples in lean cats (n=12, 6 males and 6 females).

Measurements	All Diets Baseline	All Diets 3 Hour	All Diets 6 Hour	All Diets 12 Hour
Lean Cats (6 males and 6 females)				
Cholesterol (mg/dL)	137 ± 24.8	143 ± 22.8	142 ± 22.5	135 ± 27.3
Triglyceride (mg/dL)	21 + 4.8 † ŧ	34 ± 12.4 † š	32 ± 12.6 ŧ ‡	23 ± 7.1 š ‡
Phospholipids (mg/dL)	260 ± 52	274 ± 40.3	271 ± 63.8	257 ± 48.4
Protein (g/dL)	6.2 ± 0.7 ŧ	5.8 ± 0.997 * š	6.6 ± 1 ŧ *	6.5 ± 0.99 š
NEFA (mEq/L)	0.39 ± 0.14	0.430 ± 0.19	0.390 ± 0.1	0.37 ± 0.15
Glucose (mg/dL)	81 ± 8.5	83 ± 9	84 ± 5.3	87 ± 10.9

† Significant (P < 0.05) difference in measurements within a row between 0 & 3 hour.
ŧ Significant (P < 0.05) difference in measurements within a row between 0 & 6 hour.
* Significant (P < 0.05) difference in measurements within a row between 3 & 6 hour.
‡ Significant (P < 0.05) difference in measurements within a row between 6 &12 hour.
š Significant (P < 0.05) difference in measurements within a row between 3 & 12 hour.

Table 10. Mean (± SD) concentration of lipids and protein in baseline and postprandial lipoprotein fractions in lean cats (n=12, 6 males and 6 females).

Measurements	All Diets Baseline	All Diets 3 Hour	All Diets 6 Hour	All Diets 12 Hour
Lean Cats (6 Males, 6 Females)				
VLDL				
Cholesterol (mg/dL)	3 ± 1.8 † ŧ**	8 ± 4.7 † *	18 ± 11.1 ŧ *	21 ± 11.3 **
Triglyceride (mg/dL)	10 ± 6 † ŧ **	28 ± 9.5 †	29 ± 12.3 ŧ	12 ± 5.6 **
Phospholipids (mg/dL)	5 ± 4.4 †	15 ± 6.1 † š	14 ± 10.1	7 ± 4.3 š
Protein (mg/dL)	1.7 ± 1.4 † ŧ	3.7 ± 1.7 †	3.1 ± 1.8 ŧ	1.9 ± 1.5
Total LP Mass (mg/dL)	19 ± 11 † ŧ **	55 ± 19.8 † š	64 ± 33.2 ŧ	41 ± 21.3 ** š
LDL				
Cholesterol (mg/dL)	24 ± 11.5 ŧ	36 ± 23.6	62 ± 32.4 ŧ	41 ± 20
Triglyceride (mg/dL)	5 ± 2.7	4 ± 1.7 *	15 ± 7.5 *	11 ± 13.9
Phospholipids (mg/dL)	23 ± 15.1	23 ± 12.3 *	31 ± 11.5 * ‡	17 ± 8.6 ‡
Protein (mg/dL)	11.5 ± 5.9	11.1 ± 5.0	13.01 ± 4.3	8.3 ± 4.3
Total LP Mass (mg/dL)	63 ± 30.8 **	74 ± 40.6 *	121 ± 49.8 *	77 ± 37.8 **
HDL_2				
Cholesterol (mg/dL)	62 ± 13 † ŧ **	104 ± 24.3 †	128 ± 29.6 ŧ	114 ± 21.1 **
Triglyceride (mg/dL)	3 ± 2.3 ŧ	2 ± 1.3 † * š	12 ± 7.6 ŧ *	7 ± 4.3 š
Phospholipids (mg/dL)	86 ± 18.2	91 ± 17.6	93 ± 11.9	83 ± 17.4
Protein (mg/dL)	125 ± 30.2	123.9 ± 26.3	128.1 ± 18.5	121.2 ± 26.3
Total LP Mass (mg/dL)	275 ± 61† ŧ **	320 ± 66.8 † *	361 ± 62.4 ŧ *	325 ± 60.9 **
HDL_3				
Cholesterol (mg/dL)	50 ± 10.7 † ŧ **	89 ± 12.3 †	101 ± 14.8 ŧ	109 ± 20.6 **
Triglyceride (mg/dL)	2 ± 0.9 ŧ	2 ± 1.4 *	11 ± 6.9 ŧ *	9 ± 8.7
Phospholipids (mg/dL)	91 ± 18.9	89 ± 11.6	823 ± 13.8	92 ± 12.6
Protein (mg/dL)	135.5 ± 34.8	138.5 ± 25.7	111.7 ± 32.7	128.7 ± 25.1
Total LP Mass (mg/dL)	279 ± 62.7 † **	318 ± 46.4 †	306 ± 60.5	338 ± 62.7 **

† Significant ($P < 0.05$) difference in measurements within a row between 0 & 3 hour.
ŧ Significant ($P < 0.05$) difference in measurements within a row between 0 & 6 hour.
** Significant ($P < 0.05$) difference in measurements within a row between 0 & 12 hour.
* Significant ($P < 0.05$) difference in measurements within a row between 3 & 6 hour.
‡ Significant ($P < 0.05$) difference in measurements within a row between 6 &12 hour.
š Significant ($P < 0.05$) difference in measurements within a row between 3 & 12 hour.

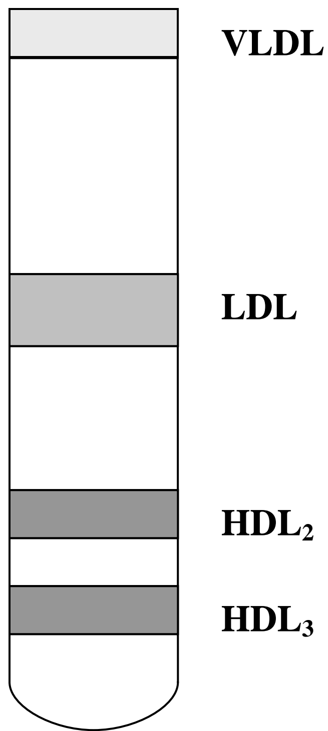

Figure 1. Feline lipoprotein density gradient after ultracentrifugation representing VLDL, LDL, HDL_2, and HDL_3 fractions.

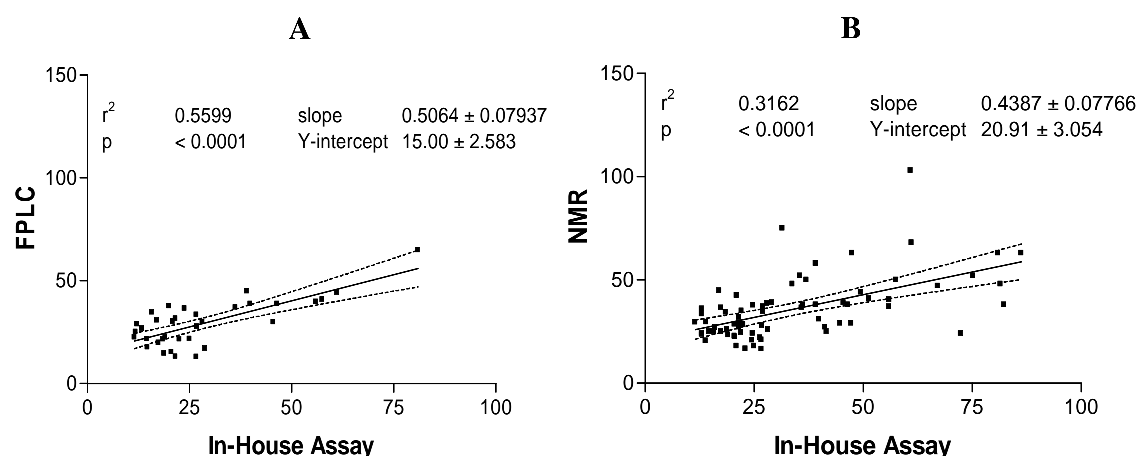

Figure 2. (A) Baseline in-house assay for total plasma triglycerides vs. FPLC determined total plasma triglycerides (mg/dL) in lean (n=12) and obese (n=12) cats and **(B)** baseline in-house assay for total plasma triglycerides vs. NMR determined total plasma triglycerides (mg/dL) in lean (n=12) and obese (n=12) cats.

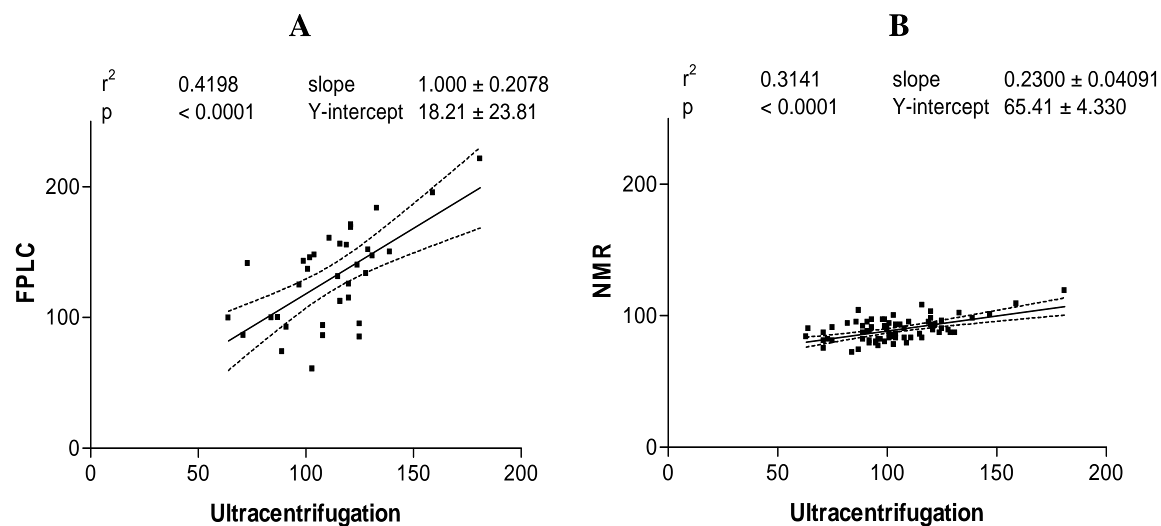

Figure 3. (A) Baseline ultracentrifugation HDL-cholesterol vs. FPLC HDL-cholesterol (mg/dL) (n=24, 12 L and 12 OB) in cats and **(B)** Baseline ultracentrifugation HDL-cholesterol vs. NMR HDL-cholesterol (12 L and 12 OB on 3 different diets) in cats.

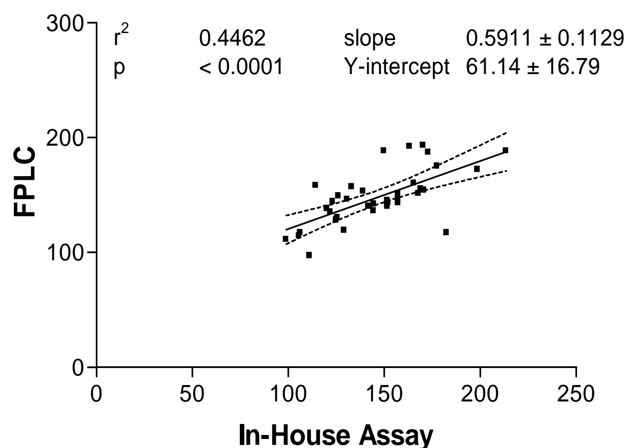

Figure 4. Baseline in-house assay for total plasma cholesterol vs. FPLC determined total plasma cholesterol (mg/dL) in lean (n=12) and obese (n=12) cats.

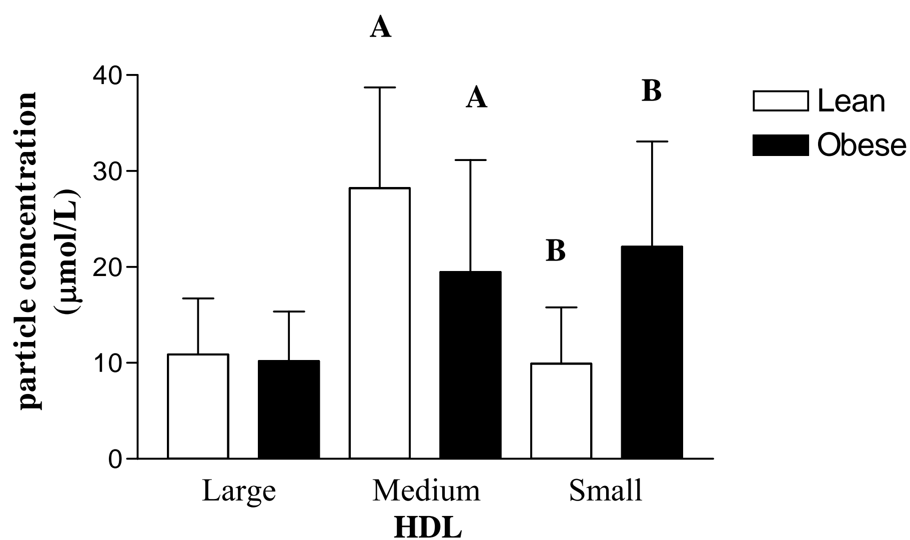

Figure 5. NMR determined HDL particle concentrations (µmol/L) in L and OB cats (n=24, 12 L and 12 OB). (A) and (B) denote significant difference (p = 0.002 and < 0.0001, respectively) between L and OB particle concentrations.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The overwhelming increase in obesity, diabetes mellitus, and cardiovascular disease seen in humans and the increase in obesity and diabetes mellitus seen in veterinary medicine in cats, has led researchers to investigate the changes occurring in lipids and the lipoprotein particles which transport lipids throughout the body and their relationship to these diseases. The purpose of this research was to determine the effects of diet and obesity on lipids and lipoprotein fractions in lean and long-term obese cats and to compare these results using the ultracentrifugation method with that of fast phase liquid chromatography and nuclear magnetic resonance.

We found that obesity does have a significant effect on both plasma lipids and lipoprotein concentrations as well as particle size using these methods. Plasma triglycerides were found to be increased with obesity by all three methods. In the VLDL fraction, all lipid and protein components were found to be increased with obesity, with triglycerides being the highest increase of the components. There were no changes in lipids and protein components in the LDL fraction with obesity, similar to previous results from our lab; however, HDL_2 cholesterol and phospholipids were significantly decreased with obesity, which is different from the previous research in our lab which showed that newly obese cats have significantly higher levels of HDL cholesterol than lean. These changes suggest that at the beginning of obesity the cat produces

more HDL cholesterol, but that over time the levels are actually decreased. A decrease in HDL cholesterol is seen in human obesity and is a risk factor for its comorbidities.

We found that changes in lipoprotein concentrations did not change overall with the type of diet the cats were fed. The most apparent diet effects in the cats were seen after ultracentrifugation in the LDL fraction of lean. Here it was evident that all lipid and protein components were increased with diet C when compared to diets A and B. Obese LDL particle size as determined by NMR showed that diet B had the largest particle size and that diet C had the smallest particle size, and this pattern was mirrored by the LDL particle concentration. The fact that diet C showed the smallest LDL particle size could be due to the fact that this diet contained unsaturated fatty acids. Diet B which was higher in saturated fatty acids exhibited larger LDL particle diameter which is also similar to human studies. The overall lack of significant diet effects in our cats indicates that dietary components have little effect on fasting plasma lipid and lipoprotein concentrations.

Triglycerides and protein were the major plasma components that were affected by feeding in the lean cats. They increased after feeding and returned to baseline concentrations after 12 hours. In the lipoprotein fractions, cholesterol, triglycerides, and total lipoprotein mass increased significantly with feeding. It is interesting to note that these components were still elevated at 12 hours post feeding. It would have been interesting to perform a similar postprandial analysis in obese cats; however, this was not possible because of their different eating pattern. The results of NMR particle concentration and size provides novel information about lipid metabolism in the cat. Our results indicate that obese cats have increased VLDL particle concentrations and particle size when compared to lean cats, which is comparable to what is seen in humans with diseased states such as with coronary heart disease and insulin-

resistance. Regarding LDL particles, obese cats showed much higher concentrations of medium-small and very small-LDL particles as well as significantly decreased LDL particle size, which is also indicated in humans with coronary heart disease and insulin-resistance. Similarities were also seen in the HDL particle concentrations in obese cats, with small HDL particle concentrations being significantly increased in the obese and medium particle concentrations being increased in the lean. It has been shown in humans that decreased HDL particle size is seen in those with insulin-resistance, and our results indicate that obese cats also have significantly decreased HDL particle size when compared to lean cats. We assume that the obese cats in this study were insulin resistant as we have previously shown that insulin sensitivity decreases with an increase in body weight. Based on the results of the NMR determined particle concentrations and size it appears that the cat develops changes in lipoproteins during obesity in a similar way to what is seen in humans with coronary heart disease and insulin resistance. However, despite these changes, obese cats do not develop atherosclerosis, whereas atherosclerosis does occur in humans. This raises the question about additional factors that are necessary for vascular changes to occur.

The comparison of results obtained with ultracentrifugation, FPLC, and NMR indicate they can all be used to measure feline lipids and lipoproteins. Our results indicate that ultracentrifugation has a stronger correlation with FPLC for both plasma triglycerides and HDL-cholesterol, than the NMR technique. In human medicine, ultracentrifugation, NMR, and FPLC have all been shown to correlate even higher; however, a much larger number of samples have been analyzed for humans.

In conclusion, this study describes a comprehensive analysis of lipids and lipoproteins in lean and long-term obese cats using established and novel methodology. The results indicate that

cats are excellent models to study changes in lipid metabolism which occur with obesity. Similar changes are seen in cats and humans, yet, the pathologic consequences are different.

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