LIPOPROTEIN LIPASE DEFICIENCY IN A COLONY OF DOMESTIC CATS

by

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Abstract

I have investigated the molecular basis of lipoprotein lipase deficiency (LPL) in a colony of domestic cats. Members of this cat colony suffer from chylomicronemia and share many of the same phenotypic features as humans with lipoprotein lipase deficiency. Biochemical analysis revealed that these cats have defective LPL catalytic activity suggesting a molecular defect in the LPL gene. Molecular analysis revealed a point mutation resulting in a substitution of arginine for glycine at amino acid residue 412. Segregation analysis, *in vitro* mutagenesis and expression studies, all showed that this DNA change is the underlying cause of LPL deficiency in this cat colony.

Cats homozygous for this mutation have reduced body mass, growth rates and increased stillbirth rates. Body composition analysis showed a reduction of body fat in the LPL deficient cats. Homozygote cats born to a homozygote queen are more profoundly affected than homozygote cats born to a heterozygote queen. The lack of free fatty acids, as a result of lipoprotein lipase deficiency, may limit the maturation of pre-adipocytes. However, the mechanism by which LPL affects body composition is not certain.

LPL deficient cats, have an elevation of serum triglycerides even when maintained on a low fat diet. They also have significant elevations of VLDL-triglycerides, VLDL-cholesterol and decreased LDL-cholesterol. Particle composition analysis indicates that normal cats have a lipid particle composition similar to that of humans with respect to VLDL and LDL. An

oral fat tolerance study indicates that homozygotes have a marked reduction in the clearance of plasma triglycerides and heterozygotes have an intermediate clearance rate.

To study the role of LPL in atherogenesis, normal cats were fed a cholesterol-enriched diet. The vascular tree and organs were evaluated by experienced pathologists to assess the severity of atherosclerotic disease. Serum lipid measurements and vessel wall lesion assessment were elevated in the cholesterol-fed cats, compared to the cats on a normal diet. The lipid profile was correlated with vessel wall measurements. This study provides the basis for evaluating the susceptibility of the LPL-deficient cats to diet induced atherosclerosis.

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Chapter 1: General Introduction

Foreword

In this thesis I have presented a compilation of experiments and data that encompasses the phenotypic observations of lipoprotein lipase (LPL) deficiency in the cat, definition of the molecular defect that results in LPL deficiency in this colony of cats, the delineation of the metabolic consequences of LPL deficiency in the cat and suggested the use of the cat as an animal model for studies of atherosclerosis. The majority of these data are published or have been submitted for publication and will be presented here in a format that reflects these efforts. This introduction consists of several sections that provide the background for the main points of the thesis.

In order to fully explore the many facets of LPL deficiency in the cat and an animal model in general, it was necessary to form several long term collaborations with laboratories that are focused in various related fields. For example, a considerable amount of information was acquired from Dr. Quinton Rogers on matters such as developing special diets, body composition analysis, and animal husbandry in general. In lipid analysis I was assisted by Li Miao in our laboratory as well as by Dr. Jean Dallongeville at the Pasteur Institute in Lille, France. Pathological expertise was contributed by Dr. Bruce and Janet McManus.

A Historical Perspective of the Cat

The domestic cat (*Felis catus* previously called *Felis domesticus)* is thought to have been first domesticated by the ancient Egyptians, perhaps at first as a way to control rodents and pests from destroying their food stores. It can also be argued that at about the same time they were being domesticated in Egypt, they were also being used in the far east for similar purposes (Robinson, 1971). However, because the Egyptians eventually elevated the cat to a "god-like" status in their culture and would go to great lengths to collect and contain these "gods," most people look to the Egyptians as the first people to have domesticated the cat. Once the Egyptians lost control of them, domestic cats quickly spread throughout the Mediterranean and eventually into Europe, and now domestic cats can be found throughout the world.

The exact species of wild cat that became domesticated is not known for certain. It is likely though that it was the African wild cat (*Felis libicus)* that was first domesticated when a few "variants of temperament" were able to tolerate the first agricultural peoples. These "variants" might have found the uneasy alliance with people a profitable one, for they might have been able to thrive in this semi-captive environment in exchange for a steady supply of food (either hand outs and/or feeding on rodents attracted to the food stores). This theory suggests that a mutually beneficial alliance or symbiotic relationship may have been a major factor in the domestication of the cat. However, this would not necessarily preclude these first "variant" cats from mixing with the gene pool of the wild populations, but the need for tameness and a friendly disposition

(important for getting along with people) may have been an important barrier that would have eventually maintained this domestic strain as a separate gene pool, which in turn would lead to the first domestic cat species as separate from the wild species.

Karyotype analysis of the various cat species demonstrates that all members of the cat family have either 36 or 38 chromosomes. The domestic cat has 38 chromosomes as do most other species, except the Geoffrey's cat, Margay, Ocelot and Tiger cat which each have only 36 chromosomes (Robinson, 1971). There have been instances in which lions and tigers have successfully mated and generated offspring, however these offspring "ligers" or "tions" *(sic)* are sterile. This ability to breed even at such extremes helps illustrate how similar the various cat species still are to one another, especially when using the inability to inter-breed as a strict criterion for speciation. Yet the exact definition of what constitutes a separate species has continued to be controversial from the days of Darwin to the present. That no true consensus exists on the reality of what constitutes a species (Dodson, 1985), is certainly exemplified by the felids.

Genetic Lessons from the Cat

The cat has been an excellent animal model used to illustrate some of the basic principles of genetics, from examples of mendelian genetics of simple traits, such as the autosomal recessive nature of LPL deficiency, to X-inactivation, variable expressivity, reduced penetrance and multifactorial traits. The coat of the cat provides excellent examples of these genetic principles (Whiting, 1918). The fact that only the female cat is capable of having a tri-colored mottled coat, termed calico (Searle, 1968) serves to illustrate the

principle of X-inactivation, in which the random inactivation of one of the two female X chromosomes containing the coat color genes results in patches of hair that are characteristic of the tri-colored calico cat. However, there are some very rare exceptions in which males have been found with a calico coat (Bamber, 1932), and likely represents a unusual translocation event or a XXY cat. Piebald spots in the coats also help illustrate the later two genetic principles of variable expressivity and reduced penetrance in that when piebald spots are present (reduced penetrance), there is a very wide range of patterns that can be termed as piebald (variable expressivity). The susceptibly to heart disease is a clear example of a multifactorial trait that is one of the most important traits to affect the longevity of modern humans. It is my hope that the cat will be helpful to study such complex multifactorial disease.

Animal Husbandry of Cats

There are many issues of handling and care of cats that had to be addressed during the course of these studies and it had a profound influence on how various experiments would be or could be conducted, as well as, ensuring the long-term survival of the colony. Some of these issues are unique to LPL deficient cats, others involve the care and handling of cats as an animal model in general.

Cats are social animals with a very intricate social structure (Kleiman and Eisenberg, 1973), that must be taken into account when housing decisions are made. We have maintained our colony of cats in a group format that was adjusted on a regular basis. The facilities at the animal care center here at UBC are spacious and greatly exceed the regulations set forth by any government

regulations. The dimensions of a typical room are approximately 10 feet wide by 10 feet deep with an access door leading to an outside run that is about 10 feet wide by 8 feet deep and is entirely enclosed by a standard wide mesh wire fence.

The cats are typically housed in groups of six, although this has varied widely depending on a number of considerations. Factors that primarily influenced the number of cats per pen included : i) Whether or not it was a breeding situation, in which case a single male would be housed with one or more females. ii) How well a particular group of cats got along with each other, in some instances troublesome males were housed individually as they would often fight with any other males present. This is not an unexpected occurrence and is likely related to the hierarchy of dominant breeding males. Siblings tended to tolerate one another quite well. iii) How well the cats were socialized, with respect to humans and strange cats. In some instances cats that weren't socialized very well would often terrorize other cats and were very difficult to work with. In some instances it was necessary to sedate the troublesome cats just to obtain a blood sample. iv) Expectant and nursing mothers (referred to as "queens') were usually housed in isolation, although there were a few instances when females giving birth at about the same time, and that got along particularly well, were housed together. Other factors influencing the housing arrangements include sickness, special diets and quarantine for newly arrived cats.

Nutrition was of particular concern for the husbandry of our cat colony. The LPL deficient cats represented a unique problem in that their condition, if they were left on a "normal" cat diet (which is often as high as 30% or more fat on a dry matter basis) would put them at risk of complications of severe

chylomicronemia. This was noted first by our colleague, and founder of this cat colony, Dr. Boyd Jones of Massey University in New Zealand, who noted that homozygotes when left on a typical cat diet, would suffer from what he originally thought was Horner's syndrome, due to neurological disturbances as well as eruptive cutaneous xanthomas (Jones et al., 1983). In this case, a group of cats were living on a farm in rural New Zealand and fed, in addition to the small animals they were able to catch, leftovers including scraps of fat or trimmings from any animal that the owners slaughtered for their own consumption (Jones, personal communication).

After Dr. Jones had maintained a small group of these cats for some time on a standard commercial cat food, he observed in those cats that died prematurely, that they seemed to die from renal failure. It was thought to be a complication of the severe chylomicronemia that afflicted these cats (Thompson et al., 1989). It was also found that, on occasion, while on this "semi-feral" diet, the affected cats suffered from a type of peripheral neuropathy that resulted in hind limb paralysis (Jones et al., 1986). In fact it was the apparent neurological problems that prompted the original owners to seek veterinarial assistance which in turn is how Dr. Jones first became aware of these cats (Jones, personal communication).

The cat has evolved as a strict carnivore (MacDonald et al., 1984.) and a high fat high protein diet is normal for a healthy cat. However we needed to minimize as many complications as we could in order to preserve the colony. Therefore, I chose to keep the cats on a commercial diet that was as low in fat as possible. We were limiting one of the main caloric sources for these cats, which was compensated in part by increasing the protein and carbohydrate content.

Normal cats are capable of eating a diet with a high level of protein but carbohydrates are not well tolerated or digested (Quinton Rogers, personal communication). However, it was the best solution possible given the risks of eating a higher fat diet.

One of my first observations was that affected queens were not very good breeders. Also they were typically very lean and did not go into estrus as often as normal queens. When bred, the affected female was not capable of producing an average size litter nor could she produce very much breast milk, if any. To circumvent this problem we would try to cross-foster any kittens born to affected queens to a normal queen, if possible. Fortunately, cats often go into estrus at times of the year in which there is a noticeable change in the light/dark cycle. In our facility, we are not able to control this light/dark cycle (due to the outdoor portion of their pen), usually we find that most females enter an estrus cycle in the fall and in the late winter/early spring. However, on occasion there were no normal females available for cross-fostering, in which case the kittens had to be completely hand-reared. This was a very labor intensive effort that was performed by me and the animal care technician (Karen Holtzman). During this process, the kittens required feeding with a kitten milk-substitute every few hours for the first six weeks of life. We succeeded in maintaining 4 kittens in this manner, but had an equal number die. One complication of hand -rearing kittens, is the danger of aspiration of the milk-substitute by the young kitten, and this was the likely cause of death on at least one occasion.

In a more natural setting, a queen, when bred, will often search out a very secluded area in which to "build" a den for the eventual birthing of her litter. In the more domesticated varieties of cats this will often depend on the cat's

particular living arrangements. In many homes the cat will seek out a dark closet or den under a bed (Bradshaw, 1993). In feral cats it may resemble more that of the wild species. In the wild, cats will often seek out a den in the form of a cave or hollowed out tree trunk during the later part of gestation and not come out to feed for the last few weeks of gestation and the first few weeks of lactation. However, in the wild the queen will usually move to a different den on a regular basis in order to avoid predators (Hale, 1969).

The behavior of the wild cat has particular relevance to the domestic cat in several ways. It provides a more accurate view of the evolutionary circumstances in which the biology of reproduction in the felid family has come to be, and of which the domestic cats are still bound. The normal gestation time for the domestic cats is 65 to 70 days. In the wild, the cats will not eat for about the last 10 to 14 days (Hale, 1969), which puts added stress on the queen in terms of nutrition for the developing fetuses. The pregnant cat will therefore gain weight in a linear manner, unlike most mammalian species which gain weight in accordance with the developing fetus (Latimer, 1936.), whereby she will increase her caloric intake beyond that which is needed by her and her developing offspring early in gestation for use later in gestation. In turn, the queen must then rely on these adipose stores as the sole source of energy to supply her with the energy for her own metabolic needs, as well as the developing kittens needs. This will occur from the later part of gestation, through the first 7 to 10 days of lactation. This will become particularly relevant in the context of LPL deficiency in which the affected queen has reduced adipose tissue stores, and lacks the means to generate an appreciable amount. This will be discussed in greater detail in Chapter 4.

Biochemical History of Lipoprotein Lipase

In 1943, during the course of studying red blood cell mass in dogs, Dr. Paul Hahn stumbled upon what would later be called the "clearing reaction." The clearing reaction was a clearance of alimentary lipemia, due to absorption of a fatty meal, after the intravenous injection of heparin. (Hahn, 1943). It was initially thought to be a physical phenomenon, a result of a physical dispersion or dissolution of chemically-unaltered particulate, and not an enzymatic one. (Robinson, 1987). Another group, after re-discovering Hahn's work about 7 years later, concluded that this clearing reaction was produced by what they termed a "clearing factor" that is released after the administration of heparin (Anderson and Fawcett, 1950). This "clearing factor" was subsequently found to be the enzymatic cause for the clearing reaction (Afinsen et al., 1952). Once established as an enzymatic reaction, studies were initiated to define how this "clearing factor" could remove the large amount of lipid transported in the circulation after a meal. As a result of these efforts, it was determined that this clearance of lipids was the result of hydrolysis of the chylomicron triglycerides present in the lipemic blood by a lipase (Robinson, 1987.) The resulting fatty acids formed a water-soluble complex with plasma albumin and facilitated the clearance of the large water-insoluble chylomicrons.

Lipoprotein lipase is an important enzyme in the regulation of lipoproteins and in lipid metabolism. LPL-mediated hydrolysis of triglycerides (TG) from circulating chylomicrons and very-low density lipoproteins (VLDL) facilitates the production and cellular uptake of fatty acids for energy

metabolism, primarily in muscle and for storage in adipose tissues (Brunzell, 1995). The lipoprotein remnants derived from VLDL and chylomicrons are further processed in the liver and are important contributors to the formation of high-density lipoprotein (HDL) (Patch et al., 1978). As a result, levels of HDL cholesterol are directly correlated with plasma LPL activity in humans (Patsch et al., 1987).

The cellular origin of LPL in the circulation remains uncertain. Although LPL is produced mainly by parenchymal cells such as adipocytes, muscle cells and macrophages, its site of primary function is the luminal surface of the vascular endothelium (Braun and Severson, 1992, and Olivecrona et al., 1993). There it is bound by the heparin-sulfate side chains of membrane glycoproteins. LPL is displaced to the plasma after intravenous injection of heparin, which allows assessment of its *in vivo* activity. Active LPL is a noncovalently-linked homodimer of two 50 kD glycoprotein chains and requires the presence and binding of apolipoprotein CII (Persson, et al., 1989 and Santamarina -Foho and Brewer, 1991).

The LPL protein is only catalytically active in its dimeric form. Other functions have recently been ascribed to the LPL dimer. One such function is the role of LPL as a ligand for receptors in the low density lipoprotein (LDL) receptor family (Beisiegel, et al., 1991). It has now been demonstrated that the LPL protein facilitates the recognition and uptake of lipoprotein into cells via very low density lipoprotein (VLDL), LDL and the low density lipoprotein-related protein (LRP) receptors (Beisiegel, et al., 1991, and Krapp, et al., 1995). The LPL protein has also been implicated, through these interactions with lipoprotein receptors, in the recruitment of lipid-rich lipoprotein particles into

the vascular wall during the formation of foam-cells in the atherosclerotic process (Goldberg et al., 1992).

There is increasing evidence of the relationship between triglyceride rich lipoproteins and progression of coronary artery disease (Koran et al., 1996). However, the role of increased triglyceride levels associated with LPL deficiency on the development of atherosclerosis is yet to be assessed in an *in vivo* experimental system. The availability of an animal model of human LPL deficiency will afford a unique opportunity to assess, *in vivo*, the biochemical features associated with LPL deficiency and to directly assess the relationship between triglyceride rich lipoproteins and atherosclerosis.

Human Lipoprotein Lipase Deficiency

Human lipoprotein lipase deficiency is an autosomal recessive disease with a world wide frequency of about one in a million, however there are instances of founder effects in which this frequency may reach up to one in five hundred (e.g. the Saguenay region of Quebec). The gene for LPL is located on chromosome 8p22 and to date, there have been over 50 mutations defined which alter the lipase activity of this enzyme and have been implicated in familial LPL deficiency (Santamarina-Foho and Brewer, 1991).

The most common presenting symptom is acute abdominal pain and is usually noticed in early childhood (Brunzell, 1995). There currently is no treatment other than maintaining the individual on a very low fat diet. This diet may be supplemented with medium chained triglycerides which are not transported in chylomicrons. Adherence to the diet is not always complete and patients often suffer from periodic episodes of pancreatitis concomitant with eating a fatty meal. This condition is characterized by grossly elevated plasma triglyceride-rich lipoprotein particles, chylomicrons and VLDL. Eruptive xanthomata frequently are found on the thighs and buttocks of affected individuals. It also is accompanied by hepatosplenomegaly and lipemia retinalis.

It has been previously believed that LPL deficiency was not a serious risk factor for the development of early heart disease (Havel, et al., 1960, Fredrickson and Lees, 1965, and Lees, et al., 1973). Recently however, it has been reported that three patients with complete LPL deficiency have developed early vascular disease. However, they also had several other risk factors that could have

contributed to the atherosclerotic process (Benlian et al., 1996). Despite the paucity of examples in which complete LPL deficiency is associated with early heart disease, there is additional evidence accumulating, which suggests that individuals partially deficient in LPL are more susceptible to early vascular disease (Reymer et al., 1995, Jukema, et al., 1996). On the surface it may seem paradoxical that an individual that is partially deficient for an enzyme may be at greater risk than someone who is completely deficient. However, as noted earlier in the introduction, the multifunctional nature of LPL *in vivo* may help explain this apparent discrepancy. The role LPL plays in the recruitment of atherogenic particles at the vessel wall of normal or partial LPL deficient individuals may ultimately be more important to the progression of atherosclerotic lesions than the constant circulation of triglyceride-rich lipoprotein particles in completely deficient individuals. Moreover, the heterozygote state for LPL deficiency may be more deleterious due to the one normally functioning copy of LPL which still participates in the accumulation of lipid-rich lipoproteins at the vascular wall (O'Brien et al., 1992, and Yla-Hertualla, et al., 1991) and the lack of a second allele which delays the clearance of triglyceride rich particles after a meal, which are then available to be taken up at the vessel wall. This delayed clearance of triglyceride rich lipoproteins in heterozygotes in the post-prandial state, which in people eating three meals a day, results in elevated levels of atherogenic lipoprotein particles for a large portion of the day. The delayed clearance would then represent the most serious risk factor for partial LPL deficiency. Of course this is speculative but it represents the basis of a hypothesis that could very likely be tested in an animal model such as the LPL deficient cat.

Chapter 2: A Mutation in the Lipoprotein Lipase Gene is the Molecular Basis of Chylomicronemia in a Colony of Domestic Cats

Foreword

This chapter is mainly comprised of data that has been published in Ginzinger et al., 1996. In this chapter, I have presented most of the phenotypic observations of LPL deficiency in our colony of domestic cats together with the definition of the underlying molecular defect as confirmed by *In Vitro* mutagenesis and expression studies.

At the point in which I began working on this project, it had been previously demonstrated that the post-heparin plasma taken from some individual animals was lacking any detectable LPL enzymatic activity (Peritz, et al., 1990). In addition, both Southern and Northern analysis indicated that there was no gross re-arrangement of the LPL gene and no altered levels of expression of the LPL mRNA in adipose tissue. It should also be understood that the majority of data presented in this chapter was generated by me. The data in this chapter that was generated, in part, by other members of our laboratory is limited to the fast protein liquid chromotography (FPLC) profile which was performed by Guoqing Liu, basic lipid measurements and LPL activity measurements were conducted by Li Miao, expression studies in the mammalian COS-1 cell line with the normal and affected cat LPL constructs

were designed by me and in part conducted by Sarah Jones under my supervision.

Introduction

In this Chapter, I present the initial experiments that were conducted to identify a causative mutation in the cat LPL gene. The cats in our colony were previously tested for post-heparin lipolytic activity (Peritz, et al., 1990) and it was thought that the absence of LPL activity was the result of a mutation in the LPL gene, similar to findings in our laboratory on human type I patients. This hypothesis was likely to be correct, due to the numerous mutations that were identified in the LPL gene in human type I patients and the relative paucity of other factors that could also result in this apparent loss of lipolytic activity. These other possibilities include ApoCII deficiency or a lipolytic inhibitor that might be present in the plasma of these cats, as well as the possibility that the LPL assay used was not able to detect LPL activity in the cat.

At the time I started testing this hypothesis, there were about 30 different mutations known to alter the function of the LPL gene in humans, and only the rare case of ApoCII deficiency (Brunzell et al., 1995). Furthermore, the affected cats certainly had a chylomicronemic phenotype that would suggest a lipolytic deficit and to the best of my knowledge there had not been a documented case of a circulating inhibitor of lipases, let alone one that can act specifically on LPL (Hepatic lipase is normal in these cats). In addition, the LPL assay has been routinely used for measuring the LPL activity in the mouse and rabbit as well as several other species. Therefore, the most likely cause of LPL deficiency in these cats was clearly a genetic alteration of the LPL gene.

Previous studies conducted in our laboratory, prior to my arrival, suggested that this putative genetic alteration wasn't the result of a gross rearrangement of the LPL gene, as detected by Southern blot hybridization, nor was it due to a gross change in the level of expression of, or size of the LPL transcript, as detected by Northern blot hybridization.

In this section I will begin to describe many of the phenotypic features of the LPL deficient cats which demonstrate the similarities to the human condition as well as illustrate the differences that are unique to the cat. Many of these observations are expanded upon in subsequent chapters but this will provide the basis of the description of the LPL phenotype in the cat.

Methods

Animals

The animals in this study are members of a colony of domestic cats that have been maintained at the animal care unit at UBC. These cats have been fed a standard commercial diet, either Hill's "prescription diet - Feline r/d" or "Whiskas with tuna" both of which have no more than 12% fat. All cats were fed *ad libitum* on both the wet and dry foods.

Plasma Lipid Analysis

Blood was obtained through the jugular vein of sedated animals (0.1 ml Ketamine/kg body weight) anticoagulated with EDTA and spun at 2000 rpm for 15 minutes in a standard table-top centrifuge. Plasma was removed for lipid determinations. Total cholesterol and total triglycerides were determined enzymatically using commercially available kits (Boehringer Mannheim, FRG.). HDL cholesterol levels were determined after precipitation of other lipoproteins with heparin manganese. LDL cholesterol levels were calculated according to the following formula: Total cholesterol minus (HDL cholesterol + Triglycerides$/2.2$). FPLC analysis was conducted with a Pharmacia LKB LCC 501 plus controller and model 500 pump using a Sephadex 6 column, using standard methods(Hara, et al., 1986).

LPL Enzymatic Activity and Immunoreactive Mass

A blood sample was taken prior to, and 10 minutes after the administration of sodium heparin (100U/kg) for determination of LPL mass and activity. LPL protein mass and lipolytic activity was determined as previously described (Peterson, et al., 1992, Iverius et al., 1985). In brief, LPL activity was calculated as the fraction of total lipolytic activity that can be blocked with the addition of a monoclonal antibody (5D2) that binds to the LPL protein rendering it inactive, or by the addition of 1M NaCl which preferentially inactivates LPL. The remaining lipolytic activity is attributed to hepatic lipase. LPL protein mass was determined by ELISA using the monoclonal antibodies 5F9 and 5D2 as previously described (Peterson, et al., 1992, Babirak, et al., 1989).

Northern Analysis

Total RNA was isolated from 12 tissue types taken immediately after the sacrifice of cats, and prepared using the standard acid-guanidium isothiocynate method of Chomcznski and Sacchi (1987). Approximately 10 ug of total RNA from each tissue was loaded per lane on a 1% formaldehyde gel and run overnight at 30 volts. The RNA ladder was cut from the gel, stained with ethidium bromide and photographed to help estimate the size of the transcripts. The gels were soaked in DEPC-treated water for about 2 hours prior to blotting overnight. The blots were baked at 80 ^{0}C for 2 hours then pre-hybridized in Church buffer with 1% BSA added, for 2 hours. The Northern filters were hybridized with a random primer labeled (Feinberg and Vogelstein, 1983) normal cat *LPL* cDNA overnight at 60 ^{0}C. The filters were washed in 2 X SSC and 0.1% SDS for 30 min. at room temperature then washed 2 times in 0.5 X SSC

plus 0.1% SDS at 60 ^{0}C for 30 minutes each. Filters were then exposed to Kodak XAR5 autoradiographic film for 1-3 days at -70 ^{0}C.

Cloning and Sequencing of the Normal Cat LPL Gene

Initial screening of the normal feline cDNA library identified a partial cDNA clone for the feline LPL gene using a human LPL cDNA clone as a probe (Wion, et al., 1987). This clone contained an insert of about 900 bp, which showed significant similarity to the human LPL gene. A full length LPL clone was not found in this library. Therefore, I used a rapid amplifcation of cDNA ends (RACE) method (Frohmann, 1990) to obtain the sequence in the 5′ region of the feline LPL gene. The RACE-PCR products obtained were blunt end ligated into the Bluescript k/s vector (Stratagene, La Jolla CA.) and sequenced by the dideoxy chain termination method (Sanger et al., 1977) (USB, Cleveland OH).

In addition, I designed a set of PCR primers based on a high degree of sequence similarity between the promoter region of mouse and human LPL genes (Zechner, et al., 1991). The 5' end primer (N25) was designed at the position -83 to -57 in the mouse LPL gene (which is completely conserved in the human gene). A second primer (N24) was synthesized complementary to the 5' UTR primer in the promoter region. A third primer (N26) 3' from primer N24 was used to prime a reverse transcribed cDNA copy from total RNA isolated from adipose tissue of a normal cat and mouse. These cDNAs were amplified using the 5' primer and primer N24 with the following conditions; 1.5 mM Mg Cl, 75 mmol dNTP's, 50 pmol primer 5' UTR and N24, 1% formamide in 100 µl reaction. Cycle parameters used for a Perkin Elmer Model 480 thermocycler were: 35 cycles at- 94° C for 1', 67° C for 1', 72° C for 2', with a final extension of 10

min. at 72° C. The PCR products were sequenced using a thermocycle sequence kit (NEB, Beverly MA.) and was confirmed to be specific to the cat and mouse LPL sequences.

Cloning and Sequencing of the Affected Cat LPL Gene

In a manner similar to the method used to generate a full length clone from the normal cat, RT-PCR was used to generate a copy of the affected cat LPL gene from adipose tissue RNA. A primer (N22) specific to the 3' end of the feline LPL gene was used to generate a reverse transcribed cDNA copy. This single strand copy was used as the template for PCR amplification with gene specific primers that span the coding region of the normal cat LPL gene. These PCR products were then used to generate single strand sequencing template for asymmetric sequencing in which one primer is limiting (1/100 the concentration of the other primer) resulting in a linear amplification of a single strand product suitable as a sequencing template. Using asymmetric PCR to generate sequencing template, the normal and affected cat LPL cDNAs, were sequenced on both strands, in duplicate, with the dideoxy chain termination method (Sanger, et al., 1977) using reagents from USB (Cleveland OH).

***In Vitro* Mutagenesis and *Cos*-1 Cell Transfections**

The human LPL gene was ligated, in the sense orientation, into the phagemid vector CDM8. The ligated recombinant DNA was transfected into MC1061/p3. Purified CDM8-LPL DNA was then transfected into a dut(-) ung(-) host (BW313/p3/F') to generate a uracil-containing clone (Zoller, et al., 1987). These single-stranded uracil-containing clones were recovered by

superinfection of the host with a helper phage (R408, Stratagene) needed to package the construct. The mutant clones were identified by oligonucleotide hybridization and verified by DNA sequencing. Expression of the mutant clones was conducted by transfection of COS-1 cells by electroporation according to previously described protocols (Henderson, et al., 1991). The cell-free medium was used to measure LPL activity.

The normal and affected (Gly412Arg homozygote) cat LPL cDNAs were obtained using standard RT-PCR techniques (Beverley, 1991). These products were sequenced twice on both strands in order to ensure that there were no Taq. polymerase errors that would result in a defective protein. Once they were confirmed, they were cloned into the pcDNA3 expression vector (In Vitrogen, San Diego, CA). These constructs were then used to transfect Cos-1 cells as per previously (Henderson, et al., 1991). The cell-free medium was used to measure LPL activity.

Assay for the 412 Mutation and Segregation Analysis

The Gly412Arg mutation does not alter any known restriction endonuclease recognition sites. However, based on the similarity of organization between the human and mouse LPL genes (Zechner et al., 1991), the nucleotide substitution at codon 412 is located 16 bases 5′ of the predicted 3' end of exon 8. In order to develop a PCR based screening protocol, sequence from intron 8 of the cat LPL gene was required. This was facilitated by PCR amplification using primers flanking exon 8. The resulting PCR product was directly sequenced by thermocycle sequencing techniques (NEB, Beverly MA). Once the intronic sequence was determined, a primer (I8-2) was developed in the intron that, in

conjunction with a mismatch primer (MM-SB) in the exon, would produce an amplification product amenable to separation on a standard agarose gel after digestion at the restriction site introduced in the mismatch primer. A fragment of 135 bp was selected to facilitate the resolution of the digestion products upon electrophoresis in a 3% agarose gel. The mismatch introduced in primer MM-SB will introduce a new restriction endonuclease recognition site (BstNI) only on the normal allele. Amplification conditions are: 1.5 mM Mg Cl, 75 mmol dNTP's, 50 pmol primer N23 and I8, 0.5 units Taq. Polymerase in 100 ul reaction volume. Cycle parameters used for a Perkin Elmer Model 480 thermocycler were: 35 cycles at- 94° C for 1', 67° C for 1', 72° C for 45". After PCR amplification of genomic DNA from members of the cat colony, the amplification products are digested with the BstNI restriction endonuclease according to manufactures recommendations (NEB, Beverly, MA). The digestion of these products results in the cleavage of 30 base pairs of the 135 base pair product only on the normal allele which is then resolved on a 3.0% agarose gel. This mismatch analysis provides a simple method to determine the genotype of each animal in the colony.

Growth Curves and Stillbirth Rates

All kittens were maintained on a similar diet from the weaning period through adulthood. The homozygous kittens were cross-fostered at day 2 by a normal queen, due to the homozygous queen's reduced ability to lactate. Each kitten was weighed daily, weights were averaged for each genotype and plotted as a function of time.

Stillbirth rates were tabulated from the entire breeding colony of LPL deficient cats at UBC and in New Zealand as well as those bred for other investigators at UBC. After the queening of a new litter the number of live offspring and the number of dead offspring were recorded. Affected queens are homozygous for the Gly412Arg mutation. The stillbirth rate is calculated as the number of stillborn offspring over the total number born and is expressed as a percentage.

Results

Isolation of the full length feline LPL cDNA

A clone was previously identified and contained an insert of about 900 bp, which is approximately 500 bp shorter than the full length human cDNA clone. To obtain the full length sequence, a 5' RACE (Frohmann et al., 1990) procedure was performed which provided all but 125 nucleotides from the predicted 5' end of the normal cat gene. To derive sequence from the 5' end, a PCR primer (N25) was developed based on the high degree of sequence homology between the promoter region of mouse and human LPL clones (Zechner et al., 1991). The anti-sense oligonucleotide primer was based on the sequence of the normal cat LPL cDNA and was positioned downstream of intron one so that a PCR product generated would be the result of amplification of cDNA not genomic DNA. Together, these oligonucleotides were used to generate PCR products from reverse transcribed adipose tissue RNA and the resulting product was of the expected size and also hybridized to the human LPL gene. The complete coding region plus approximately 200 bp of 5' UTR was generated for the normal cat LPL cDNA.

Sequence Analysis

The cat cDNA sequence contains 1512 nucleotides and includes a predicted open reading frame of 451 amino acid residues (Figure 2.1). The sequence includes 34 nucleotides of the 3' untranslated region and 125 bp of 5' UTR. A three amino acid insertion at the beginning of the predicted mature

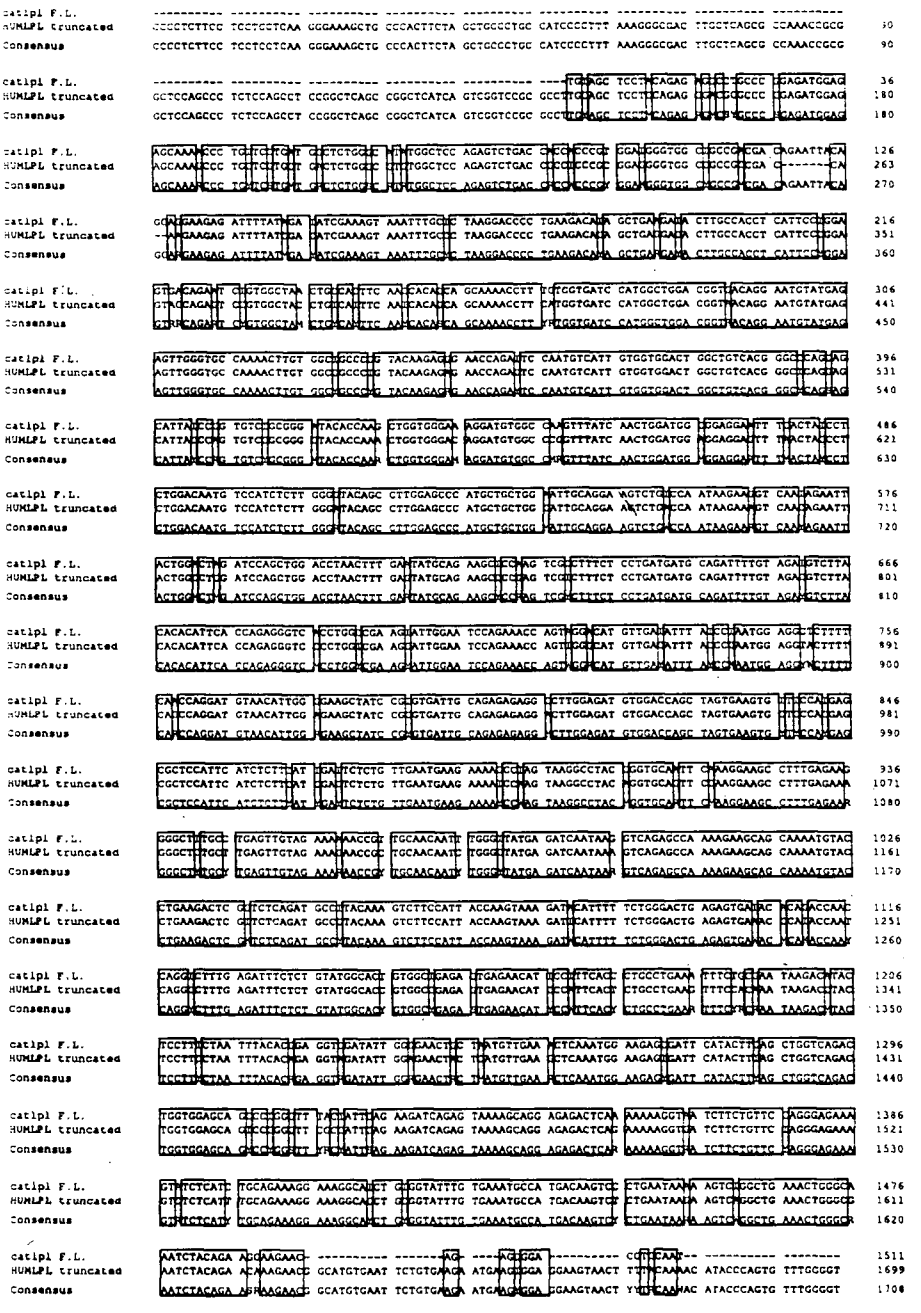

Figure 2.1. Nucleotide sequence comparison between cat and human cDNAs. This indicates the high level of similarity (90%) between these two species, which is the highest level observed of all the LPL cDNAs that have been published to date. The cat is likely to use a similar ATG start site as the human. The cat sequence contains an insertion of 9 nucleotides (nucleotides 74-85 in the cat sequence) at the beginning of the predicted mature peptide, as seen in the bovine. The cat nucleotide sequence predicts a 451 amino acid peptide of about 50 kD. Nucleotide sequence of the cat has been submitted to GenBank acc. # U42725, the human sequence is taken from the published cDNA18 under GenBank acc. # M15856.

insertion at the beginning of the predicted mature peptide of the cat cDNA is present, similar to bovine LPL (Senda et al., 1987) but not observed in the human LPL cDNA (Wion, et al., 1987).

Nucleotide and amino acid sequence comparison between the human and cat LPL cDNAs reveals nucleotide and predicted amino acid similarity of 90% and 94.5% respectively. Comparison of the predicted amino acid sequence of LPL from mouse (Kirchgessner, et al., 1987), rat (Brault, et al., 1992), bovine (Senda, et al., 1987) and guinea pig(Enerbach, 1987), reveals about 85% identity to human LPL cDNA. Chicken LPL contains 15-17 additional C-terminal residues and 73-77% sequence identity with mammalian LPL's, (74% with human) (Cooper et al., 1992).

Sequence comparison of the normal and affected cat cDNAs identified a single DNA change (G to A transition) at position 1234 of the cat LPL gene which corresponds to amino acid residue 412 in the cat (409 in the human LPL cDNA) and results in a substitution of arginine for glycine (Figure 2.2). The identification of the Gly412Arg substitution was the only difference identified between the normal and affected cat LPL cDNAs. This glycine residue is highly conserved across species and within members of the lipase gene family (Figure 2.3). The paralogous residue is also conserved in the human (Datta et al., 1988, Stahnke et al., 19987, and Cai, et al., 1989) and rat hepatic lipase (Komaromy, et al., 1987) (HL) and the canine pancreatic lipase genes (Mickel, et al., 1989).

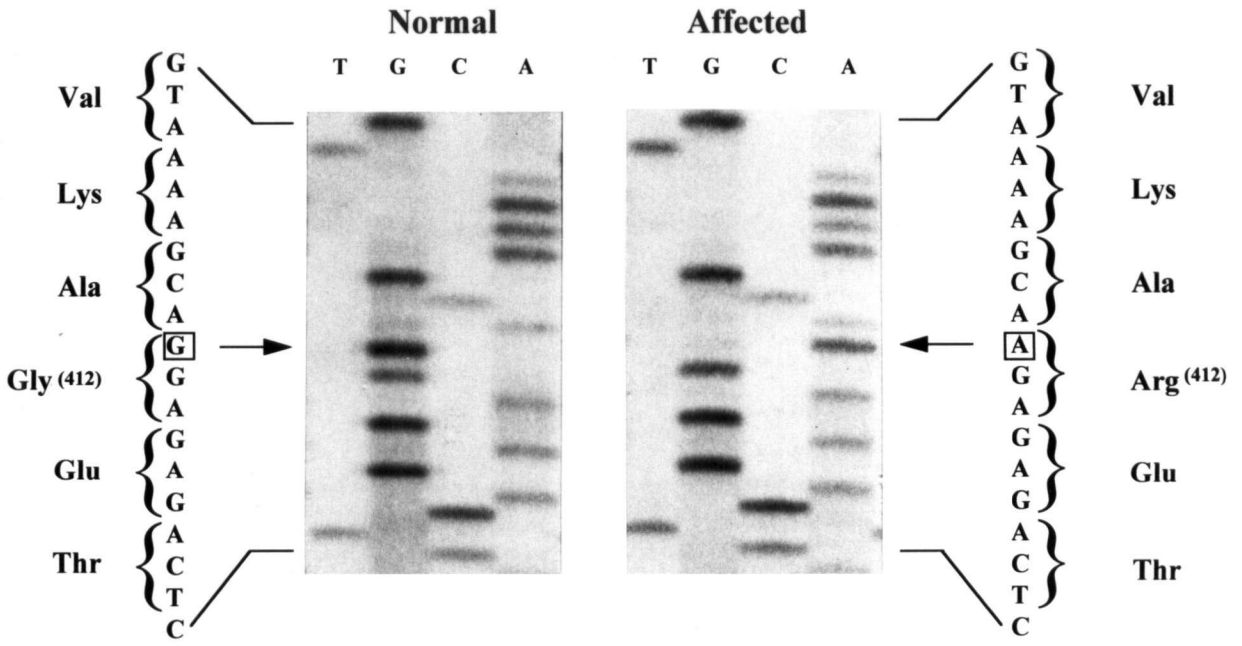

Figure 2.2. Nucleotide sequence of a region of exon 8 of the normal and affected cat cDNAs showing a G to A transition at position 1234 resulting in a substitution of arginine for glycine at residue 412 in the C-terminus of the LPL protein.

						(409)				
Human LPL	Ile	Arg	Val	Lys	Ala	Gly	Glu	Thr	Gln	Lys
Normal Cat LPL	*	*	*	*	*	*	*	*	*	*
Mouse LPL	*	*	*	*	*	*	*	*	*	*
Bovine LPL	*	*	*	*	*	*	*	*	*	*
Rat LPL	*	*	*	*	*	*	*	*	*	*
Guinea Pig LPL	*	*	*	*	*	*	*	*	*	*
Chicken LPL	Val	*	*	*	Ser	*	*	*	*	Gln
Human HL	*	*	*	*	*	*	*	*	*	Gln
Rat HL	*	Trp	*	*	*	*	*	*	*	Thr
Dog PL	*	Thr	*	Gln	Lys	*	*	Glu	Lys	*
Affected Cat LPL	*	*	*	*	*	Arg	*	*	*	*
						(412)				

Figure 2.3. Amino acid sequence comparison as predicted from the LPL cDNA sequences of seven species and two hepatic lipase and one pancreatic lipase gene family members. The residue that corresponds to 412 in the cat (Glycine) is conserved in all species and in hepatic lipase and pancreatic lipase genes, suggesting that this residue in important for the functioning of these lipases.

Northern Analysis

The human LPL transcript consists of two mRNA species of about 3.3 and 3.7 kb that arise from alternative sites of 3' terminal polyadenylation (Braun, et al., 1992 and Wion et al., 1987). Similarly, the cat LPL gene demonstrates multiple transcripts with a primary transcript of 3.4 kb and in addition two other transcripts of about 8 kb and 1.6 kb (Figure 2.4).

Multiple mRNA transcripts have been seen in all other species except the rat, which has a single 3.6 kb transcript (Kirchgessner, et al., 1987 and Semenkovich , at al., 1989). The bovine cDNA detects a 1.7 kb transcript in addition to a primary transcript of 3.6 kb (Senda et al., 1987). Northern analysis of a wide spectrum of tissues sampled from normal, heterozygous and homozygous LPL-deficient cats reveals no differences in transcript size and levels of expression between genotypes (Figure 2.4). The most abundant feline transcript is seen in the heart, with lower levels of expression in other tissues including skeletal muscle, adipose, mammary gland, ovary, pancreas, hind-brain, kidney, lymph node and lung. LPL mRNA was not observed in bone marrow, liver and frontal cortex. These studies reveal that this mutation does not induce any obvious changes at the transcriptional level.

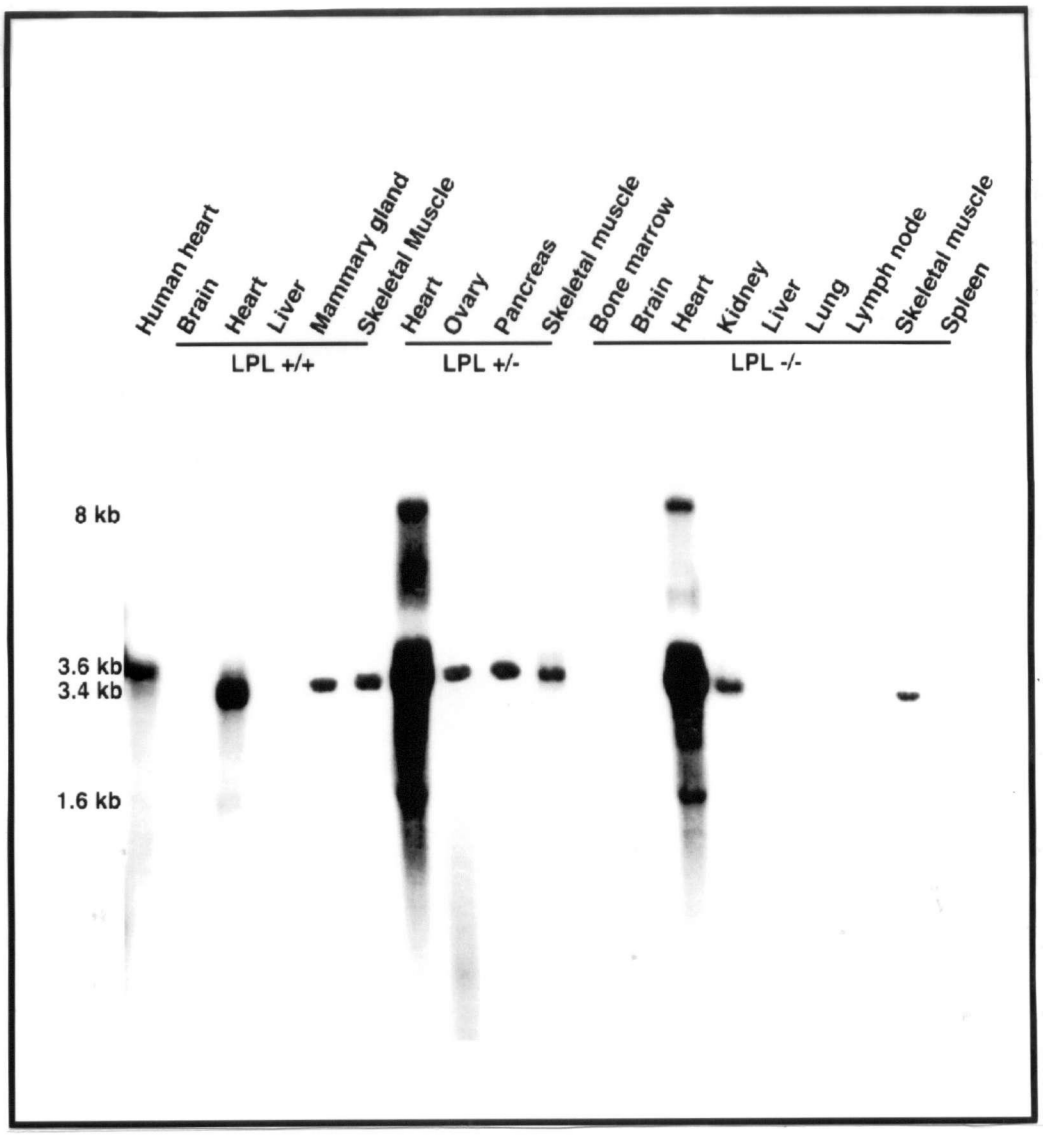

Figure 2.4. Northern analysis of total RNA from 12 different tissues from cats of all genotypes for the Gly412Arg mutation. The total RNA concentration in LPL +/+ heart was approximately half that of all other lanes as determined by hybridization with an b-Actin probe (data not shown). There is no alteration in transcript size or relative level of expression in cats of different genotypes. The highest level of expression occurs in the heart. LPL mRNA is also seen in: skeletal muscle, mammary gland, ovary, pancreas, hind-brain, kidney, lymph node and lung. The primary transcript is 3.4 kb with two other transcripts of about 8 kb and 1.6 kb.

Phenotypic Characterization of Cats with the Gly412Arg Mutation

These cats homozygous for the Gly412Arg mutations manifest with a phenotype similar to that seen in humans with LPL deficiency including lactescent plasma due to chylomicronemia, peripheral xanthomata, lipemia retinalis and failure to thrive (Table 2.1) (Figure 2.5). Biochemical analysis has revealed that cats homozygous for the Gly412Arg mutation lack both detectable immunoreactive LPL mass and catalytic activity (Table 2.2). Similar to the mouse, the cat has most of its total cholesterol in the form of HDL (Chapman, 1980). However, the cat has distinct HDL_2 and HDL_3 subfractions similar to that of human (Demacker, et al., 1987.) but not observed in the mouse. Lipoprotein analysis of affected cats revealed markedly elevated triglyceride levels when fed a low fat diet (12% fat) ($p< 0.05$) (Table 2.3). Heterozygous male cats also had significantly ($p< 0.05$) higher triglyceride values than normal cats. FPLC analysis of plasma lipoproteins of male homozygotes compared to normal siblings revealed an increase in VLDL triglyceride, consistent with a defect in LPL catalytic activity (Figure 2.6). For cats of each genotype and sex, the total cholesterol (TC) to HDL-C ratio was measured and found to be elevated specifically in homozygotes for this mutation (Table 2.3).

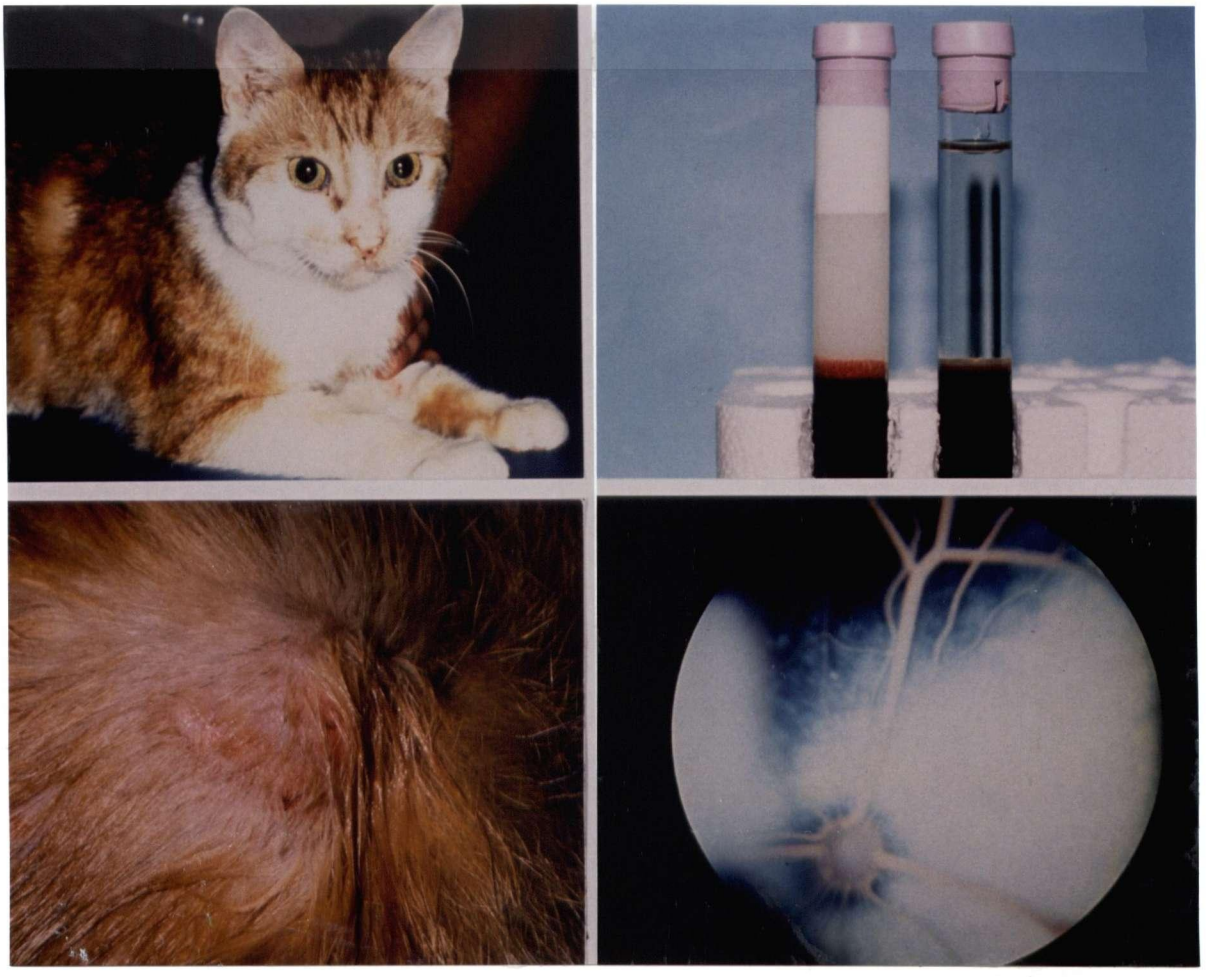

Figure 2.5. A cat homozygous for the Gly412Arg mutation with no immediately obvious phenotypic abnormalities when fed a normal diet (upper left). Overnight fasting plasma samples (upper right) indicate obvious chylomicronemia in the LPL deficient homozygote cat (left) compared to normal (right). A subcutaneous xanthoma on a LPL deficient cat, similar to that seen in affected humans (lower left). Retinal examination reveals obvious lipemia retinalis in the affected cat (lower right).

Phenotypic features of lipoprotein lipase deficiency in humans and the cat.

	Human	Cat
Clinical features	Chylomicronemia Cutaneous Xanthomata Lipemia retinalis Pancreatitis Hepatosplenomegaly Failure to thrive	Chylomicronemia Cutaneous Xanthomata Lipemia retinalis Failure to thrive
Inheritance	Autosomal recessive	Autosomal recessive
Genetic defect	> 50 different mutations in the LPL gene	G412R mutation in the LPL gene

Table 2.1. Phenotypic features of lipoprotein lipase deficiency in humans and the cat.

LPL mass and activity according to genotype in LPL deficient cats (mean ± SD)

Genotype	LPL Mass*	LPL Activity **
	ng/ml	mU/ml
LPL -/- (n=5)	7 ± 10	0
LPL+/- (n=10)	62 ± 18	84 ± 31
LPL+/+ (n=8)	151 ± 23	272 ± 70

* as detected by monoclonal antibody 5D2

** 1 nmole/ffa/min = 1mU/ml

Table 2.2. LPL mass and activity levels in the LPL deficient and normal cats (mean ± S.D.).

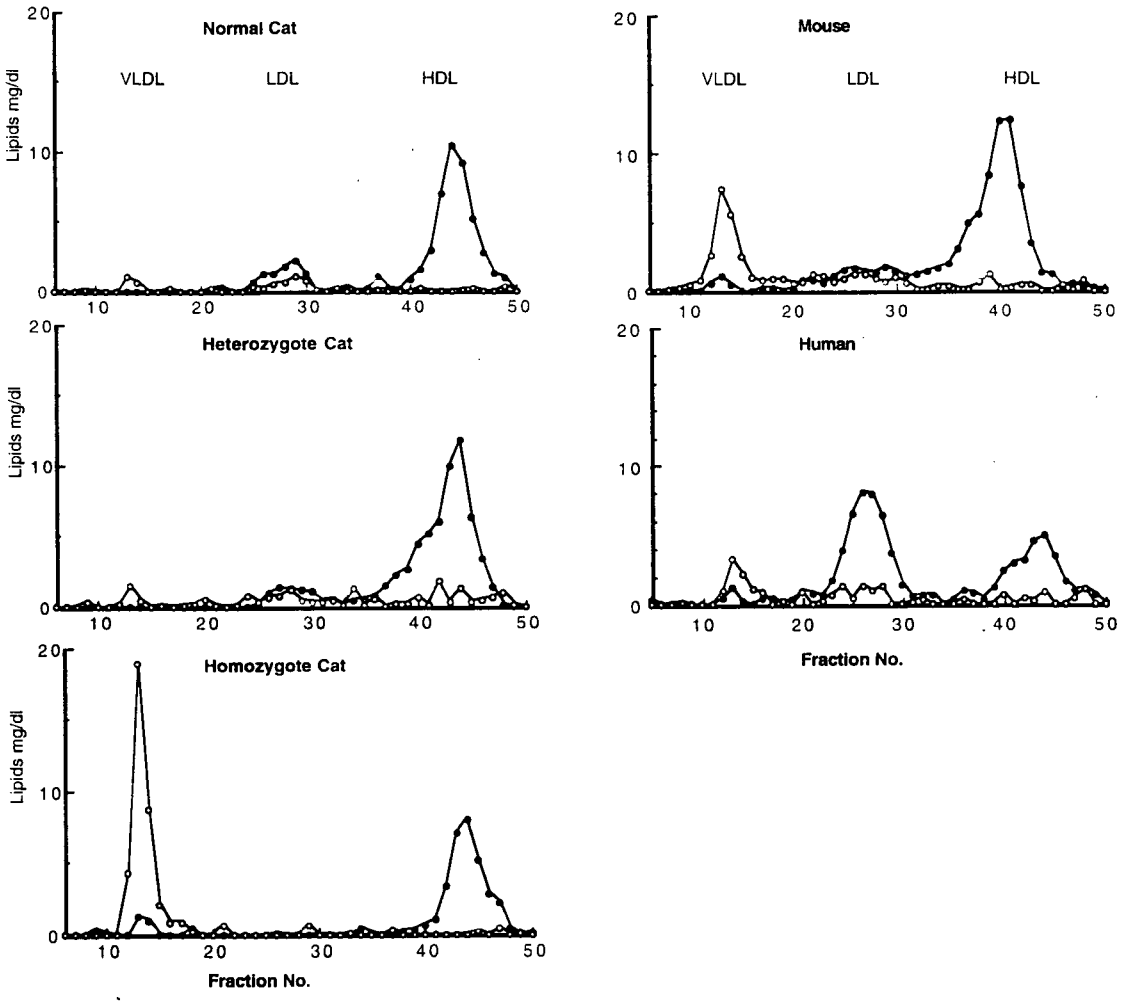

Figure 2.6. An FPLC profile from a normal, a heterozygote and a homozygote cat for the Gly412Arg mutation. --•-- represents the amount of cholesterol per fraction (mg/ml), while the --o-- notation represents the amount of triglyceride per fraction (mg/ml). Plasma samples were taken after an overnight fast and subjected to FPLC using two Superose 6 columns. Significant triglyceride peaks in the homozygote cat are evident. HDL cholesterol levels are reduced in the homozygote compared to normal. Comparison of the normal cat FPLC profile to that of the human and mouse indicates that the cat has a lipid profile similar to the mouse.

Fasting feline lipid values on a low fat diet (12%)

mmol/l	Triglycerides	Total Chol.	HDL-chol.	LDL-Chol.	TC / HDL-C Ratio
Male	mean	mean	mean	mean	
Homozygotes (n=4)	6.78 ± 3.68 **	4.37 ± 3.16	2.55 ± 0.49	0.00	1.71
Heterozygotes (n=8)	0.27 ± 0.14*	4.41 ± 1.46	3.40 ± 0.97	0.65 ± 0.37	1.30
Normal (n=6)	0.12 ± 0.10	3.33 ± 0.53	2.59± 0.37	0.75 ± 0.35	1.29
Female					
Homozygotes (n=3)	5.87 ± 2.58**	2.98 ± 1.48	2.12 ± 0.47	0.00	1.59
Heterozygotes (n=4)	0.26 ± 0.13	3.15 ± 0.48	2.68 ± 0.46	0.48 ± 0.21	1.18
Normal (n=3)	0.24 ± 0.17	2.43 ± 0.30	2.00 ± 0.31	0.51 ± 0.03	1.22

* = $p < 0.05$ compared to normal

** = $p < 0.05$ compared to normal and heterozygotes

all other comparisions are not significant

Table 2.3. Fasting lipid values of cats of each genotype while maintained on a low fat commercial cat food (~12% fat by wt.).

Cats homozygous for the Gly412Arg mutation demonstrate diminished growth rates and an increased rate of stillborn offspring. A cohort of male cats consisting of five normal, eight heterozygotes and four cats homozygous for the mutation were weighed daily, with weights averaged for each genotype and plotted as a function of time (Figure 2.7). Homozygous kittens have significantly reduced weights at birth ($p < 0.01$), at 20 weeks ($p < 0.001$) and 36 weeks ($p < 0.0001$) of age relative to both normal and heterozygous kittens, while consuming the same low fat diet.

In addition, kittens born to females homozygous for the gene defect have a significantly increased rate of stillbirth compared to offspring from matings of normal or Gly412Arg heterozygous females ($p < 0.002$) (Table 2.4). Normal (LPL +/+) females mated with male Gly412Arg homozygotes resulted in no stillbirth among 16 offspring. In contrast, matings between homozygous LPL-deficient females and heterozygote males revealed 13/32 stillbirths (no wild type males were used because we were tring to increase the number of affected cats in the colony). These findings suggest that the LPL genotype of the pregnant female is most likely the contributing factor to this increased stillbirth rate, although the genotype of the kitten might also have contributed.

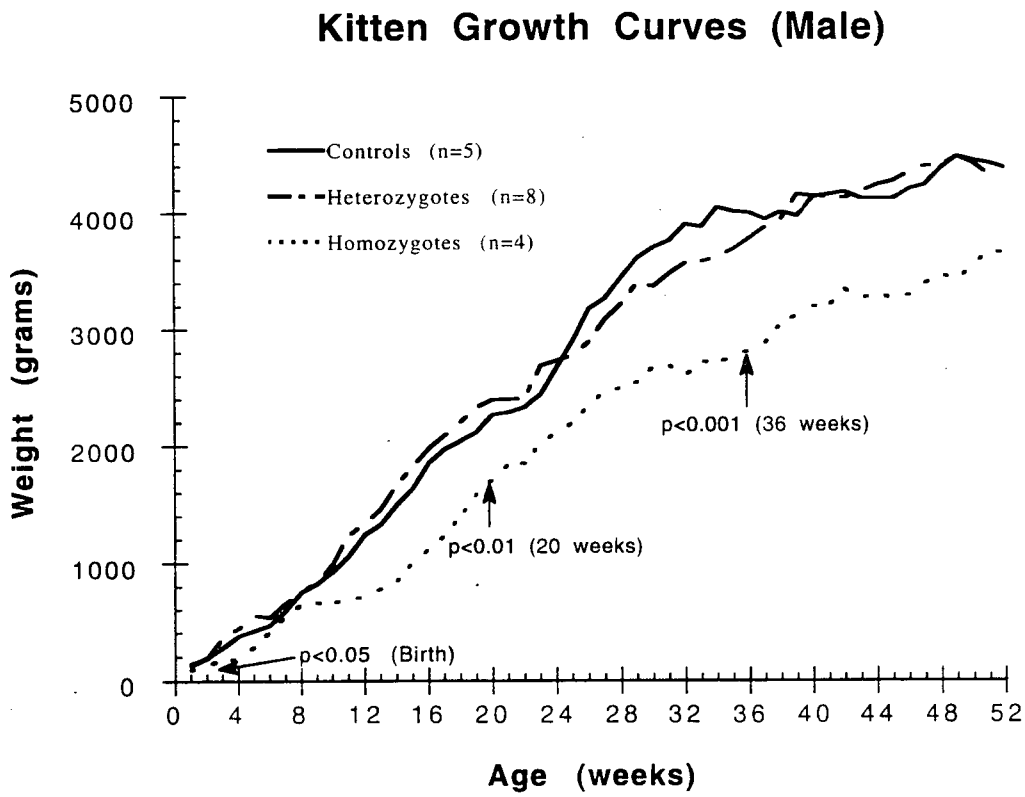

Figure 2.7. LPL deficient kittens have reduced birth weights and maintain a reduced growth rate into adulthood.

		Frequency of Stillbirths (%)	
		Males	
		Heterozygotes	Homozygotes
	Normal	1/16 (6%)	0/16 (0%)
Females	Heterozygotes	5/35 (14%)	0/7 (0%)
	Homozygotes *	13/32 (41%)	3/8 (37%)

* $p < 0.002$ compared to normal Queens

Table 2.4. Frequency of stillborn kittens born to Queen's of each genotype and sired by either heterozygous or homozygous males. The number of stillbirths is expressed as a percentage of total kittens born , numbers in parenthesis.

Functional Assessment of Gly412Arg Substitution in the Feline LPL Gene

The glycine in position 412 of the cat gene is equivalent to glycine at position 409 in the human gene (Wion, et al., 1987). Using site directed mutagenesis this mutation was recreated in the human cDNA. In addition, using standard RT-PCR techniques, we isolated both a normal and affected cat LPL cDNA. Expression studies after transfection of *Cos*-I cells, which don't normally express a measurable amount of LPL activity, revealed that LPL catalytic activity was completely abolished due to this substitution using both the human and cat constructs. (Table 2.5).

This particular mutation does not alter any restriction endonuclease recognition site. Nonetheless, the development of a PCR-based mismatch primer approach allowed direct detection of the Gly412Arg mutation (Figure 2.8). Analysis of this mutation in 50 members of this cat kindred revealed in all instances that those cats (n=11) with chylomicronemia and complete LPL deficiency were homozygotes for the Gly412Arg mutation, while heterozygotes (n=23) had an intermediate biochemical phenotype (Figure 2.9).

In Vitro Mutagenesis and Expression Studies

	LPL enzymatic activity mU/ml (mean ± S.D.)
Normal Cat LPL cDNA (n=5)	39.73 ± 6.4
Affected Cat LPL cDNA (Gly412Arg) (n=5)	0
Normal Human LPL cDNA (n=3)	129.38 ± 24.2
Gly409Arg Human LPL cDNA (n=3)	0

Table 2.5. *In Vitro* mutagenesis of the normal cat or human LPL constructs with either codon 412 mutated in the cat construct, or codon 409 in the human constructs, respectively. Constructs were expressed in COS-1 cells and medium was collected for enzymatic activity measurements.

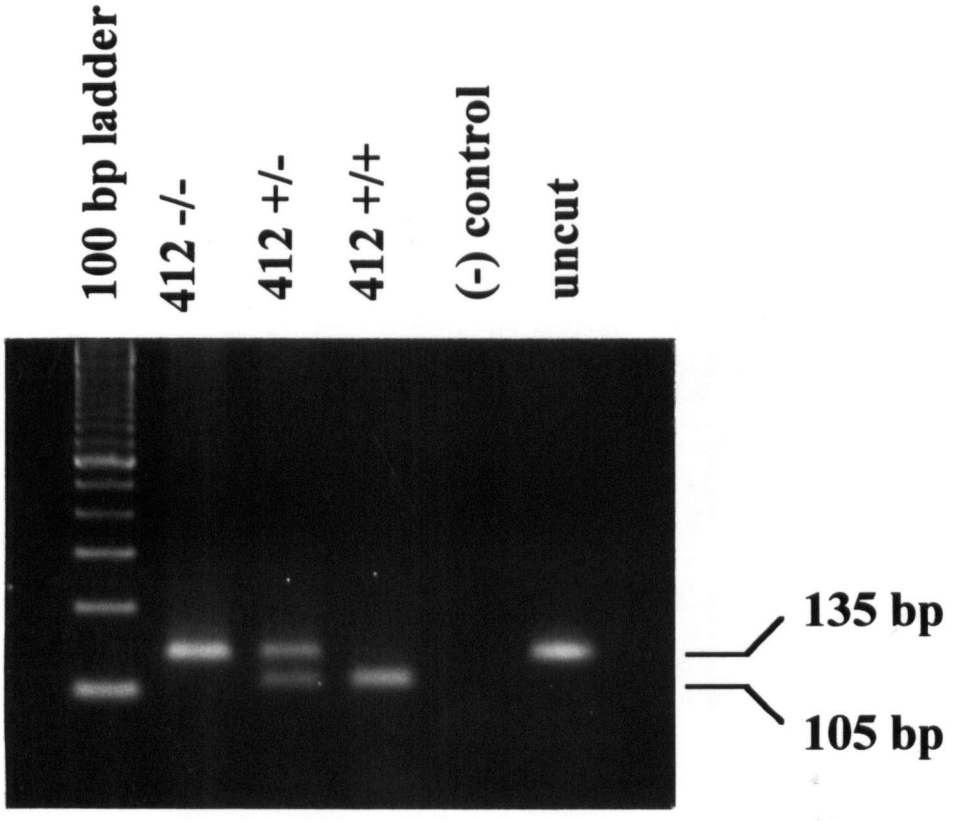

Figure 2.8. PCR mismatch analysis of the Gly412Arg mutation. After amplification of genomic DNA from cats of each genotype, the PCR product was digested with the restriction enzyme BstNI for 2 hours at 50 ^{0}C and resolved on a 3% agarose gel. Those alleles carrying this mutation are not digested with this enzyme and yield a 135 bp band (412-/-). The alleles that contain the normal sequence are digested by this enzyme and produce a band that runs at 105 bp (412+/+).

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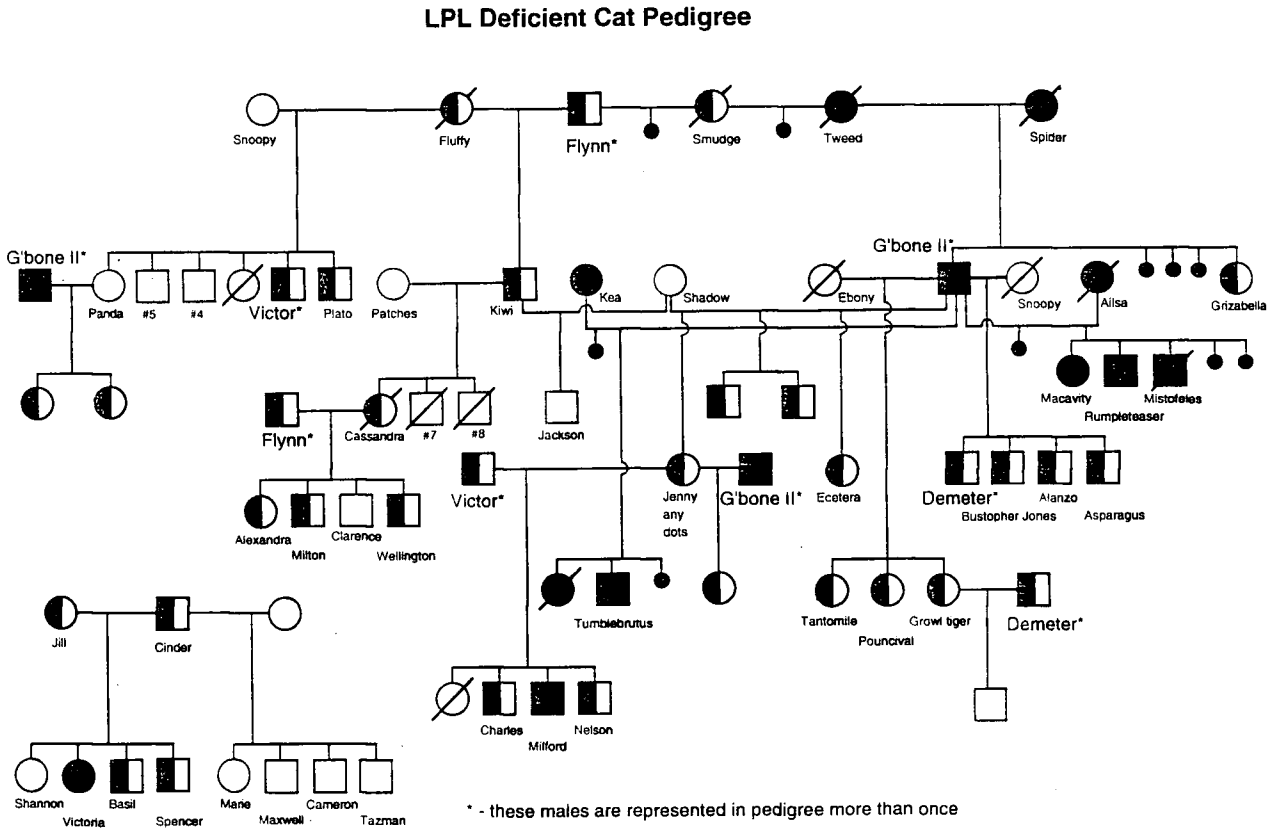

Figure 2.9. Pedigree of the cat colony. All cats homozygous for the Gly412Arg mutation have chylomicronemia and have no LPL activity (filled in boxes). Cats that are heterozygotes for this mutation (half filled) have an intermediate biochemical phenotype.

Discussion

Affected cats with chylomicronemia have a clinical phenotype very similar to humans with LPL deficiency. Here we show that the affected phenotype in this cat colony is caused by a point mutation in amino acid residue 412 (Gly412Arg) in the C-terminus of the cat LPL gene. This residue corresponds to codon 409 in the human LPL gene and is highly conserved in the LPL genes of at least ten other species and two related lipase gene family members, hepatic lipase and pancreatic lipase (Figure 2.3). Apart from sharing a similar clinical phenotype, there is the highest degree of conservation of DNA sequence identity (90%) and amino acid identity (94.5%) between human and feline LPL species compared to at least 8 other species cloned to date (Wion et al., 1987, Zechner, et al., 1991, Senda, et al., 1987, Brault, et al., 1992, Enerback, et al., 1987, Cooper, et al., 1992, Datta, et al., 1988, Komaromy, et al., 1987).

Further assessment of the significance of this amino acid substitution by both site-directed *in vitro* mutagenesis, expression studies and segregation analysis of this mutation with the affected phenotype confirms this Gly412Arg mutation as the cause of LPL deficiency in this colony of cats.

LPL comprises two functional domains with a larger N-terminal region containing the catalytic triad, and active site channel and loop residues(Brunzell, 1995, Olivecrona, et al., 1993, and Henderson, et al., 1993). The smaller C-terminal domain is likely to be involved in lipid binding (Chappell, et al., 1994), interaction with heparin (Ma, et al., 1994, and Hata, et al., 1993) and the low density lipoprotein-related receptor (LRP) (Beisiegel et al., 1991). Most mutations underlying human LPL deficiency have resided in exons 4, 5 and 6, close to the residues in the catalytic triad (Brunzell, 1995., Santamarina-Foho, et

al., 1991). A mutation at residue 410 of the human gene has also been shown to cause familial LPL deficiency (Previato, et al., 1994). However, the mechanism whereby this Gly412Arg substitution causes LPL deficiency is not yet known. This residue lies within a stretch of 7 residues highly conserved in 8 LPL species, as well as HL. Furthermore, these specific residues are postulated to be involved in lipid binding and binding to LRP (Krapp, et al., 1995). The absence of any immunoreactive LPL mass (using monoclonal antibody 5D2 which only detects LPL dimers) in homozygous cats also suggests that this mutation may significantly alter the conformation of the LPL protein resulting in rapid dissociation of active LPL dimeric protein into inactive monomers.

One intriguing observation is the higher frequency of stillbirths in offspring of female homozygotes which appears to be independent of the LPL genotype of the developing kitten but related to the genotype of the mother. LPL is crucial for the uptake and mobilization of energy in the form of free fatty acids which are a critical energy source for the developing fetus (Thomas and Lowy, 1983). In a pregnant cat with complete LPL deficiency, this function may be severely compromised and underlie the intrauterine growth retardation of the developing kittens. This hypothesis is supported by studies in guinea pigs, which revealed that in the last trimester, levels of free fatty acids, derived from maternal triglyceride-rich particles, are transported across the placenta at a greatly increased rate (Thomas and Lowy, 1983). The inability of the mother to mobilize fatty acids at this stage may be an important factor in the higher rate of stillbirths. There are no comparable reports of impaired reproductive fitness of human females with complete LPL deficiency. This may be due to alternative

pathways for mobilizing free fatty acids in humans or earlier miscarriages which have not been formally ascertained.

Homozygous kittens also grow at a significantly reduced rate from that of normal kittens and heterozygotes. It is possible that this may also be the result of decreased availability of energy derived from fatty acids for the growing kitten. Persistence of this finding into adulthood is marked by a profound generalized decrease in total body fat (data not shown), which in the case of a homozygous pregnant female may further exacerbate the difficulty of mobilizing fatty acids from adipose stores.

There have only been a few studies of the lipid transport system as well as the assessment of atherosclerosis in the domestic cat (Demacker, et al., 1987, 1988., and Manning and Clarkson, 1970). Clearly for the elucidation of the function of lipases which are involved in the metabolism of triglyceride rich lipoproteins and the metabolism of HDL sub-fractions, an animal model is needed in which the lipoprotein pattern and lipid transport system is comparable to that of human. In this respect, it has been proposed that the cat may be an appropriate animal model because it is the only animal from many different laboratory and domestic animal species tested, which has two separate HDL sub-fractions together with a distinct LDL fraction (Demacker, et al., 1987).

Ultracentrifugation reveals that the cat has two HDL bands that are similar to the average density boundaries of human HDL_2 and HDL_3 respectively. Furthermore, fasting cat VLDL and IDL has a similar apoprotein composition to human VLDL/IDL containing proteins such as apoB100, apoB48, apoE and apoC. Both cat and human HDL_2 contain apoA1 as the major lipoprotein. Cat serum has also been shown to cross-react immunologically to

antihuman apoB, A1 and E (Demacker, et al., 1987, and 1988). The cat, in contrast to the mouse which has approximately 4-5 times the amount of LPL in post-heparin plasma as human (Liu, et al., 1994), has levels of plasma LPL activity that are similar to that seen in the human (Peritz, et al., 1990).

Studies in transgenic mice suggest that variations in LPL activity are associated with altered lipid and lipoprotein levels which would predict an altered susceptibility to atherosclerosis (Liu, et al., 1994). Furthermore, studies of patients heterozygous for mutations in the LPL gene have revealed decreased levels of HDL_2 and Lp-A1 which may be associated with an increased risk for atherosclerosis (Reymer, et al., 1995). This is particularly pertinent, as some LPL mutations (e.g. Asp291Ser) may be seen in approximately 2% of the general population (Reymer, et al., 1995). To date, however, no studies have been reported which examine the frequency of atherosclerosis due to either complete or partial LPL-deficiency.

The availability of a feline model of LPL deficiency resembling the human disorder and sharing some mechanisms of lipid transport, offers a novel opportunity to assess the role of LPL in lipoprotein metabolism in a well characterized, experimental, *in vivo* system on a genetic background that is relatively homogeneous. In addition, the effects of this mutation on growth will allow further *in vivo* studies of the role of LPL in body fat accumulation. The availability of a gene-targeted mouse resulting in complete LPL deficiency would provide similar opportunities for studies of the heterozygote, but studies in the homozygote are not possible as death occurs within the first 2 days of life (Coleman et al., 1995) without hand rearing with milk containing only medium chained fatty acids (Weinstock, et al., 1995). Because the feline lipid transport

system has significant similarities to the human and the fact that a naturally occurring animal model for human LPL deficiency has been defined with a clinical phenotype that closely parallels that in the human, the study of these LPL deficient cats provides additional and highly complementary information to the studies of the gene targeted LPL deficient mice. Thus, as the Watanabe Heritable Hyperlipidemic (WHHL) rabbit serves as a paradigm for assessing LDL receptor function and therapeutic strategies, the LPL deficient cat could represent an important *in vivo* model for evaluating the relationship between elevation of triglyceride rich lipoproteins, atherogenic risk and potential efficacy of therapeutic agents including gene therapy.

Chapter 3: Lipoprotein Profile and Biochemical Characterization of Lipoprotein Lipase Deficient Cats

Foreword

In this chapter I have performed, together with our colleague Jean Dallongeville in Professor Jean-Charles Fruchart's laboratory at the Pasteur Institute in Lille France, a detailed lipid profile that will enable us to fully appreciate the metabolic consequences of lipoprotein lipase deficiency in the cat. The data presented in this chapter are the result of a collaborative effort over the past two years, and most biochemical analyses were performed by either Eric Bauje in Professor Fruchart's laboratory or Li Miao in our laboratory. The oral fat load and some of the dietary studies were conducted by Dr. Quinton Rogers in the laboratory of Dr. Robert Eckel while on sabbatical. Furthermore, breast milk analysis was conducted in the laboratory of Dr. Sheila Innis by Roger Dyer. The study designs and data analysis were conducted by me. This chapter is the basis of a manuscript that I have written and submitted to the *Journal of Lipid Research*.

Introduction

Lipoprotein lipase is the rate-limiting enzyme for triglycerides hydrolysis of chylomicrons and VLDL (Olivecrona 1987, Eckel 1989, Glaser 1992, Brunzell 1995). This enzyme works as a homo-dimer (Peterson 1992) at the surface of the endothelium of extrahepatic capillaries, providing cells with fatty acids. Lipolysis of triglyceride rich lipoproteins (TRL) results in the formation of denser remnant particles that are taken up by the liver via cellular receptor. In this process, lipoprotein lipase has an additional important role in that it anchors TRL remnants to the cellular matrix before receptor mediated uptake (Pedersen 1983). TRL and their remnants have been under scrutiny for their putative role in atherosclerosis (Koran et al, 1996).

LPL deficiency results in a complex phenotype, predominated by a drastic elevation of chylomicrons and a concomitant decrease in LDL and HDL-cholesterol levels (Brunzell 1995). Evidence has accumulated to suggest that LPL deficiency, particularly in the heterozygous form, may be atherogenic (Jukema et. al., 1996). Although, partial LPL deficiency is highly prevalent in the general population studies of atherosclerosis are complex in humans with this disease.

A number of animal species have been used to study the pathogenesis and potential treatment of lipoprotein metabolism and the development of atherosclerosis (Ross R 1993 , Armstrong 1990). The most useful animal models have thus far been : mice, rats, dogs, pigs and rabbits. The animal models for LPL deficiency and hypertriglyceridemia are much less common. So far, LPL deficient mice have been engineered to address different questions of LPL

metabolism. Although mice have proved to be an extremely useful model for lipid and lipoprotein metabolism as well as atherosclerosis development studies, these models have some limitations. Attempts to knock out the LPL gene in the mouse have resulted in homozygous mice that can't easily survive beyond the first few days of life (Coleman et. al., 1995. and Weinstock et. al., 1995).

We have previously described the underlying genetic defect in the lipase gene resulting in chylomicronemia in a colony of domestics cats originating from New Zealand (Ginzinger et. al., 1996). This colony of cats represents, to date, the only viable animal model of complete lipoprotein lipase deficiency available for study. Furthermore, some homozygote cats have survived for 10 years or more. In contrast to the severe neonatal mortality observed in mice, we have found in the cat, that LPL deficiency results instead in an increased stillbirth rate among homozygote females and a reduced growth rate for homozygote kittens (Ginzinger et al., 1996). Nevertheless, the availability of a model such as this will provide complementary information about LPL deficiency and will become the basis of further studies to investigate lipid metabolism as it relates to heart disease and therapeutics such as gene therapy. It is the metabolic consequences of LPL deficiency in the cat that is the primary focus of this study, and will enable us to determine the extent of similarities and differences between the cat and human in order to better utilize the cat as an animal model for LPL deficiency.

Experimental Procedures

Lipid Measurements

Lipids were determined enzymatically using commercially available kits; Triglycerides (Samson) Triglycerides GPO-PAP (Boeringer Mannheim, Mannheim Germany), Cholesterol (Allain) (Cholesterol C system, Boeringer Mannheim, Mannheim Germany) and phospholipids (Phospholipids PAP 150 bio Merieux, 69280 Maray l'Etoile, France).

Lipoprotein Preparation

Blood was collected in tubes containing EDTA to reach a final concentration of 0.1% EDTA. Plasma was separated by centrifugation (2500 rpm) for 20 min. at 4C. Lipoproteins were separated under standard procedure by a combination of ultracentrifugation (at d=1.006g/ml) and phophostungtate precipitation. HDL-cholesterol was measured after precipitation with phosphotungstate using commercially available reagents (Cholesterol HDL, CHOD/PAP, Boehringer Mannheim, Mannheim Germany).

VLDL was separated by ultracentrifugation, using a Beckman TL100 rotor, from 0.5 ml of plasma by a single spin at density 1.006 g/ml. 0.5 ml of NaCl 0.9% was added to 0.5 ml of plasma and spun in a polycarbonate tube (18?hrs at 100,000 rpm 20C) in a Beckman 100.2 Ti rotor. The tube was sliced and the remaining 0.5 ml infranate fraction was analyzed for lipids as per previously. TRL(d<1.006 g/ml) fraction was measured by subtracting infranate values from plasma values and LDL was quantitated by subtracting HDL values from

infranate values. Triglycerides (Triglycerides GPO-PAP, Boehringer Mannheim, Mannheim Germany) and cholesterol (Cholesterol C system, Boehringer Mannheim, Mannheim Germany) measurements were determined enzymatically.

Gel Filtration Chromatography

Gel filtration chromatography was performed using a Superose 6HR 10/30 column (Pharmacia, Pharmacia LKB Biotechnology, S-751 82 Uppsala Sweden). The column was allowed to equilibrate with phosphate buffered saline (10mmol/L) containing 1 mmol/L EDTA, 130uL of plasma were eluted with the buffer at room temperature at a flow rate of 0.2 ml/min. Elution profile was monitored at 280nm and recorded by analog recorder chart tracing system column (Pharmacia, Pharmacia LKB Biotechnology, S-751 82 Uppsala Sweden). The effluents were collected in 0.26 ml fractions. Calibration was carried out with human VLDL ($d<1.006$ g/ml), LDL ($1.010<d<1.063$ g/ml), HDL ($1.063<d<1.21$ g/ml) and Bovine albumin Fraction V (Sigma, Sigma Chemical Co. St. Louis, MO.)

Oral Fat Load

The oral fat load was performed in male cats of each genotype. The cats were food deprived for 48 hours at which time a base line blood sample was taken and triglyceride values were determined. After the baseline blood sample was obtained, a small meal containing 2.5 grams of fat per Kg. body weight was made available to each cat individually; the meal was consumed in no more than 10 minutes. After eating the meal each cat had a blood sample drawn at

intervals of 1, 2, 3, 5, 7, 12, 24 and 48 hours. Water was available ad libitum throughout the course of this experiment.

Breast Milk Analysis

Breast milk was collected from queens as close to the same date after parturition as possible. Prior to milking, each queen was injected sub-cutaneously, with oxytocin (0.1mg/kg body wt.) to stimulate the release of milk. Each sample was collected in 60 µl capillary tubes which were filled by squeezing the nipple to obtain a drop of milk that could then be drawn up into the capillary tube. After a capillary tube was filled it was immediately blown into a 2.0 ml cryo-vial and flash frozen in a dry ice-ethanol bath. By repeating this procedure several times it was possible to collect 200-400µl of breast milk per sample. Samples were stored at -80^{0}C until fatty acid analysis could be performed.

Fatty Acid Analysis

Breast milk lipids were extracted and triacylglycerols were extracted from other lipid classes by thin layer chromatography as per previously, (Innis, et. al., 1995). The composition of the breast milk fat was analyzed based on the methods of Kuksis, (1984), and Christie, (1986). In brief, fatty acids were converted to their respective methyl esters using 14% boron trifluoride in methanol/benzene/methanol (25:20:25, by vol.) at 100^{0}C, for 30 minutes. Fatty acid methyl esters were separated using a Varian 3400 gas-liquid chromatography equipped for analysis with capillary columns and quantitated

using a Varian Star data system (Varian Canada Inc., Georgetown, Ontario, Canada).

Other Methods

Agarose gel electrophoresis was performed according to Noble et al (Noble 1968) with a Beckman electrophoresis kit. In brief, 5 uL of lipoprotein particles of d<1.006 g/ml plasma fraction were applied to a 0.5% agarose gel. Electrophoresis was performed for 1 hour in a barbital buffer (pH8.6) at 100 volts. Gels were stained in Sudan Black B and scanned with an optical densitometer.

Results

Lipid And Lipoprotein Profiles

Plasma lipids are presented in Table 3.1 according to LPL deficiency status. Complete LPL deficiency (LPL-/-) is associated with increased levels of plasma triglycerides (P>0.00029), VLDL-triglycerides (P>0.00027), HDL-cholesterol (P>0.032) and a trend toward decreased levels of LDL-cholesterol (P>0.065 for +/+ vs. -/- and P> 0.011 for +/- vs. -/-). The mean values of plasma triglycerides increased from a value of 0.25±0.04 g/l in normal male cats (n=7) to 0.33±0.08 g/l in heterozygote male cats (n=8) (P>0.016) and to 2.11±1.2 g/l in homozygote male cats (n=8) (P>0.002). A similar trend was seen in female cats in which plasma triglycerides increased from 0.26±0.2 g/l in normal (n=6) to 2.02±1.45 g/l in homozygotes (n=4) (P>0.052). This pattern was also observed for VLDL-triglycerides that vary from a value of 0.13±0.03 g/l in normal male cats to 0.19±0.05 g/l (P>0.013) in heterozygote male cats to 1.85±1 g/l (P>0.002) in homozygote male cats and from 0.18±0.17 g/l in normal female cats 1.85±1.5 g/l in homozygotes. The HDL-cholesterol increased in male but not female LPL deficient homozygote cats from 0.56± to 0.73±0.14 g/l (P>0.028). This increase in HDL-cholesterol was accounted for by an increase in the both the HDL2 and HDL3 fractions. On the other hand, LDL-cholesterol decreased from 0.20±0.10 g/l in normal male cats to 0.14±0.08 g/l in homozygote male cats and from 0.22±0.16 in normal to 0.12±0.05 g/l, in homozygote female cats. These findings are confirmed by the cholesterol and triglycerides FPLC profiles of cats (Figure 3. 1). The principal difference among groups was a clear increase in triglycerides and cholesterol in the largest FPLC fractions and a decrease in cholesterol in the

fractions corresponding to LDL in the homozygote cats as compared with normal and heterozygote cat profiles.

Feline Lipid Profile -Ultracentrifugation

Sex:	Male			Female			Both Sexes		
LPL genotype:	LpL(+/+)	LpL(+/-)	LpL(-/-)	LpL(+/+)	LpL(+/-)	LpL(-/-)	LpL(+/+)	LpL(+/-)	LpL(-/-)
n= :	7	8	8	6	8	4	13	16	12
Cholesterol (g/L)	.79±.15	.88±.11	.98±.22	.88±.33	.99±.34	.84±.18	.83±.24	.93±.25	.93±.21
Triglycerides (g/L)	.25±.04[a,c]	.33±.08[b,e]	2.11±1.2[c]	.26±.20[h]	.25±.08[g]	2.02±1.45[h]	.26±.13[o]	.29±.09[n]	2.08±1.22[o,n]
VLDL-TG (g/L)	.13±.03[b,d]	.19±.05[b,i]	1.85±1[d]	.18±.17	.13±.06[i]	1.85±1.5	.15±.12[k]	.16±.06[o]	1.85±1.1[k,o]
LDL (g/L)	.20±.10	.22±.08	.14±.08	.22±.16	.23±.13	.12±.05	.21±.13	.23±.11	.13±.07
HDL (g/L)	.56±.04[a]	.64±.14	.73±.14[a]	.62±.16	.70±.22	.66±.17	.58±.11[l]	.67±.18	.70±.14[l]
HDL2 (g/L)	.13±.05f	.16±.07	.22±.05f	.17±.09	.20±.13	.14±.06	.15±.07	.18±.10	.19±.07
HDL3 (g/L)	.43±.05	.48±.10	.51±.10	.45±.08	.50±.11	.52±.11	.44±.06[m]	.49±.10	.51±.10[m]

Statistics; student t-test, all superscripts in common have the following values:

P-values
a; p=0.02
b; p=0.01
c; p=0.003
d; p=0.002
e; p=0.03
f; p = 0.008
g; p=0.04
h; p=0.05
i; p=0.04
o; p=0.0003
k; p=0.0003
l; p=0.03
m; p=0.03
n; p=0.0004

Table 3.1. Summary of a lipid profile in cats of each genotype as assessed by ultracentrifugation of fasting serum. Values are given in g/L and statistical analysis are as indicated using a student t-test.

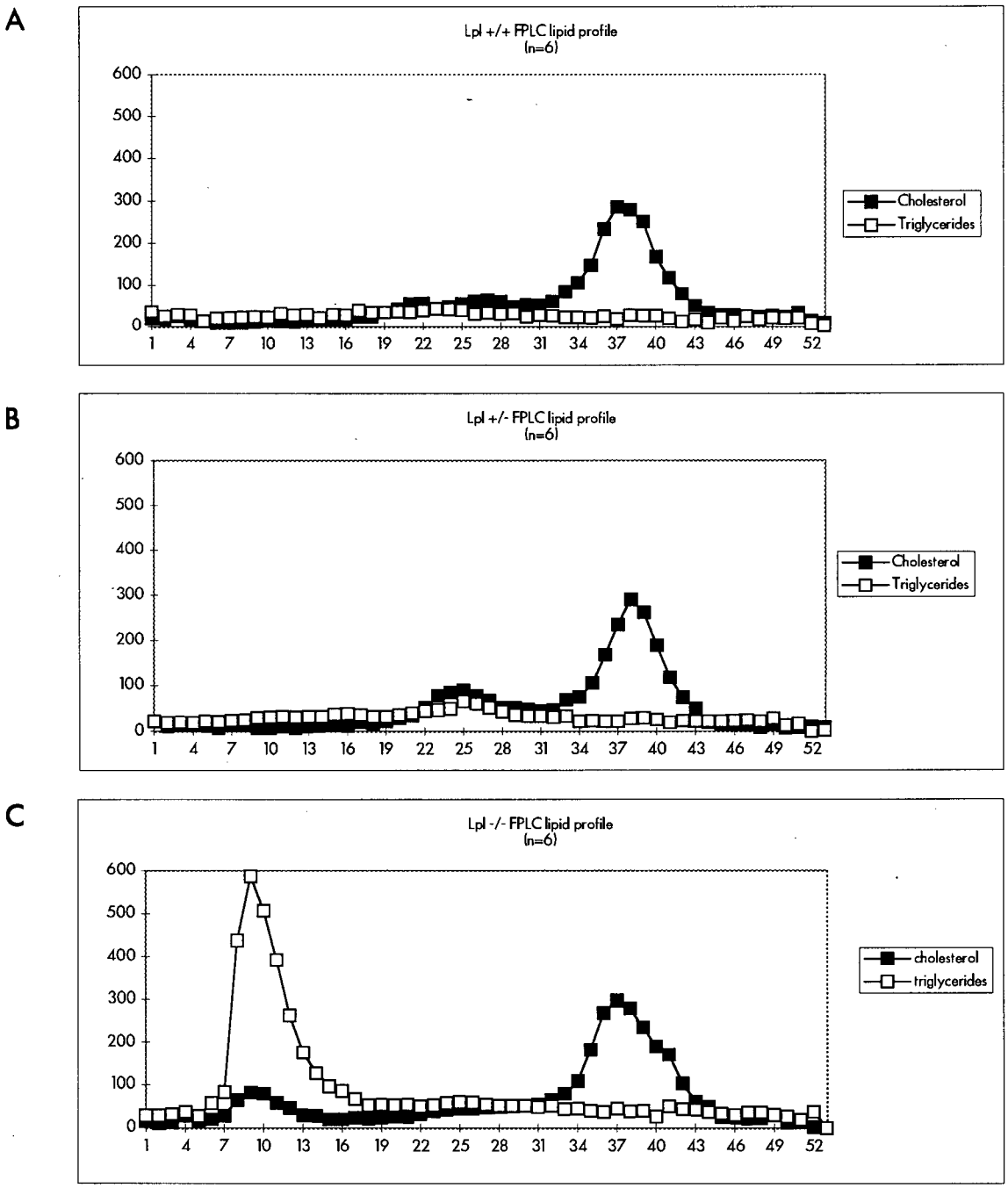

Figure 3.1. FPLC profile of fasting cat plasma. The X-axis is the fraction number and the Y-axis is in g/L. In normal cats (panel A) the predominate lipid particle is in the range for HDL-cholesterol. The heterozygotes (panel B) is for the most part indistinguishable from the normal, with the possible exception of a slight increase of triglyceride in fractions 22-26. The most obvious feature of the homozygotes (panel C) is the tremendous increase of triglyceride in fractions 7-14, which corresponded to the VLDL particles.

Lipoprotein Composition

The VLDL, LDL and HDL lipid composition are summarized in Table 3.2 and illustrated in Figure 3.2 according to LPL deficiency status. As expected and comparable to humans, VLDL were triglyceride-enriched lipoparticles. There is no statistical significant difference in VLDL triglycerides, cholesterol and phospholipid relative composition among the different genotypes. LDL particles were cholesterol-enriched as compared with VLDL. The LDL composition differed among LPL genotype status, such that LDL particles from LPL deficient homozygote cats contained relatively more triglycerides (34%±17) and less cholesterol (37%±26) than those of heterozygotes (18%±7, TG; and 49%±16, Chol.) and normal cats (16%±6, TG; and 46%±14, Chol.). The relative content of LDL phospholipids content remained unchanged among groups. The HDL relative lipid composition differed according to LPL deficiency status and gender. HDL from homozygote male cats were relatively enriched in cholesterol (41%±18) and depleted in phospholipid (58%±18) as compared to HDL of normal male cats (26%±5 and 74%±5 respectively) ($P>0.053$ and $P>0.044$ respectively). Also, there are significant differences in HDL composition based on gender, in that, all males (n=23) have relatively less cholesterol (32%±13) and more phospholipids (67%±13) compared to all females (n=18) (44%±10 and 56%±11, respectively) ($P>0.003$ and $P>0.0004$, respectively). This was seen both within a given genotype for each sex and when pooled regardless of genotype (Table 3.2).

Table 3.2. Summary of particle composition of normal and LPL deficient feline lipids.

TABLE 3.2 : Particle Composition

	Males				Females				Both sexes (by genotype)			
	n	Chol.	Triglyc.	P.pholipids	n	Chol.	Triglyc.	P.pholipids	n	Chol.	Triglyc.	P.pholipids
VLDL												
LPL +/+	7	11±7	74±10	14±9	6	10±4	81±11	10±8	13	10±6	77±10	12±9
LPL +/-	8	13±6	74±5	13±10	8	20±14	74±17	6±8	16	17±11	74±12	10±9
LPL -/-	8	13±8	68±8	19±7	4	21±15	70±17	9±8	12	16±11	69±11	15±8
All genotypes (by sex)	23	12±8	72±8	15±9[k]	18	17±12	75±15	8±8[k]				
LDL												
LPL +/+	7	45±14	16±6[a]	39±10	6	47±16	16±8[e]	38±9	13	46±14	16±6[f]	38±9
LPL +/-	8	44±12	20±5[b]	36±11	8	54±19	16±9	29±16	16	49±16	18±7[g]	33±14
LPL -/-	8	36±26	36±17[a,b]	29±14	4	39±30	31±18[e]	30±16	12	37±26	34±17[f,g]	29±14
All genotypes (by sex)	23	41±17	24±15	34±11	18	48±21	19±12	32±14				
HDL												
LPL +/+	7	26±5[c,n]	0±1	74±5[d,p]	6	39±8[n]	1±1	61±7[p]	13	32±9[h]	1±1	68±9[i]
LPL +/-	8	29±3[q]	0±1	71±3[r]	8	44±10[q]	1±1	57±11[r]	16	37±11	0±1	64±11
LPL -/-	8	41±18[c]	1±2	58±18[d]	4	51±16	1±1	49±15	12	44±17[h]	1±1	55±17[i]
All genotypes (by sex)	23	32±13[l]	1±1	67±13[m]	18	44±10[l]	1±1	56±11[m]				

Statistics; Student T-test: Superscripts in common have the following p-values:

Comparisons by genotype;			Comparisons by sex;	
a ; p= 0.013	d ; p=0.044	g ; p=0.007	k ; p=0.007	n ; p=0.006
b ; p=0.035	e ; p=0.075	h ; p=0.040	l ; p=0.003	p ; p=0.002
c ; p=0.053	f ; p=0.003	i ; p=0.028	m ; p=0.0004	q ; p=0.003
				r ; p=0.005

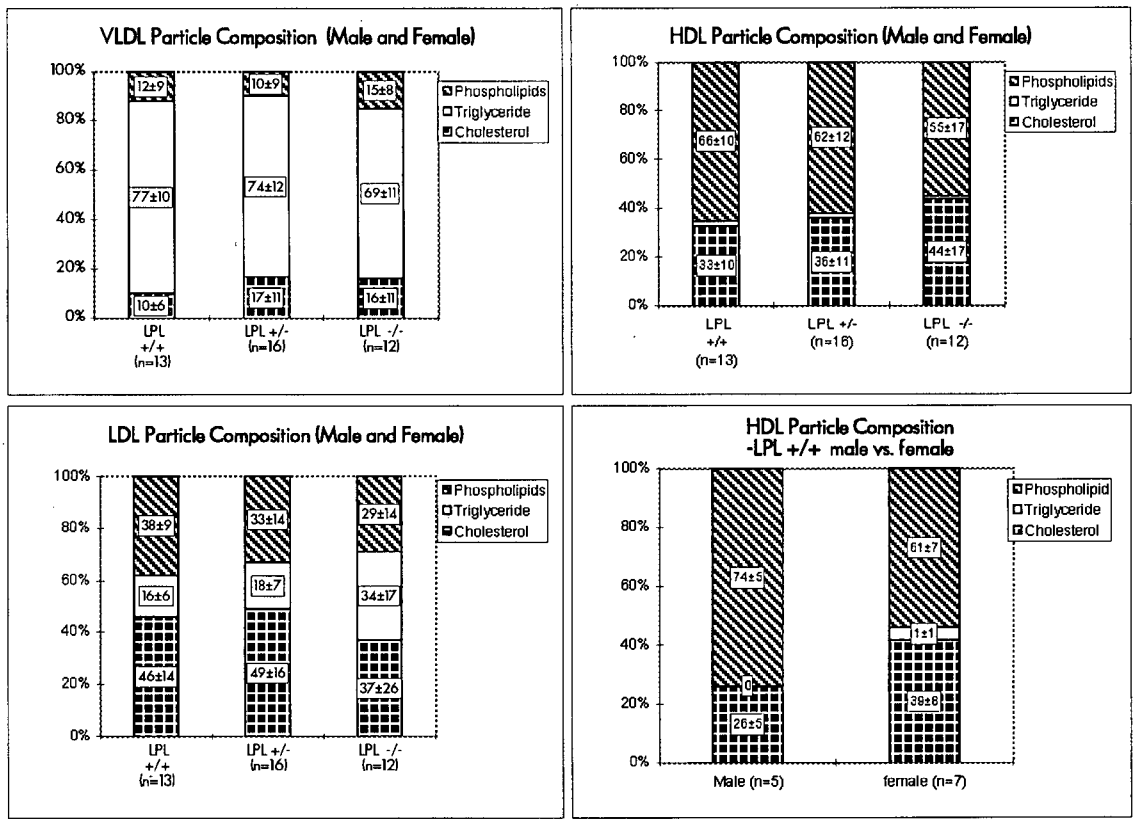

Figure 3.2. Particle composition of normal and LPL deficient feline lipids. VLDL particles are composed similarly for all genotypes (upper left panel) despite the high total amount of VLDL in the homozygotes. LDL particles are composed of more triglyceride at the expense of cholesterol in the homozygotes (lower left panel). Females tend to have more cholesterol in the HDL particle (lower right), however, there is no significant difference in the composition of HDL particles between different genotypes (upper right).

Agarose Gel Electrophoresis

Agarose gel electrophoresis of the plasma d<1.006 g/ml and d>1.006 g/ml density fraction are illustrated in Figure 3.3. The d<1.006 g/ml of normal, heterozygotes and homozygotes cats migrated as a major band in pre-b position and an additional band was observed at the origin in the LPL -/- cats corresponding to chylomicrons. The d>1.006 g/ml density fraction migrated as 2 bands: one major band in pre-α position and a minor band in β position.

Apolipoprotein profile

The VLDL and HDL SDS-PAGE apolipoprotein profiles are illustrated in Figure 3.4 and 3.5 respectively. Apolipoprotein A-I is the major apolipoprotein of normal cat's VLDL followed by apo B. In the other hand, apo B is the most abundant apolipoprotein of the LPL deficient homozygote cats followed by apo A-I. Apo E was detectable as a faint band among different genotypes. The principal difference, however, between homozygotes and normal cats was the consistent presence of apoCs' in VLDL of LPL homozygote cats, most notably a band that corresponds with ApoCIII. Apo B48 was found, inconsistently, in LPL deficient cats, most probably depending on the fasting state and diet (Figure 3.5).

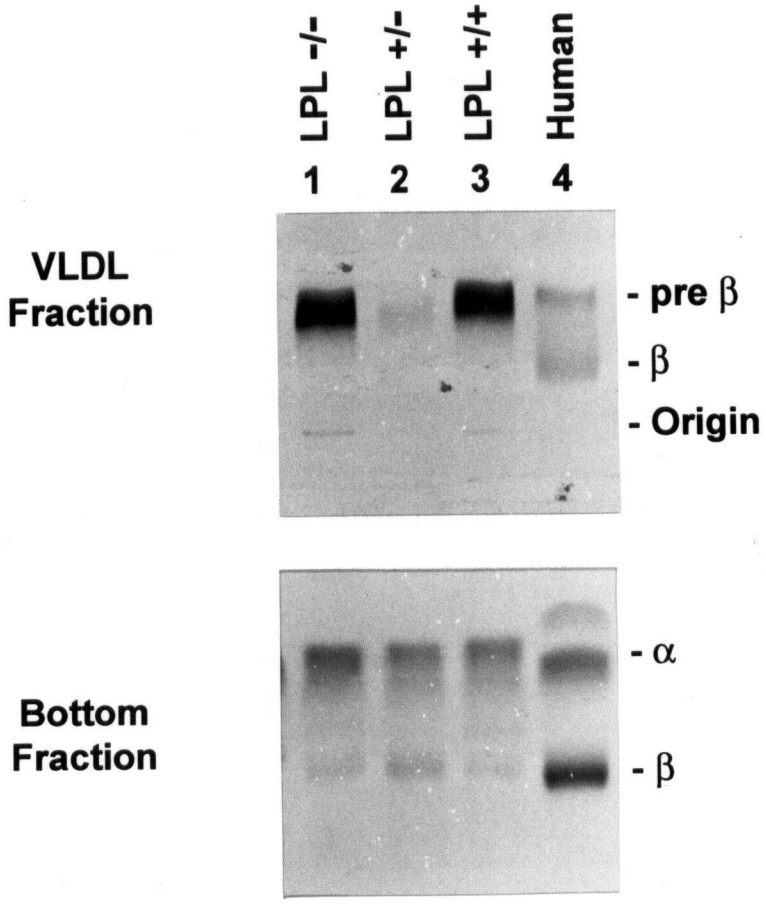

Figure 3.3. Agarose gel analysis of feline lipids. Plasma has been loaded from cats of each genotype (lanes 1-3), and lane 4 is plasma from a normal human. In the upper panel is the the VLDL fraction, in lane 1 (a homozygote), chylomicrons are evident at the origin. The lower panel is the bottom fraction, which includes the HDL particles, there is very little difference between each of the genotypes.

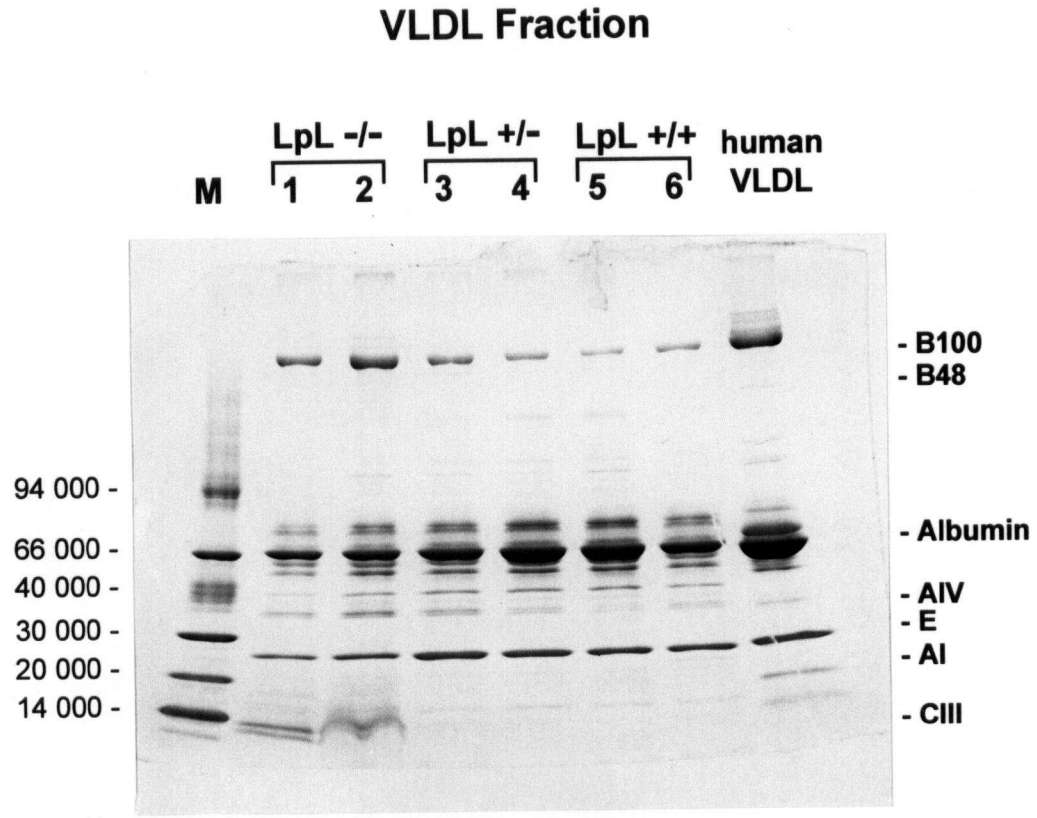

Figure 3.4. SDS-PAGE analysis of the VLDL fraction of normal and LPL deficient cats compared to a normal human. In lanes 1 and 2 there are large amounts of proteins comparable to Apo CIII. Apo B48 is also present in some cats indicating that the cat has Apo B editing similar to the human and that the homozygote deficient cat has a delayed clearance of chylomicrons.

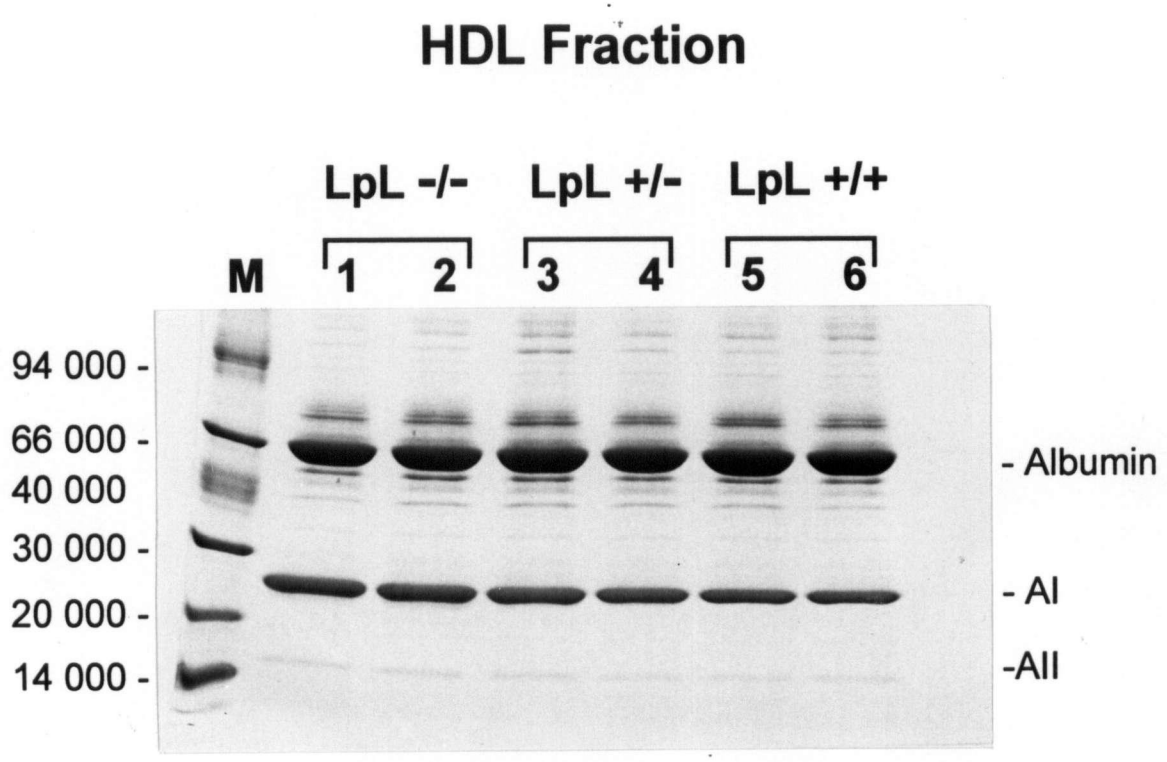

Figure 3.5. SDS-PAGE analysis of the HDL fraction of feline lipids. The cat has identifiable ApoAI and ApoAII proteins similar to the human and there are no differences between genotypes.

Fat Tolerance Test

The results of the fat tolerance test are illustrated in Figure 3.6. Mean plasma triglycerides of normal cats increase only moderately from a fasting baseline value of 44 g/L to a peak values of 97 g/l 3 hours after the oral fat load. Then triglyceride levels decrease progressively to return to the baseline value after 7 hours and remained stable up to 48 hours. The heterozygotes (n=4) had fasting triglyceride levels ranging from 27g/l at baseline to 208g/l after 5 hours. All heterozygotes demonstrate a gradual increase in triglyceride levels after the fat load. The postprandial triglyceride profile differs significantly from that of controls. The peak value was four times the fasting value compared to about 1.5 times base line in controls, and the peak time was delayed to 5 hours from about 2 hours in controls. Triglyceride levels reached the fasting baseline value 12 hours after the fat load. This difference was even more pronounced in LPL deficient homozygotes with postprandial values increasing 8 fold and peak time delayed to 7 hours after fat ingestion. Twelve hours after the fat load the triglyceride levels were still more than 2 fold the fasting baseline value. Moreover, after a 48 hour fast, the homozygotes never reached a triglyceride level as low as the baseline in a normal cat. Thus the area under the postprandial triglyceride curve was significantly greater in LPL homozygotes than heterozygotes and normal cats. Furthermore, this may explain some of the tremendous variation observed in triglyceride values obtained after an "overnight fast," such that some animals may have been sampled at about 14 hours post-prandial and other may have been sampled 20 hours after their last meal. At this position on the curve the value could vary considerably.

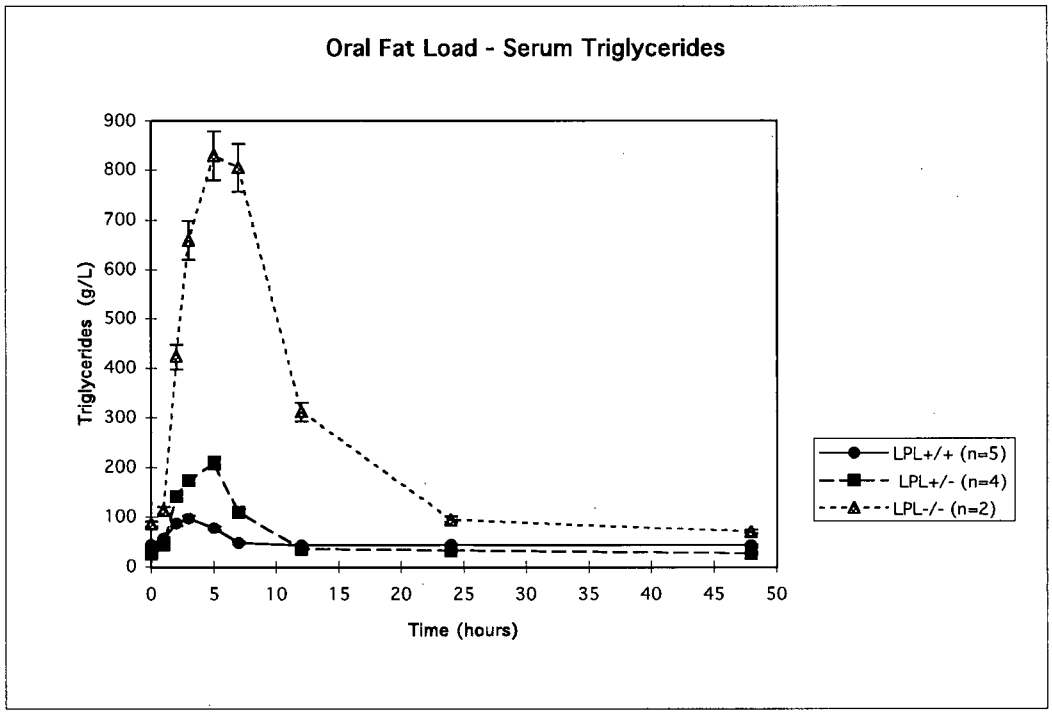

Figure 3.6. Serum triglyceride values of normal and LPL deficient cats prior to, and as measured over the indicated time intervals following an oral fat load administered by feeding a small meal of a high fat commercial cat food after food deprivation of 24 hours. All meals were comsumed within 10 minutes of being fed the test meal. The LPL heterozygotes have a delayed clearance compared to that of normal cats and the homozygotes have a dramatically reduced clearance rate relative to that of both normal and heterozygote cats.

Breast Milk Analysis

The fatty acid analysis of breast milk from queens of each genotype indicates that there is a shift in composition of milk from the homozygotes. Despite a small sample size there is a significant shift in fatty acid composition of the homozygotes milk compared to that of normal and heterozygotes. In the former, there is an increase of short chain fatty acids and a decrease in long chain fatty acids (Figure 3.7). Furthermore, the ability of homozygotes to produce milk is reduced and the total amount of fatty acids is reduced to about 4 grams per deciliter compared to about 16 g/dl. In addition, we have found that it is not very common for a homozygote queen to breast feed a litter beyond a few days without the kittens losing weight, although a single kitten can be nursed for much longer.

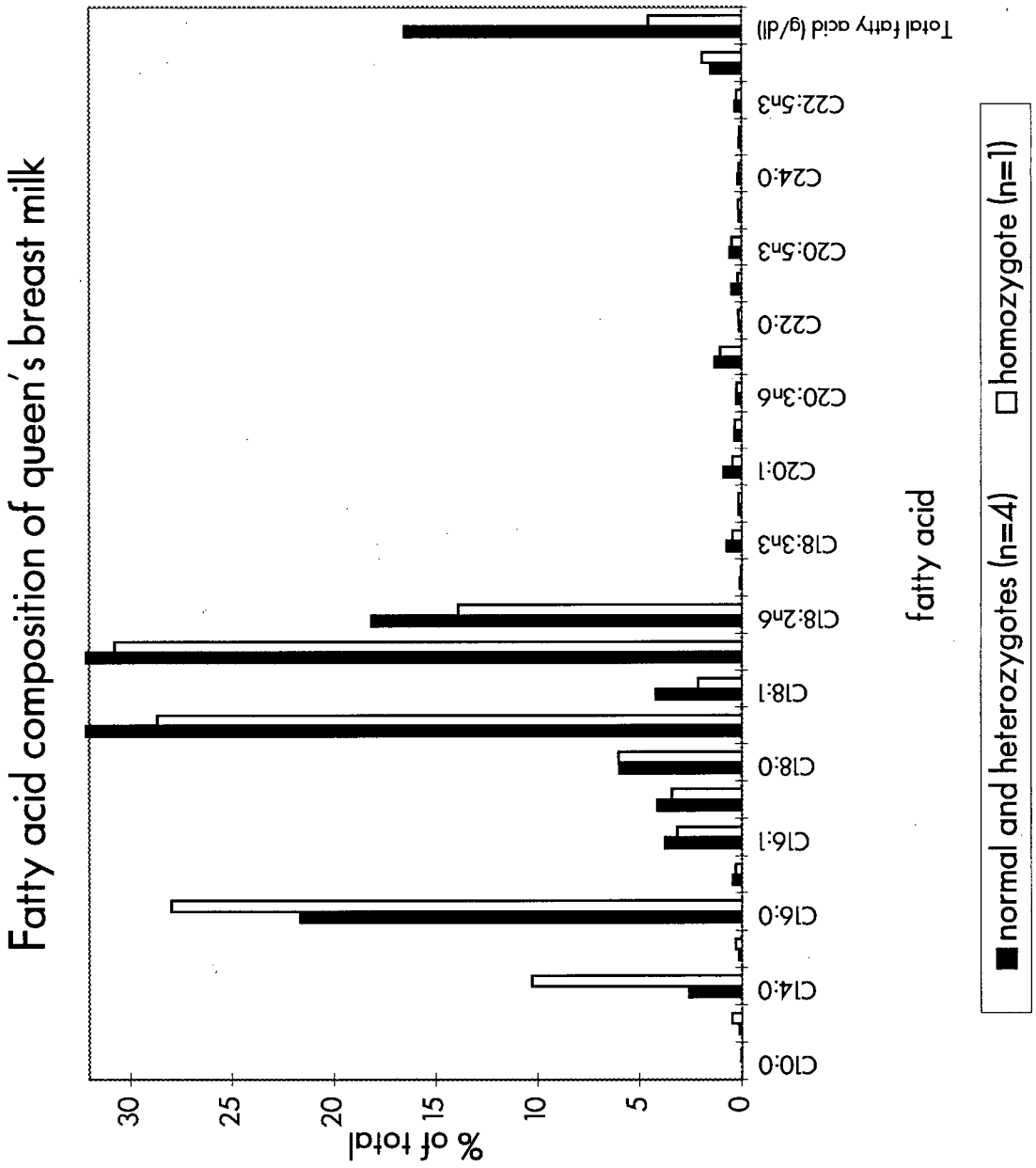

Figure 3.7. Fatty acid composition of breast milk from lactating queens. Cats were sampled twice over a four day period from 24 to 28 days post partum. The homozygote queen has a different composition of fatty acids in the breast milk in which there is a shift toward more short chain fatty acids at the expense of the long change fatty acids. The homozygote also has a reduced total amount of fat in her milk compared to that of normal cats.

Discussion

In this chapter, I have described the lipoprotein profile in normal and LPL deficient cats as well as aspects of lipid metabolism in the context of LPL deficiency which demonstrates that the LPL deficient cat is a useful model to study lipid metabolism. I have evaluated the lipid metabolism in the cat with LPL deficiency, both partial and complete, in a very controlled setting and under circumstances that are not possible for humans. In this colony of cats, the diet is strictly controlled and body weights continuously monitored. The cats, in addition to adhearing to the same diet, are housed in similar pens which limits all cats equally in how much exercise they can get. These measures have been taken in order to reduce as many factors as possible that can influence the lipid profile.

Although complete LPL deficiency in humans is not very common (about 1 in a million world wide) the carrier frequency is much greater. When including those individuals that have a mutation in the LPL gene resulting in reduced lipolytic activity, the prevalence of lipolytically compromised people is perhaps as high as 4-5% of the population (Reymer et al, 1995). It has been shown that pregnant women suffering from complete LPL deficiency are not only potentially at risk for lethal pancreatitis or complications that endanger the fetus, but when they are capable of a healthy delivery, they tend to have an abnormal breast milk composition (Steiner et. al., 1985.)

There are several studies in which the cat has been included, with several other species, to investigate a particular aspect of lipid metabolism (e.g. Chapman et. al., 1981, and Terpstra et al., 1982). There are only a few studies that

have looked at various aspects of lipid metabolism in the cat as the main focus of the study (Demacker et al., 1986&1987 and Watson et al., 1995). The lipid profile we present here for the normal cat is in agreement with and expands on that of Demacker et al., (1987) and complements that of Watson et al., (1995). The normal domestic cat has many similarities, and some differences, to humans with respect to lipid metabolism. The cat has VLDL triglyceride levels that are lower than those of human and HDL and LDL levels that are about 1.5 times and one fifth, respectively, that of humans. In cats, as in humans, LPL deficiency is associated with increased levels of plasma triglycerides and VLDL-triglycerides and decreased levels of LDL-cholesterol. However, in contrast to Type I humans, which have reduced HDL cholesterol compared to normal lipidemic individuals, LPL deficient cats have increased HDL- cholesterol values. This difference is however, consistent with studies in human subjects with primary hypertriglyceridemia fed an oral fat load, in which it was demonstrated that there is an inverse correlation between CETP levels and HDL-C levels (Foger et al, 1996). Furthermore, in the case of CETP deficiency due to mutations in the CETP gene (Inazu, et al., 1990), and in other species which are CETP deficient (Tall, 1993) there is drastic elevation of HDL-C. Moreover, studies in the rabbit after immunological blockage of CETP also showed that HDL is elevated in the absence of serum CETP activity. (Whitlock et al., 1989. and Abbey et al., 1989). All of the above examples of CETP deficiency help illustrate that the lipid profile observed in the LPL deficient cat, despite some differences to that of human LPL deficiency, are consistent with what would occur in the human if the role of CETP is taken into account.

Human hypertriglyceridemia is associated with critical changes in lipoprotein composition. VLDL becomes cholesterol enriched and triglyceride depleted whereas HDL become triglyceride enriched and cholesterol depleted (Eisenberg et al., 1985). CETP plays a critical role in the transfer of cholesterol ester from HDL to VLDL and the converse transport of triglycerides (Tall, 1993). The net transfer of lipid from either side is dependent on the total amount of transfer protein in hypertriglyceridemic individuals and in the level of substrate (VLDL triglycerides) in normotriglyceridemic subjects (Mann et al., 1991).

Recently the normal cat has been demonstrated to be lacking any measurable serum CETP activity, at least when using human HDL as a substrate (Watson et al., 1995, and unpublished data from Gary Shen in Winnipeg). CETP deficient animals, such as the mouse (especially in conjunction with transgenic techniques) have nonetheless been valuable model organisms for studying human lipid metabolism. In the present study, changes can be observed which allow us to identify the relative effect of CETP deficiency on the lipoprotein compositional changes in the context of normal lipidemia and hypertriglyceridemia. In this respect, despite a 20 fold increase in plasma triglyceride levels there were no significant changes in VLDL composition in LPL deficient cats, which indicates that there must be more particles.

Thus, the LDL levels decrease in LPL deficient cats indicating a reduced production of LDL and/or an increase clearance of LDL lipoprotein in these cats. Several hypotheses may explain this observation. First, the decrease in LPL deficient cats supports the concept that LDL is a product of the catabolism of VLDL, and that the production of LDL is reduced in LPL deficient cats. However, that LDL are still produced indicates that the alternative lipolytic

pathway is less efficient than LPL. Hepatic lipase (HL) has been shown to hydrolyze VLDL. HL activity in the cat is about twice that of human (unpublished observations). However, phospholipid is a better substrate for HL than triglycerides, thus explaining the lipoprotein change observed in the LDL fraction. Secondly, an accelerated clearance of LDL might explain the reduced levels observed in LDL deficient cats. Such acceleration has been demonstrated in hypertriglyceridemic subjects (Janus et al., 1980).

The normal cat has been considered an "HDL-animal" due to a report in which the cat had about 5 times as much cholesterol in the HDL fraction than the LDL fraction (Demacker et al, 1987). In our study, however, we found only about 2.5 times as much cholesterol in the HDL fraction than the LDL fraction. Nonetheless, the cat still had a majority of the cholesterol in the HDL particles. It is likely that the elevation of HDL in the LPL deficient cat is a consequence of not expressing, in the circulation, any measurable CETP activity. In this context, the increased levels of HDL-cholesterol on one hand and the increased relative content of triglycerides in LDL are possible consequences of these metabolic deficits (LPL and CETP). In the absence of CETP activity, cholesterol accumulates in HDLs and does not transfer to triglyceride-rich lipoproteins, resulting in cholesterol enriched HDL. Moreover, since the CETP mediated flux of cholesterol ester towards VLDL is absent in this animal model, less cholesterol enters the VLDL to LDL cascade resulting in cholesterol-depleted and triglyceride-enriched LDL in normal cats. An alternative explanation to this observation would be that all lipoproteins (VLDL, LDL and HDL) compete for the remaining available lipolytic pathways, most likely hepatic lipase, thus leading to decreased lipolysis and delayed clearance of HDL. According to this

hypothesis VLDL are less efficiently lipolized giving rise to triglyceride-enriched LDL.

Oral fat tolerance studies with human LPL deficient and partially deficient patients have illustrated the reduced post-prandial triglyceride clearance of those with compromised LPL hydrolytic activity (Patch et al., 1986., and Pimstone et al., 1996). It is the prevalence of these TRL particles that have recently been put under close scrutiny as risk factors for coronary artery disease (Goldberg, 1996, Jukema et al., 1996). In this chapter I have demonstrated that the LPL deficient cat has a greatly reduced clearance rate of plasma serum triglycerides (Figure 3.6). The homozygotes had a higher baseline value than normals, and demonstrated a profound elevation of triglyceride levels that peaked at about 5 hours after the meal. After about 12 hours these cats still had over 3 fold higher TG levels than their baseline values. But by 24 hours they had cleared the majority of the circulating triglycerides, possibly as a result of the action of HL or perhaps an up-regulation of a receptor mediated uptake pathway such as the VLDL receptor. However at this point this remains speculative.

I have also found that homozygote cats, when maintained on a "low fat"(12% by wt.) cat chow, will have about 20 fold higher triglyceride level than normal and that even after 72 hours of fasting these cats do not reach the baseline of normal cats (data not shown; up to 48 hours is shown in Figure 3.6). Furthermore, if given a high fat diet (39% by wt.) ad libitum, the homozygotes reach serum triglyceride levels as high as 18,000 mg/dL (data not shown). It was also noted that in response to a high fat diet the LPL and HL activity levels would increase about 2-fold over the course of 6-8 weeks on a diet with 30% fat

(by wt.)(data not shown). The LPL heterozygote cats have also shown a similar response to an oral fat load compared to human LPL heterozygotes or those with partially compromised LPL activity (e.g. Asp9Asn, Ser291Arg.) (Figure 3.6). These data help illustrate the effect an intermediate lipolytic activity has on the post-prandial response to an oral fat challenge. This also indicate that cats heterozygous for LPL deficiency closely resemble humans with reduced LPL activity.

In a report of a human type I patient, it was shown that she had greatly reduced total amount of fat in her breast milk which in turn had an altered fatty acid composition (Steiner et al., 1985). Remarkably, the LPL deficient cat shows a similar pattern (Figure 3.8). Even with only a few animals tested, due to the difficulty of obtaining a significant amount of breast milk from a cat, the shift toward shorter chain fatty acids is clearly evident. This unbalance of fatty acid composition and total reduction in the amount of fat in the milk may represent the contribution of LPL to the production of breast milk. In the normal breast, LPL is the major enzyme responsible for the liberation of fatty acids from chylomicrons for the production of triglycerides to be exported in breast milk (Scow, et al., 1977). The chylomicrons are the major serum lipid carrier and contain predominantly long chain fatty acids, where as short chain fatty acids are capable of being transported in the circulation bound to serum albumin. The chylomicrons are thought to bind to the plasma membrane in the capillary lumen at which point LPL hydrolyzes the triglycerides for uptake into mammary cells (Scow et al., 1980). In the case of LPL deficiency, the human and cat are unable to liberate the longer chained fatty acids that reside in the chylomicrons, and therefore the milk will have a reduced total amount of fat. It

is then necessary to rely on secondary pathways resulting in an over-representation of smaller chain fatty acids which can be utilized. This finding also highlights the similarity of the LPL deficient cat to the human.

In this chapter, I have described detailed lipoprotein analysis in normal and LPL deficient cats as well as several aspects of lipid metabolism in the context of LPL deficiency. In cats, as in humans, LPL deficiency is associated with increased levels of plasma triglycerides, VLDL-triglycerides and VLDL-cholesterol. This difference is small between normal and heterozygote cats, but is much more pronounced when stressed by an oral fat load, highlighting the impaired clearance of heterozygote animals. These manifestations of LPL deficiency are almost completely comparable to those of the human LPL deficient state. In a CETP deficient animal such as the cat, our LPL deficient cats show remarkable similarity with the human LPL deficient state. Hence, the LPL deficient cat has become a useful model to study lipid metabolism, and potential therapeutics of human LPL deficiency. This model will also provide insight into the study of triglyceride rich lipoproteins as it relates atherosclerosis, in that TRL have been implicated in coronary artery disease (CAD), yet CETP is thought to be protective of CAD. It is interesting to speculate about which factor will ultimately be found to be more important in the progression of heart disease.

Chapter 4: Altered Body Composition in LPL deficient cats

Foreword

In Chapters 2 and 3 I have described much of the phenotype associated with LPL deficiency in our colony of cats. However, in this chapter I will explore in more detail one additional manifestation apparently related to the lack of LPL activity in these cats. In describing the decreased growth rates and increased stillbirth rates in the LPL deficient homozygote cats in chapter 2, I have speculated that this might be the result of lack of energy in the form of free fatty acids that are crucial for the growing fetus and later in life for the proper growth of the cat. In this section of the thesis I am extending that line of reasoning, the hypothesis is then, if the cats with a demonstrated reduced growth rate (as presented in Chapter 2) were not developing any body fat, then this lack of body fat could in turn account for the apparent growth deficit. In other words, the apparent reduced growth rate of LPL homozygote kittens can be accounted for by the lack of adipose tissue.

In order to assess this hypothesis I have conducted body composition experiments in collaboration with Dr. Quinton Rogers at the University of California at Davis. For the deuterium dilution experiments, all deuterium analysis of deuterium labeled blood was performed in the laboratory of Dr. Quinton Rogers at the University of California at Davis. The deuterium injections and blood sampling of the cats were performed by me with the assistance of Zabeen Ladna at the UBC animal care center. All data analysis has

been conducted by me and this chapter is forming the basis of a manuscript I am currently writing and I hope to submit for publication to the *Journal of Lipid Research*.

Introduction

During the earlier studies in chapter 2, in which I identified the underlying genetic defect in the lipoprotein lipase gene resulting in the chylomicronemia phenotype of the affected cats, I also described an increase in stillbirth rates among homozygous females and a reduced growth rate for homozygous kittens. In this chapter, I present data illustrating that the LPL deficient cats have a greatly reduced body fat content, which seems to be influenced *In utero* by the genotype of the queen. Furthermore, this reduction of body fat is enough to account for the apparent growth retardation of homozygote kittens. I have also observed that some of the cats, almost exclusively homozygotes, appear to be very "scrawny" as measured by a body condition scoring system. Together with the reduced growth rates and poor breeding performance of the homozygous queens, I formulated a hypothesis that would help explain these manifestations of LPL deficiency in the cat. The following hypothesis is based on aspects of the molecular and biochemical pathways whereby LPL deficiency might result in these additional phenotypic observations: if there is a lack of free fatty acids available to a developing kitten (either *in utero* or neonatally) as a result of either maternal insufficiency and or *in situ* insufficiency, then that kitten will be at greater risk for spontaneous abortion and will be hindered in generating fully mature adipocytes which will manifest as a reduced growth rate or a reduced percent body fat for that kitten.

This hypothesis is based on two observations; one is the observed increased rate of stillbirths in homozygous mothers and the second is the altered body composition of LPL deficient cats. These two observations share a

common molecular and biochemical basis. However, formal evidence indicating a causal relationship is beyond the scope of this thesis and will become the basis of future studies.

LPL is highly expressed in adipose tissue and it is a likely point to begin to see if the LPL status influences the adipose tissue in a manner that would result in the phenotypes in question. Expression of LPL in the adipose tissue is important for the delivery of fatty acids to adipose tissue in times of sufficient caloric intake so that energy in the form of fatty acids can be stored in order to provide energy at a later time in which there might not be a wealth of energy. Throughout evolution of the mammalian species it has been important for the survival of the species to obtain sufficient energy to satisfy the caloric needs of the individual organism, thus helping insure the potential for reproduction. As such, an adequate system evolved whereby energy is sufficiently stored in times of feast, that can then be liberated in times of famine. One of the many functions of LPL is hydrolyzing the triglycerides in the circulation, thus liberating fatty acids for uptake into adipose tissue for storage in times of plenty as well as hydrolyzing triglycerides for the uptake and subsequent oxidation by muscle tissue when needed as an energy source. This balance between metabolic needs and long term storage is a dynamic process.

It has also been shown that LPL is a critical marker indicating the differentiation of adipocytes in cell culture (Abumrad et al., 1991). Furthermore, free fatty acids, the hydrolytic products of LPL, are critical for the transformation of pre-adipocytes to fully mature adipocytes. It is easy to speculate that LPL plays an important role in the maturation of adipocytes. It possesses the hydrolytic capacity to generate the fatty acids that may provide

the signal triggering the development of the mature adipocyte. As stated earlier in my hypothesis, the LPL deficient cat will be useful to help define this role in adipocyte maturation. Without LPL producing a substantial amount of free fatty acids during the development of a homozygous kitten, my hypothesis suggests that these kittens will not have the proper signals, in the form of free fatty acids, to induce the conversion of pre-adipocytes into mature adipocytes. This might further be exacerbated by the genotype of the queen, such that, if the queen is also a homozygote, then she may compound the difficulty of attaining a sufficient level of free fatty acids, whereby normal adipocyte development would be very difficult to achieve. Moreover, the homozygote queen is drawing on her own adipose stores to generate fatty acids for transport across the placenta for use by the developing kittens, as well as for her metabolic needs. It is this particular aspect of the homozygote queens inability to supply the developing fetus with adequate energy that may result in the late gestational death of the kittens.

It has been previously demonstrated by studies of placental transfer of maternally derived fatty acids, that the placenta is a major source of free fatty acids for the developing fetuses in the guinea pig (Thomas and Lowry 1986). Furthermore, free fatty acids are important for the maturation of pre-adipocytes (Abumrad et al., 1991). In fact, one fatty acid in particular, arachidonic acid, has been shown to be critical for the maturation of pre-adipocyte cells lines such as Ob1771 and 3T3-F442A, and rat adipose precursor cells in primary culture (Gaillard et al., 1989). What is most interesting about this study is that cats lack the enzyme delta-6-desaturase, an important enzyme in the pathway for generating arachidonic acid *de novo*. Therefore must rely on exogenous

sources, such as diet or maternal delivery, in the case of a developing kitten, to supply them with arachidonic acid. In the case of a LPL deficient cat, which is unable to efficiently transport long chained fatty acids, including arachidonic acid, they might reduce the chance that pre-adipocytes will achieve the necessary stimulus to mature.

Before these hypotheses can be fully tested, it is first necessary to establish the techniques to accurately measure the body composition of the cats. In this chapter I will describe the approach I have employed to analyze the body composition of these cats. By using a deuterium dilution method that has been used in studies of body composition of many other species (Munday, 1994), I evaluate the amount of body fat that each individual cat has and then correlate this with the genotype of each cat. These data also provide the foundation for hypotheses to help delineate the role of LPL in the increased mortality and growth curves previously described in this colony of cats.

Methods

Deuterium dilution analysis.

The cats were food and water deprived for 24 hours, a sample of blood was taken for a base line measurement via the jugular vein. A bolus of 0.1 to 0.4 g/Kg body weight of D_2O 99.99% pure, (Metabolic Solutions Inc.) was injected and allowed to equilibrate for 4 hours. During this time the cats remained food and water deprived. After the D_2O had equilibrated, a venous blood sample was taken and analyzed.

The method used to determine the body composition was adapted from Ballevre et al., 1994 and Schoeller et al., 1980. The deuterium (D_2O) dilution method was performed in most instances two separate times with two methods of measuring the deuterium. The data presented here was generated using mass spectrophotometry to determine which portion of the water molecules present in the plasma was in the form of D_2O and was performed in the laboratory of Dr. Quinton Rogers according to Byers et al., (1979).

Results

The deuterium dilution method capitalizes on the fact that muscle tissue has a greater water content than does adipose tissue and when injecting a tracer such as deuterium it will equilibrate into the various tissues at different rates depending on the water content of the tissue. After equilibration, a blood sample is taken and analyzed for the proportion of water in the form of deuterium compared to that of a base-line sample. When entered into a formula that has been previously derived by (Schoeller et al., 1980) results in a reasonable estimate of the percentage of the lean body mass that is composed of adipose tissue.

Normal cats that were tested using this method had 18.6 ± 5.6% (n=12) of the lean body mass in the form of adipose tissue (Table 4.1). The cats heterozygous for the LPL mutation had a somewhat lower percent body fat (PBF) 15.2 ± 4.3% (n=9). The most dramatic difference was observed in the cats that are homozygous for the mutation in the LPL gene (n=14), which had 6.1 ± 5.4% body fat ($p < 0.00001$ compared to normal). Furthermore, cats that are homozygotes and were born to a homozygous queen (n=6), had the lowest PBF, 2.0 ± 2.5% compared to 9.2 ± 4.9% for homozygotes born to heterozygous queens ($p < 0.01$) (Table 4.2).

Cat	Sex	LPL-Genotype	Dam's-genotype	Sire's - genotype	Body Wt (kg)	% Body Fat
LPL +/+ (n=12)						
Marie	F	"+/+"	"+/+"	"+/-"	2.7	17.4
Panda	F	"+/+"	"+/+"	"+/-"	2.7	12.2
Shadow	F	"+/+"	"+/+"	"+/+"	3.6	22.5
Shannon	F	"+/+"	"+/-"	"+/-"	2.4	16.5
#04	M	"+/+"	"+/+"	"+/-"	4.2	16.6
#05	M	"+/+"	"+/+"	"+/-"	4.1	12.5
94-569	M	"+/+"	"+/+"	"+/+"	4.3	15.8
94-575	M	"+/+"	"+/+"	"+/+"	5.3	23.8
94-576	M	"+/+"	"+/+"	"+/+"	4.6	20.1
94-577	M	"+/+"	"+/+"	"+/+"	5.5	25.8
Jackson	M	"+/+"	"+/+"	"+/-"	4.3	11.2
Timmy	M	"+/+"	"+/-"	"+/-"	5.4	28.7
				Mean:	4.1	**18.6**
				Std.dev.:	1.1	5.6
LPL+/- (n=9)						
Alexandra	F	"+/-"	"+/-"	"+/-"	2.3	16.5
Etcetera	F	"+/-"	"+/+"	"-/-"	3.3	20.7
Pouncival	F	"+/-"	"+/+"	"-/-"	3.3	21.5
Tantomile	F	"+/-"	"+/+"	"-/-"	2.7	14.5
Bob	M	"+/-"	"+/+"	"-/-"	3.7	9.6
Griz	M	"+/-"	"+/+"	"-/-"	4.0	12.4
Kiwi	M	"+/-"	"+/-"	"+/-"	3.9	17.3
Plato	M	"+/-"	"+/+"	"+/-"	3.5	9.4
Victor	M	"+/-"	"+/+"	"+/-"	4.9	15.0
				Mean:	3.5	**15.2**
				Std.dev.:	0.8	4.3
LPL-/- (n=14)						
Kea	F	"-/-"	"-/-"	?	2.5	0.4
Macavity	F	"-/-"	"-/-"	"-/-"	2.2	4.2
Priscilla	F	"-/-"	"-/-"	"-/-"	2.3	0.0
Griddlebone II	M	"-/-"	"-/-"	"+/-"	4.2	6.1
Tumblebrutus	M	"-/-"	"-/-"	"-/-"	3.1	0.0
Rumpleteaser	M	"-/-"	"-/-"	"-/-"	3.2	1.6
95C-002	F	"-/-"	"+/-"	"-/-"	2.5	12.2
95C-004	F	"-/-"	"+/-"	"-/-"	2.8	12.3
Victoria	F	"-/-"	"+/-"	"+/-"	2.9	10.5
95C-003	M	"-/-"	"+/-"	"-/-"	5.0	13.6
Casey	M	"-/-"	"+/-"	"-/-"	3.8	6.9
Milford	M	"-/-"	"+/-"	"-/-"	4.2	14.0
Rum Tum	M	"-/-"	?	?	2.2	1.5
Sammy	M	"-/-"	"+/-"	"-/-"	3.2	2.6
				Mean:	3.2	**6.1**
				Std.dev.:	0.9	5.4

Table 4.1. Body composition analysis of cats as assessed by a deuterium dilution method which estimates the percent of lean body mass which is composed of adipose tissue. The homozygotes on average have much less body fat than either normal or heterozygotes (6.1% vs. 18.6% or 15.2% respectively).

Kitten Genotype	Mean % body fat	p-value	
"+ / +" (n=12)	18.6		
"+ / -" (n=9)	15.2	"+/+" vs. "+/-"	0.15
"- / -" (n=14)	6.1	"+/+" vs. "-/-"	0.000006
"- / -" born to -/- Dam (n=6)	2.0	"+/+" vs. "-/-"	0.000004

Dam's genotype (w/in -/- kitten group)		p-value
"+ / -" (n=7)	9.2	"+/- Dam vs -/- Dam
"- / -" (n=6)	2.0	0.007

Table 4.2. Statistical analysis of the body composition data indicates that the homozygote cats have a significant reduction in the percent body fat, regardless of gender. Furthermore, the kittens that are born to homozygous queens have a greater reduction of body fat.

Discussion

Lipoproteins are the major carriers of triglycerides and cholesterol and the major purpose of transporting lipids is for their utilization in tissues. Fat, in the form of triglycerides, has a high caloric content on a dry matter basis so it seems reasonable to think that over the course of evolution, mammalian development has been favored an organism that could consume as much food as possible while it is available and store it so that it could be readily available when these resources are absent. LPL plays a critical role in this process, in which hydrolysis of triglycerides allows for critical energy sources (i.e. fatty acids) to be metabolized by muscle tissues or stored in adipose tissue.

The cat is somewhat peculiar in the way it utilizes energy during reproduction and is interrelated with its breeding habits. In the introduction I discussed the unique breeding habits of the cat and how they will be involved in later discussions. I mentioned that the reclusive nature of the queen has influenced the way she nourishes the developing fetuses. At the beginning of pregnancy, a normal queen will usually store more energy than is needed for her immediate metabolic needs, which will then be made available at a later critical moment, just prior to parturition. In the later stages of gestation she relies on these energy stores, in the form of adipose deposits, to be liberated for her metabolic needs as well as for those of her litter. Most mammalian species gain weight in accordance with the developing offspring, which when plotted, is an exponential curve. The cat is unique in that it gains weight in a linear fashion, perhaps reflecting the storage of adipose tissue early in gestation and the release of these stores just before delivery.

This energy, when stored early in pregnancy, will then keep the mother and kittens supplied with energy through out late gestation and into the nursing stage. In the context of LPL deficiency, it is possible that the queen is incapable of liberating an appreciable amount of free fatty acids, hence preventing her from storing enough energy early in gestation which would in turn result in the increased mortality I have described in chapter 2. Furthermore, this queen would also lack the ability to supply the developing fetus, via the placenta, with a sufficient amount of free fatty acids needed for the maturation of adipocytes. Similarly, the growing kitten may not be capable of liberating enough fatty acids that can be used for incorporation into adipose tissue, resulting in the appearance of a reduced growth rate. It is possible that, with the absence of adipose tissue being generated in the homozygous kitten, that there is as much lean tissue as in a normal sibling.

One explanation may be that the local delivery of fatty acids across the placenta via LPL is critical for the proper development of the kittens in utero (Thomas and Lowy, 1983). Without LPL activity in the placenta of homozygous queens there will not be a sufficient amount of fatty acids to meet the requirements of the developing kitten (e.g. increased stillbirth rates). Also, in the proportion of kittens that do manage to survive to birth, they may be deprived of enough fatty acids to allow for the proper maturation of adipocytes (e.g. reduced percent body fat). This hypothesis is supported by the findings that LPL plays an important role in the differentiation of pre-adipocytes into mature adipocytes (Gaillard et al., 1989) and that LPL expression has been used as a marker for adipocyte differentiation (Abumrad et al., 1991). Moreover, in cell-culture studies, the addition of long chain fatty acids (such as those that

LPL would normally liberate) are critical for differentiation of pre-adipocytes into mature adipocytes (Gaillard et al., 1989). As such, a homozygous LPL deficient kitten, developing *in utero* of a LPL deficient queen, may be in the unique situation where the queen, incapable of delivering an adequate amount of free fatty acids via the placenta, limits the pre-adipocyte maturation of all kittens. In addition, a homozygous queen in combination with a developing homozygous kitten, without a *de novo* pathway to generate fatty acids such as arachidonic acid, will further limit the maturation of adipocytes in that kitten. This may be an example of an *in utero* environmental effect having a profound impact on the adult status of an animal.

In this chapter I have documented that LPL deficient cats have a marked reduction in body fat. Moreover, there seems to be a maternal influence on the severity of the reduction in percent body fat. Future studies will focus on the mechanism by which the *In utero* environment is capable of influencing the ultimate adult body composition of the kitten.

Chapter 5 : Diet-Induced Atherosclerosis in the Domestic Cat

Foreword

In this chapter I have established the foundation for additional studies to investigate the role of LPL in the progression of atherosclerotic lesions by determining if the normal cat is susceptible to diet induced atherosclerosis. This work was conducted in collaboration with Dr. Bruce McManus's laboratory from the Department of Pathology, UBC, at St. Paul's Hospital. This study was designed and implemented by me. I received assistance in designing the atherogenic diet from Dr. Quinton Rogers of the Department of Veterinary Sciences at the University of California at Davis. I also developed the sacrifice protocol and all animal sacrifices were conducted by me and the animal care technicians at UBC. All dissections were conducted by me and Janet McManus, sectioning of some organs and vessels were conducted by Susie Clee and Kate Ashbourne. Subsequent pathological sample preparation was performed by Janet McManus and all histological samples were reviewed by an experienced pathologist, Dr. Bruce McManus. This chapter forms the basis of a manuscript which has been accepted for publication in the journal of *Laboratory Investigation* (Ginzinger et al., In press).

Introduction

Evidence has accumulated over many years implicating elevated levels of serum low density lipoprotein (LDL) cholesterol as a risk factor for premature atherosclerotic coronary artery disease (CAD) (Goldstein and Brown, 1977); conversely, it is well established that elevated levels of high density lipoprotein (HDL) cholesterol are protective against CAD. More recently, the lipid-rich very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) particles and post-prandial lipoproteins, together termed triglyceride rich lipoproteins (TRL) have been implicated as additional risk factors. Yet it is still somewhat controversial as to what role these particles play in the atherosclerotic process (Zilversmit et al, 1979; Havel, 1994). Furthermore, it has been demonstrated that polymorphisms located near the LPL gene are associated with increased severity of CAD (Wang et al, 1996). One particular mutation (Asn291Ser), estimated to occur at a frequency of 5% in the general population, is associated with reduced levels of HDL cholesterol in patients with premature atherosclerosis (Reymer et al, 1995). In people that are carriers of mutations in the LPL gene, it has been demonstrated that they have a significant delay in the clearance of triglyceride rich lipoproteins (Miesenbock et al, 1993). Clearly, these studies suggest that there is a need to further evaluate the role of LPL in the atherosclerotic process.

In order to further characterize the LPL deficient cat as an animal model for atherosclerosis, and its relevance to the human, it was necessary to first determine the natural susceptibility to atherosclerosis in the normal cat.

Early attempts to induce atherosclerosis in the domestic cat by feeding a high fat, high cholesterol diet had not been very successful (Cirio L, 1928; Yuasa D, 1928). From these initial studies and a study focused on the incidence of atherosclerotic lesions in older animals (Lindsay and Chaikoff, 1955), it was concluded that the cat was not particularly susceptible, if not resistant, to atherosclerosis. However, in 1970, Manning and Clarkson were able to convincingly demonstrate that diet-induced atherosclerosis was possible in the cat fed a cholesterol-enriched diet. Still, the domestic cat has not subsequently been utilized as an animal model for atherosclerosis research.

The cat can be used to study diet-induced atherosclerosis without additional manipulation necessary with other carnivorous animals. For example, canines are only susceptible to atherosclerosis when their thyroid function is suppressed (Mahley, et al., 1974; Geer, et al., 1965). The mouse is commonly used in atherosclerosis studies (Breslow, 1993), but only inbred strains such as the C57BL/6J (Paigan, et al., 1985) or transgenic models like those with the LDL receptor knock out (Ishibashsi et al, 1993), Ath-1 gene (Paigen et al, 1987), apoE deficient (Plump et al, 1992), or cholesterol ester transferase protein (Marotti et al, 1993) are particularly susceptible to lesion development. Other than inbred or transgenic strains of mice, the mouse is naturally resistant to diet-induced atherosclerosis. The rabbit has also been very useful for studies of atherosclerosis and lesions are easily induced with cholesterol feeding (Buja et al, 1983; Imai et al, 1966). However, the rabbit is a strict herbivore and does not naturally eat a "Western style" diet.

The cat, on the other hand, is a strict carnivore and typically eats a diet very high in protein and fat, one which would most closely resemble that of "at

risk" North Americans. Furthermore, detailed lipid profiles (Demacker et al, 1987; and chapter 3) suggest that the cat has a profile similar to that of humans, more so than several other animal models. However, the major difference between the feline and human lipid profiles is that the cat has over 2.5 times as much HDL cholesterol than LDL cholesterol and is CETP deficient.

The size of vessels in the cat make them very amenable to a combination of structural, functional, metabolic and molecular evaluations and to genetic or other manipulations. Thus, the cat may be a valuable new model to complement studies of atherosclerosis in other model systems.

In consideration of the single previously published report on diet-induced atherosclerosis in the cat, we felt it was imperative to corroborate and extend the findings of Manning and Clarkson (1970). Moreover, while confirming that diet-induced atherosclerosis is feasible in the normal cat, before further pursuit of the LPL deficient cat as a model, concurrent qualitative, quantitative, and distributive assessments of atherosclerotic lesions in the normal cat were performed. For this report, we used a diet enriched in cholesterol to assess feline susceptibility to atherosclerotic lesion formation. This has allowed us to perform a thorough pathological analysis of induced atherosclerotic lesions. As well, we obtained lipid profiles that will be useful in identifying atherogenic lipoprotein components predictive of lesion severity. These studies will form the basis of an in-depth analysis of genetic contributions to the atherosclerotic process in this model system.

Methods

Animals used in this study were mixed breed short or long hair domestic cats ranging in age from 1 to 4 years old. Four of the cats used in this study were from the cat facility at University of California, Davis, CA, which is a pathogen free facility. All other cats were obtained from the SPCA. This study followed accepted procedures reviewed and established by the Committee on Animal Care at the University of British Columbia.

The cholesterol-enriched diet used in these studies was formulated by Bioserv Inc. Frenchtown NJ as follows: 30% fat (20% saturated, 10% unsaturated), 20% carbohydrate, 30% protein (casein), 10% moisture and 3% cholesterol (by weight) (Figure 1). The food was pelleted (1/8" in diameter by 1/2" long) and provided ad libitum. To enhance the palatability of the diet, it was sprayed with chicken broth (Campbell's Soup Co.) and dried in an oven at a low temperature for approximately 20 minutes to reduce the moisture and prolong shelf-life. The cats were weaned onto the diet over a 4-week period in which 1/4 of their previous diet (Whiskas with tuna) was replaced each week with the atherogenic diet. The cats were then allowed to proceed on the diet for 2, 3, 4, 6, 7, or 8 months, after which they were sacrificed.

Body weights were recorded weekly over the course of the experiment. Blood samples were taken via the jugular vein on a monthly basis after an overnight fast. Blood was centrifuged at 2000 rpm for 15 minutes, plasma was removed, aliquoted and stored at -800C until lipid measurements were performed. Total and HDL-cholesterol were measured by standardized kits (Boehringer-Mannheim). LDL-cholesterol was calculated using the standard

formula TC - (HDL + [Triglycerides/2.2]).

At the end of the indicated time periods on the atherogenic diet, the animals were sedated with Ketamine and blood was drawn for lipid measurements. They were then maintained in a sedated state by administering halothane and isopentane while the animal was exsanguinated via a heart puncture. An intravenous saline drip was used to flush as much blood as possible from the tissues before sectioning. The cats were finally killed by the administration of a lethal dose of sodium pentobarbital.

Necropsies were performed immediately after sacrifice according to a detailed triage protocol (Figure 5.1). Most tissues were divided into 3 pieces which were 1) fixed in phosphate-buffered formalin and processed in paraffin, 2) flash frozen, or 3) flash frozen in OCTTM. Small subsets of arterial and other tissues were fixed in glutaraldehyde, and formalin-fixed and frozen in OCTTM. All frozen samples are stored at -80°C.

The arterial tree was photographed and sectioned as illustrated in Figure 5.1. The thoracic aorta was opened by anterior (ventral) midline incision to photograph the intimal surface enface. Following standard processing, samples from the aorta were stained with hematoxylin and eosin to define general architecture including foam cells and atheromata, and with Movat's pentachrome to identify elastin, collagen, glycosaminoglycans, and smooth muscle cells. Oil red O (ORO) was used to stain for neutral lipid.

All tissues were evaluated blindly by an experienced cardiovascular pathologist and graded for presence of foam cells or lipid-rich lesions (0-5+/5+). Whereby, a 0 represents no foam cells and a 5+ represents the most foam cells or lesions. In addition, all vessels were qualitatively scored (0-5+/5+) for intimal

thickening. Mean values were calculated for the entire vascular tree.

Morphometric analysis of intimal area was performed on all vessel segments stained with Movat's pentachrome. Sections were viewed on a Nikon microscope fitted with a camera lucida attachment, projected onto a Summa Sketch II tablet (Summagraphics, Seymour CT) calibrated for the Bioquant System IV morphometry program (R&M Biometrics Inc. Nashville CT) on a Packard Bell Pack Mate I computer. Tracings were made of the length of the internal elastic lamina, length of laminae adjacent to thickened intima, and the total area occupied by intimal thickening in any part of the intima in which the intimal thickness exceeded 0.015 mm. Arterial branchpoints were excluded from analysis. The mean intimal area was calculated for each section and the results averaged for each site of the vascular tree including brachiocephalic, common carotid, iliac/femoral, and subclavian vessels and the ascending, descending, thoracic, abdominal, and lower abdominal aorta (Figure 5.1). The averages from each site were then meaned to provide an index of intimal area for the entire vascular tree, thus providing a figure representative of the intimal thickening throughout the arterial tree for each cat.

Excess lipid content was evaluated utilizing Oil red O staining in a quantitative manner utilizing the Bioview Color Image Analysis System. The system was adjusted to a reference sample prior to each set of measurements. The total area of Oil red O positivity (μm2) was measured by spectral analysis of an entire aortic and brachiocephalic cross-section of two tissue sections from each vascular region under study and expressed as μm2 per μm length of luminal endothelial surface.

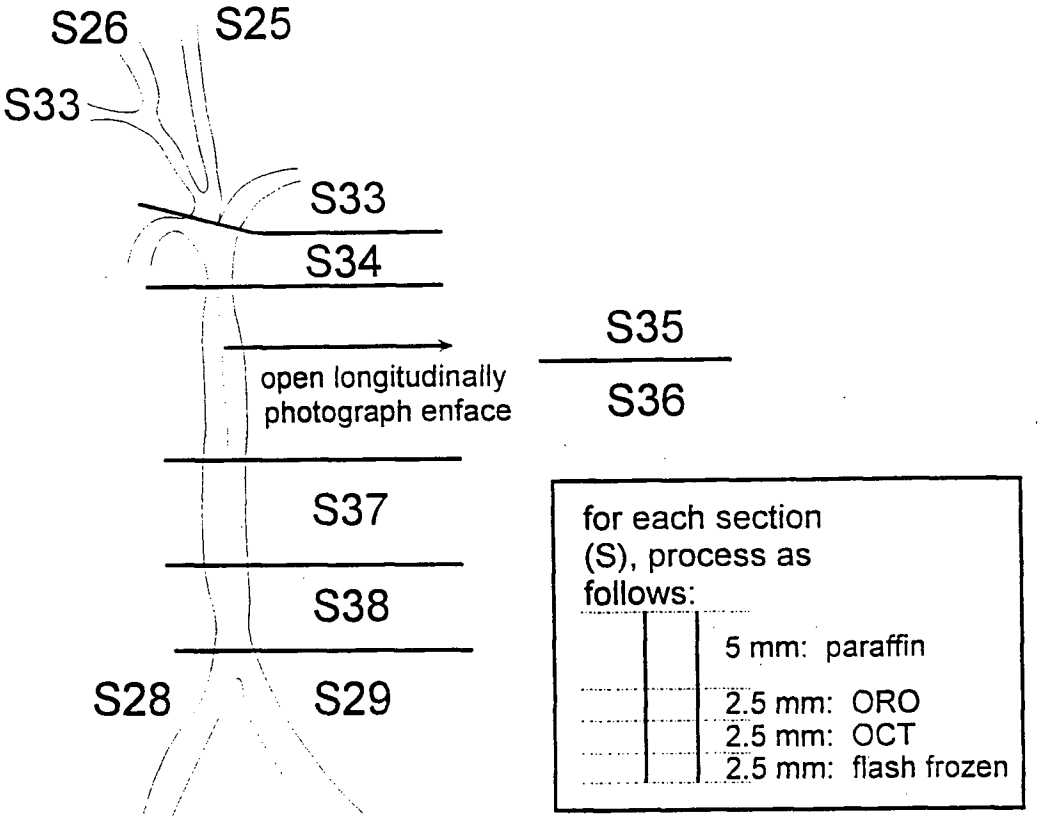

Figure 5.1. Triage protocol for the arterial tree. Sections S35 and S36 were cut open longitudinally along the vertebral surface and photographed before further sectioning

Results

Diet

The cholesterol-enriched diet included an addition of 3% cholesterol and a moderate increase of fat to 30% which is not unusually high for a normal cat diet. (Figure 5.2). All cats tolerated the cholesterol-enriched diet quite well. In the first 3 months, a taurine deficiency was identified in the formulation of the cat diet and the palatability of the diet was sub-optimal. From 3 months onwards, taurine was supplemented in the drinking water, and chicken broth was added to the food. In the remainder of the experiment, the cats found the diet more appealing and body weights were generally maintained (Figure 5.3).

Serum lipids

Blood lipid values were significantly increased over baseline values ($p<0.05$) for all cats on the cholesterol-enriched diet (Table 5.1). Calculated LDL values were very low or could not be detected at baseline, however the mean levels following the cholesterol-enriched diet increased significantly for nearly all cats. One individual cats had a 1000% mean increase or more in total triglyceride levels and in TC:HDL ratio. The cats could be divided into two groups; the "responders" or "non-responders" based on serum lipid response to the diet. (Figure 5.4). When the two groups were compared, all values except for the HDL-cholesterol and the LDL-cholesterol were statistically increased in the responder group (Figure 5.5).

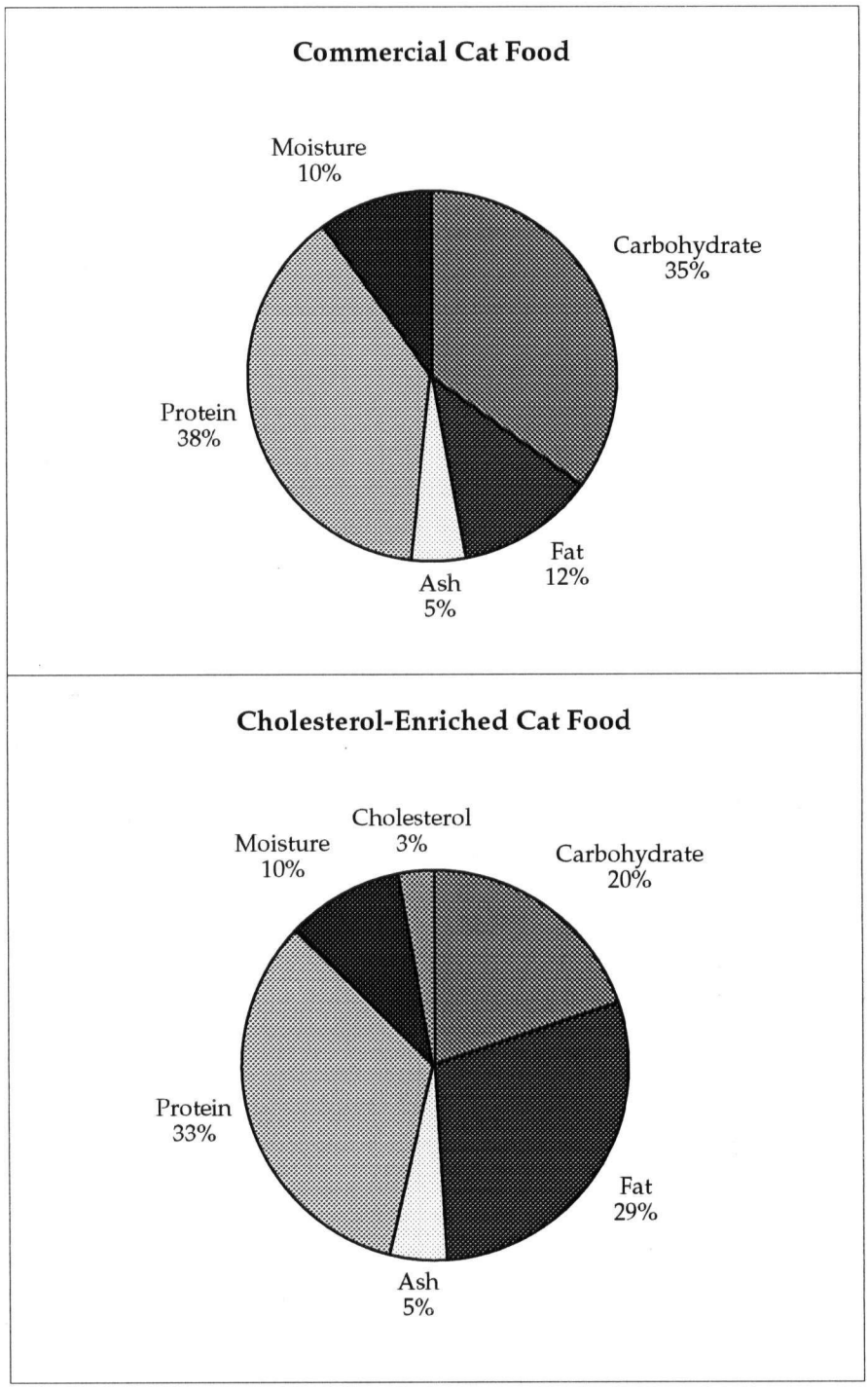

Figure 5.2. Percent composition of nutrients in the normal (Whiska's with Tuna) and cholesterol enriched (Bioserv Inc.) diets of the cat.

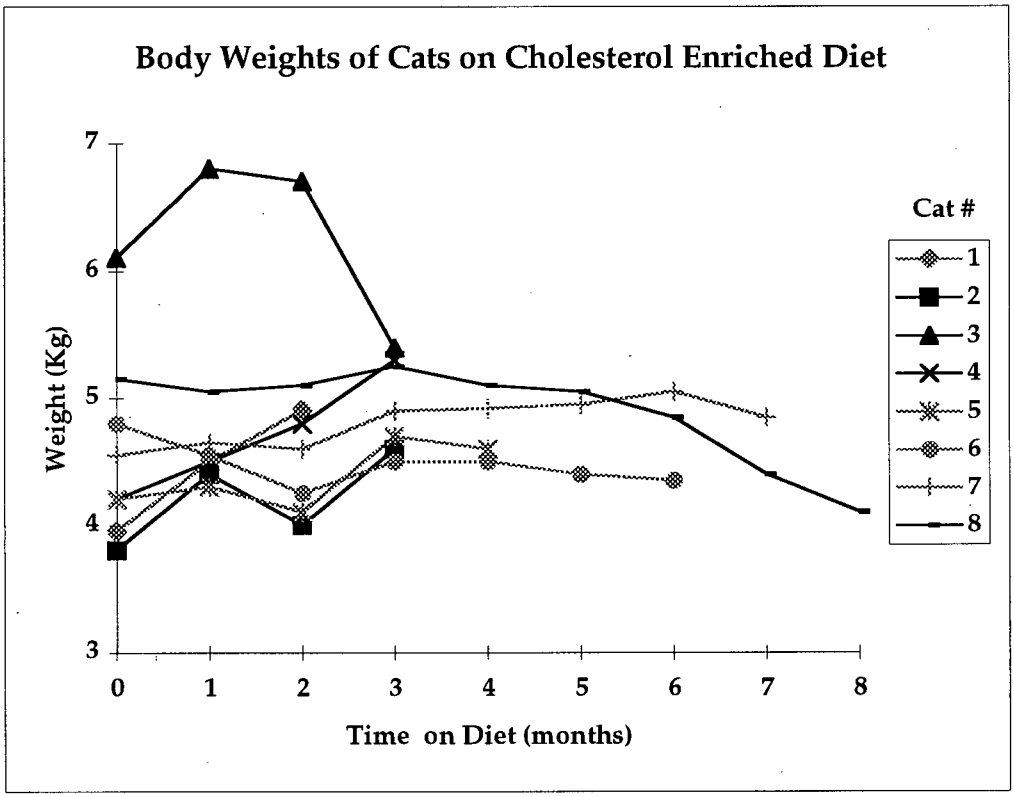

Figure 5.3. Body weights of cats on the cholesterol-enriched diet. Most cats tolerated the diet well as reflected in the time course of body weights.

Table 5.1. Serum lipid values at baseline and following a cholesterol enriched diet for the specified time.

		Total Cholesterol (g/L)			HDL-Cholesterol (g/L)		TC : HDL Ratio			Triglycerides (g/L)			LDL (g/L)
Cat	Time on Diet (mo.)	Baseline	Mean Post-Diet	% increase	Baseline	Mean Post-Diet	Baseline	Mean Post-Diet	% increase	Baseline	Mean Post-Diet	% increase	
1	2	87	540	521	81	149	1.07	3.59	234	14	157	1018	320
2	3	55	357	548	55	162	1	2.21	534	17	43	371	475
3	3	106	959	805	91	76	1.116	15.9	1265	34	154	352	107
4	3.5	64	250	290	64	98	1	2.61	161	18	98	446	69
5	4	100	528	428	89	79	1.12	7.61	577	26	49	88	10
6	6	131	175	40	130	151	1.01	1.16	15	41	29	-28	427
7	7	45	186	310	50	87	0.9	2.24	149	26	66	155	4
8	8	137	160	17	127	141	1.08	1.14	6	32	33	2	815
Mean		**90.6**	**394.3**	**370.2**	**85.9**	**117.7**	**1.04**	**4.56**	**367.8**	**26**	**78.6**	**300.4**	**278.4**
Std. Dev.		**34.2**	**274.4**	**264.7**	**30.3**	**36.3**	**0.08**	**5.03**	**421**	**9.4**	**52**	**339.5**	**285.5**

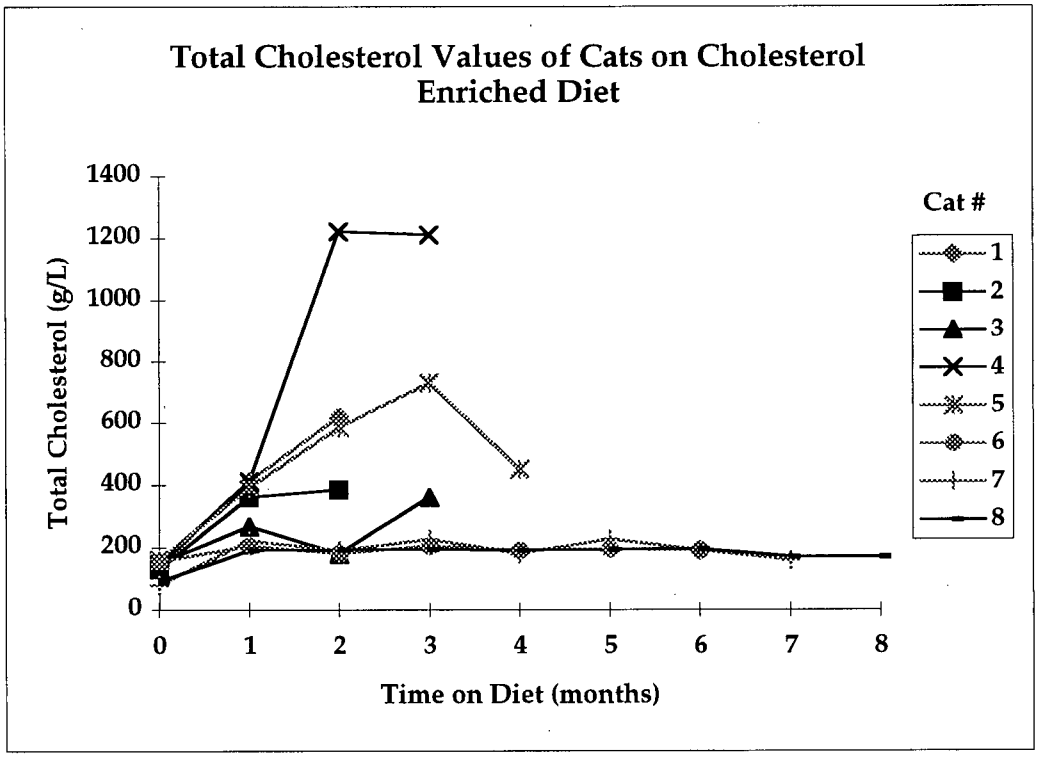

Figure 5.4. Total cholesterol levels of cats on the cholesterol-enriched diet. Blood was taken once a month over the time course the cats were on the diet. Note cats 6 and 8 appear to be non-responsive to the diet as compared with the response of cats 1-5 and 7.

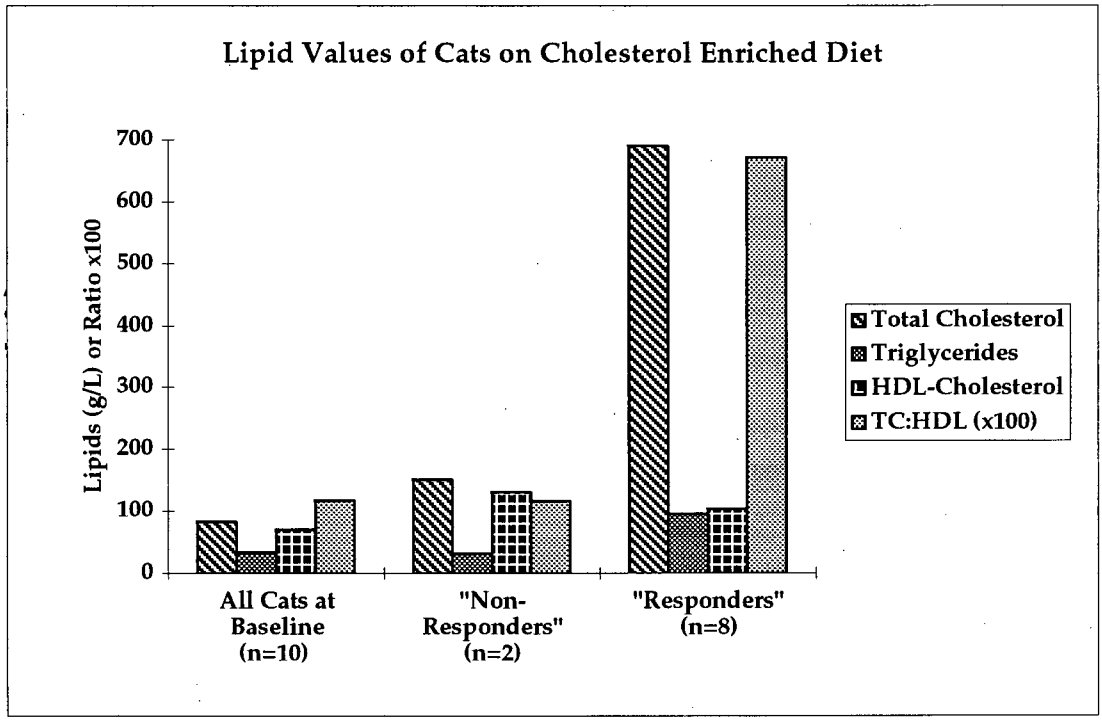

Figure 5.5. Blood lipid increases due to the cholesterol enriched diet with values at baseline for all cats and the "non-responders" (cats 6 and 8) and "responders" (cats 1-5 and 7) differentiated. Total cholesterol, triglycerides, HDL-cholesterol and the total cholesterol (TC):HDL-cholesterol in the responders are all increased significantly over the baseline value taken before the cats were on the diet as well as over the non-responder cats.

Qualitative and semi-quantitative pathologic analysis

The organs primarily affected by the diet were the adrenal glands, kidneys, liver, and spleen (Table 5.2). The organs from cats on a normal diet had no increase in foam cells or fat insudation as compared to the cats on the cholesterol-enriched diet (Figure 5.6). These observations are concordant with those of Manning and Clarkson (1970).

When the vessel tree was examined histologically, all vessels had superficial intimal foam cells and intimal thickening present in cats on the cholesterol-enriched diet (Figure 5.6 and 5.7). The intimal thickening ranged from very mild to notable localized lesions in the cholesterol-fed cats. The intima was visible in cats on the normal diet, however the tissue was matrix-rich, and foam cells were not present (Figure 5.6 and 5.7). The foam cell grade was statistically higher in the responder cats than the non-responders (0.69 vs. 0.10, $p=0.001$). However the intimal thickening grade was not significantly different (0.76 vs. 0.46, $p=0.14$).

Quantitative morphometric analysis

Quantitative morphometric analysis of the vessels performed to obtain intimal area revealed that those cats on a cholesterol-enriched diet had a mean measurable intima that was greater than cats on a normal diet ($p<0.04$) (Table 5.3). This was true for multiple sites along the aorta (the ascending, thoracic and the lower abdominal), as well as the brachiocephalic/subclavian vessels (Figure 5.8). Disease was not prominent in the carotid and femoral/iliac vessels. When the responders were compared to the non-responders,

Cat	adrenal	kidney	liver	spleen
1	0	3	5	4
2	2	2	0	0.1
3	3	3	3	0
4	3	3	5	3
5	2	0.5	1	2
6	0	0.5	3	0.5
7	0	0.5	3	0.5
8	1	0.5	2	0
Mean of cats on cholesterol-enriched diet	1.375	1.625	2.75	1.2625
1N	0	0	0	0
2N	0	0	0	0
Mean of cats on normal diet	0	0	0	0
p Value	0.015	0.004	0.0015	0.02718

Table 5.2. Lipid infiltration grade (0-5+/5+) in the organs after a cholesterol enriched diet

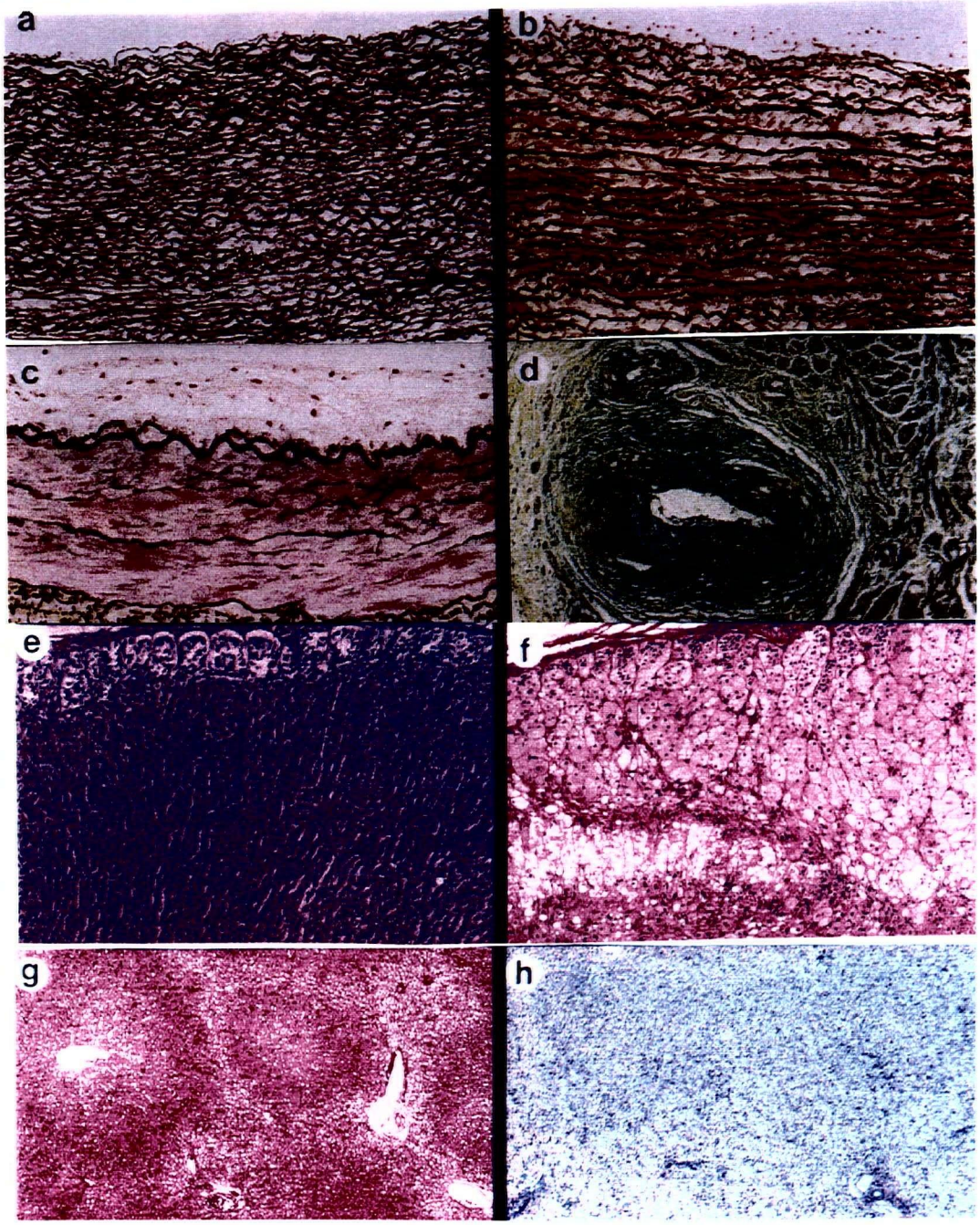

Figure 5.6. There is minimal intimal thickening in the cat on the normal diet (a), and the significantly increased thickness of aorta in the cat on a cholesterol-enriched diet (b). The femoral artery just below the aortic bifurcation in a cat fed the cholesterol-enriched diet for four months has an eccentric atheromatous lesion present (c). The same cat also had a lesion in the brachiocephalic trunk (d). Adrenal (e,f) and Liver (g,h) sections from cats fed a normal diet (e,g) and a cholesterol-enriched diet (f, h). Note the apparently increased steroidogenesis (adenomatous appearance) in the sections from the cats fed the cholesterol-enriched diet. Section of ascending aorta from a cat on a normal diet (e) and from one on the cholesterol-enriched diet (f). Hematoxylin and eosin, x50 (e,f,g,h); Movat's pentachrome, x33 (a,b,c,d).

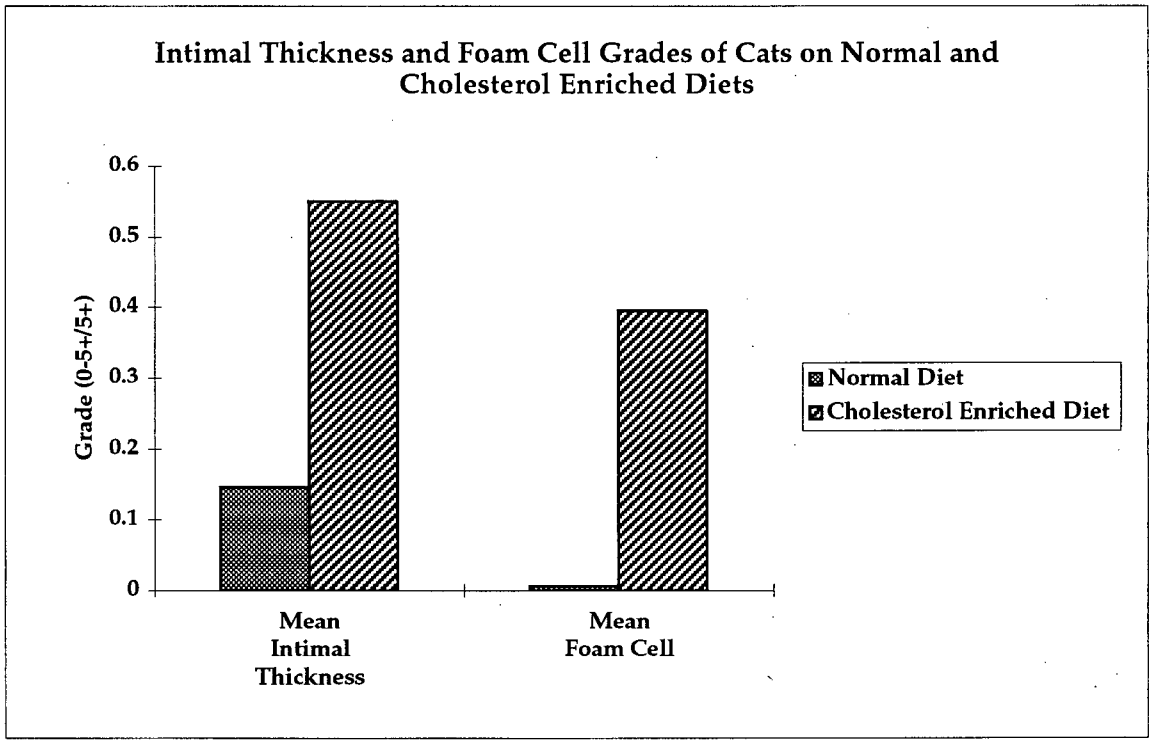

Figure 5.7. Intimal thickening (grade 0-5+/5+) and foam cell grade (0-5+/5+) in cats on a normal diet and on the cholesterol-enriched diet. Both thickness and foam cells are increased significantly in the cats on the cholesterol-enriched diet ($p<0.05$).

Cat	Time on Diet	Ascending Aorta	Decending Aorta	Thoracic Aorta	Abdominal Aorta	Lower Abdominal Aorta	Mean Aorta	Brachiocephalic/ Subclavian	Femoral	Carotid	Mean Vessel
1N	normal diet	0	0		0	0	0	0		0	0
2N	normal diet	0	0.021	0	0.053	0.006	0.019	0.009	0.005	0.004	0.016
Mean		**0**	**0.01**	**0**	**0.0265**	**0.003**	**0.0095**	**0.0045**	**0.005**	**0.002**	**0.008**
Std.Dev.		**0**	**0.015**	**0**	**0.0374**	**0.0042**	**0.0134**	**0.0064**		**0.0028**	**0.0113**
	Chol.-enriched										
1	2			0.01	0.045	0.1	0.032	0.135	0.0076	0	0.05
2	3	0	0	0	0.084	0.073	0.032	0.02	0	0	0.022
3	3	0.13	0.028	0.068	0.073	0.02	0.064	0.07	0.05	0	0.055
4	3.5	0.062	0.032	0.039	0.071	0.076	0.056	0.066	0	0	0.043
5	4	0.083	0	0	0.0043	0.0028	0.018	0.003	0	0	0.011
6	6	0.219	0	0	0.0038	0.0063	0.0458	0.0061	0	0	0.029
7	7	0.368	0.072	0	0		0.11	0.088	0	0	0.076
8	8	0.043	0	0	0.035	0	0.016	0.01	0.004	0	0.011
Mean		**0.1293**	**0.0189**	**0.0146**	**0.0395**	**0.0397**	**0.0467**	**0.0498**	**0.0077**	**0.0000**	**0.0371**
Std Dev.		**0.1264**	**0.0274**	**0.0254**	**0.0343**	**0.0418**	**0.0307**	**0.0478**	**0.0173**	**0.0000**	**0.0230**

Table 5.3. Quantitative morphometric analysis of the blood vessels.

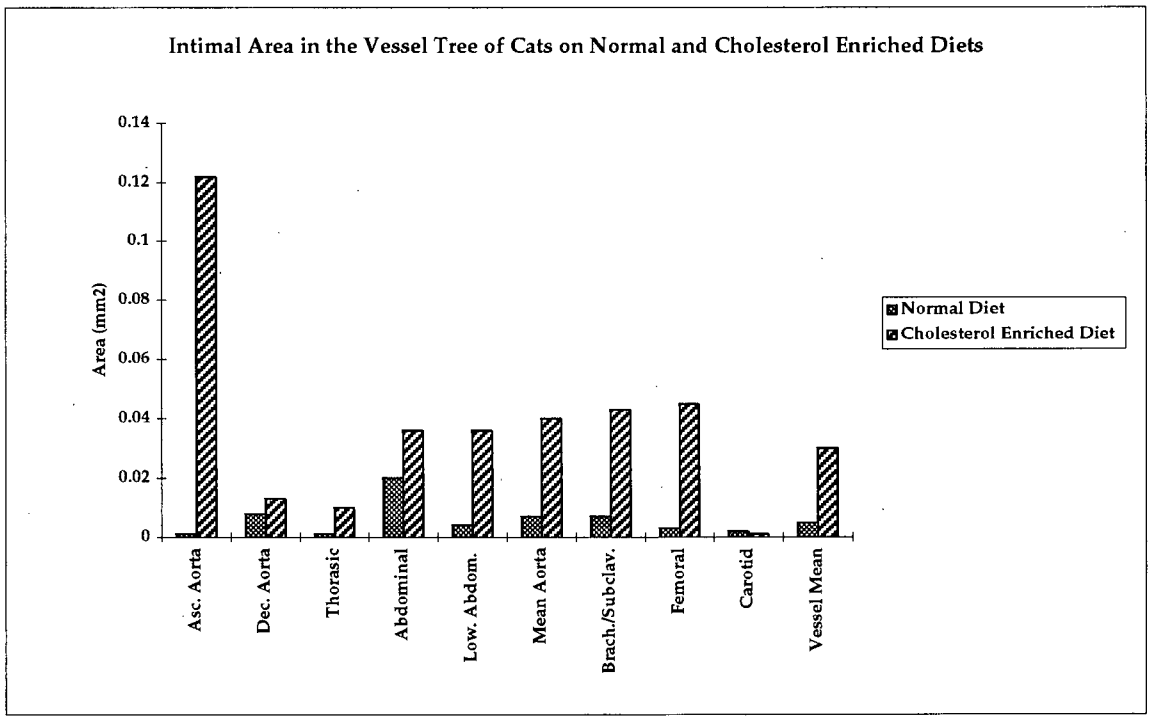

Figure 5.8. Intimal area (mm2) in the vessel tree from cats on a normal diet and on a cholesterol-enriched diet. Sites from the vessel tree were measured, with the ascending aorta, lower abdominal aorta, and brachiocephalic/subclavian sites having the most significant differences present. The mean aortic measurement and the mean vessel measurement were statistically significant as well ($p<0.05$).

the differences were site-dependent. In particular, the thoracic, abdominal, and lower abdominal demonstrated a statistically significant increase in measurable intimal area in the responders as compared to the non-responders. All other sites showed a similar trend except for the ascending aorta.

Lipid content of vessels

The ORO stain was utilized to localize lipid deposits in the aorta wall. All sections showed positive staining of lipids in adventitial fat deposits, a useful set of internal positive controls (Figure 5.9). The total areas of positive staining (μm2) in aortic intima per μm of length of luminal endothelial surface were calculated. The cholesterol-fed cats had a higher amount of ORO staining in their aortic sections than did the cats on the normal diet (20.7 vs. 0.3; $p<0.05$). The responders also showed a trend towards an increase over the non-responders (29.5 vs. 7.9; $p=0.12$), but this did not reach statistical significance.

Correlations

Correlations were performed between measured blood lipid moieties and vessel wall lesion assessment (Table 5.4). The highest correlations were evident for the % increase in total cholesterol, triglycerides, and % increase in triglycerides with the lesion assessments. In particular, the intimal thickness and foam cell grades as well as the quantitative measurements in the thoracic and lower abdominal aorta, the brachiocephalic/subclavian, and the mean for the vessel tree were highly correlated with these lipid measurements.

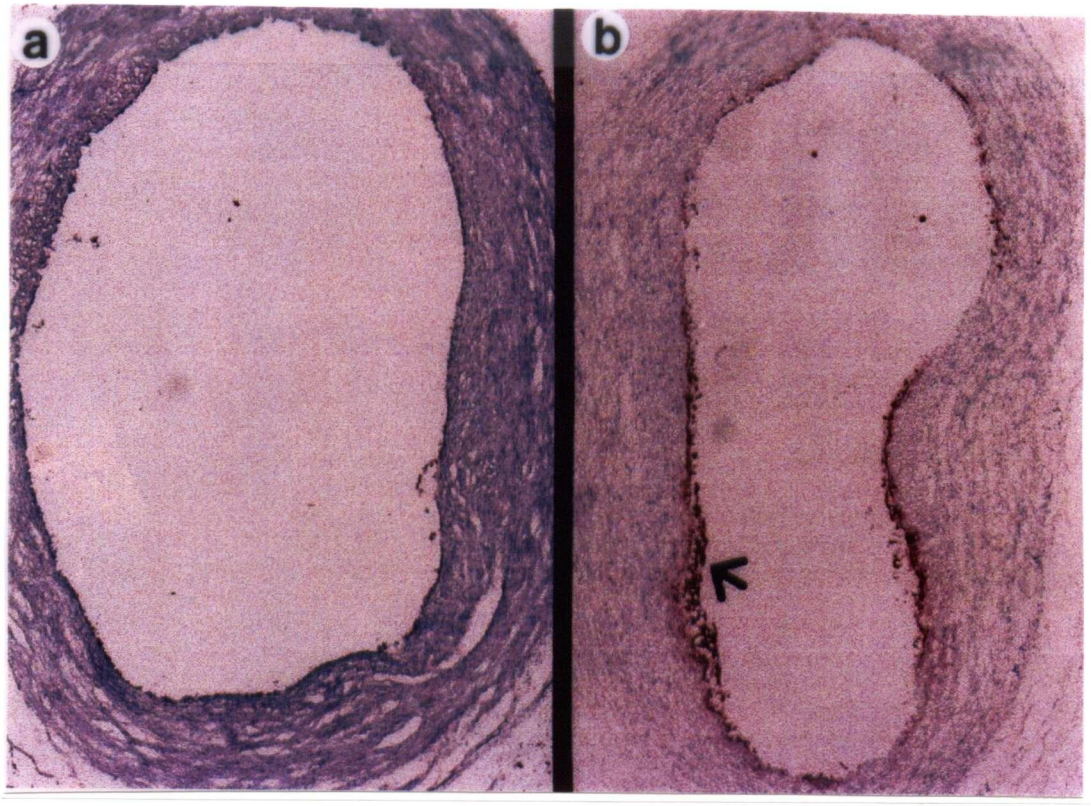

Figure 5.9. Oil red O staining in the brachiocephalic trunk from a cat on a normal diet (a) reveals little fat in the aorta while the cat on the cholesterol-enriched diet (b) has a marked increase in fat (red staining).

Vessel Wall Measurement	Total Cholesterol	% Increase Total Cholesterol	HDL-Cholesterol	Triglycerides	% Increase Triglycerides
ORO	0.14	0.3	0.44	0.57	0.86
Intimal Thickness Grade	0.38	0.61	0.27	0.57	0.55
Foam Cell Grade	0.44	0.51	0.21	0.72	0.86
Ascending Aorta Intimal Area	-0.33	0.03	-0.19	0.16	-0.15
Descending Aorta Intimal Area	-0.01	0.24	-0.46	0.18	0.01
Thoracic Aorta Intimal Area	0.72	0.62	-0.36	0.74	0.34
Abdominal Aorta Intimal Area	0.39	0.5	0.2	0.43	0.47
Lower Abdominal Aorta Intimal Area	0.24	0.51	0.43	0.61	0.91
Mean Aorta Intimal Area	0.2	0.37	-0.18	0.38	0.16
Brachiocephalic/ Subclavian Intimal Area	0.37	0.5	-0.03	0.85	0.83
Mean Vessel Intimal Area	0.38	0.53	-0.1	0.67	0.49

Table 5.4. Correlation coefficients between lipid values and vessel wall lesion measurements.

Discussion

In this study it has been demonstrated conclusively that the cat is suitable for studies of diet-induced atherosclerosis and that our methods of defining lesion severity are valuable in evaluating early lesions. The morphological methods serve as a means by which serum lipid levels and lipoprotein alterations can be used to predict lesion severity. Detailed documentation of the appropriate diet, severity and distribution of atherosclerotic lesions in the normal cat provides the foundation by which we can assess the role of LPL deficiency in lipoprotein metabolism and the susceptibility to diet-induced atherosclerosis in our naturally occurring feline model.

The atherogenic diet formulated for this study was effective at producing atherosclerotic lesions in the normal cat. Atherosclerosis was evident as early as 8 weeks on this diet, as reflected in grades of intimal thickness and foam cells, and in quantitative measurements of intimal area and lipid insudation (Oil red O staining). These findings are in support of those of Manning and Clarkson (1970) where they found atherosclerotic lesions in all five cats that were fed a similar diet. Compared to human lesions, the morphological lesions found in the cat ranged from Type I to Type III as determined by the Report on the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association (Stary et al, 1995). In preliminary data, further evaluation of the cat model on a cholesterol-enriched diet to 10 months, lesions up to Type V (fibroatheroma) were seen (data not shown).

The techniques we employed to quantitate the atherosclerotic lesions in the cat enabled us to correlate the severity of lesion development with mean

blood lipid parameters and percent increase of lipids while on the atherogenic diet. The lipid moiety most reflective of the severity of lesions were the absolute triglyceride levels and % increase triglyceride levels. Ongoing studies will further delineate the most pro-atherogenic lipoprotein or particle in the cat.

As noted, the cat has not been utilized as an animal model for atherosclerosis despite the demonstrated susceptibility to diet-induced atherosclerosis (Cirio, 1928; Yuasa, 1928). Although there are many useful animal models currently available, the cat has a lipid profile that has many similarities to that of humans, particularly with respect to HDL sub-fractions (Demacker et al., 1987). The HDL-subfractions have been clearly implicated in the protective roles of certain lipid moieties in atherogenesis. Non-HDL particles (apoB-containing particles) are bound to lipoprotein lipase present in the subendothelial matrix and appear to be pro-atherogenic. Unlike the mouse (and most other models), the cat is a strict carnivore and during a "natural" life it will eat a diet that is quite similar to a Western style diet. The cat, much as other animal models, is not normally found to have significant spontaneous CAD unless genetically manipulated.

These studies help confirm that the cat can be a useful animal model for studies of atherosclerosis. The model will be useful to determine how genetic factors including complete or partial LPL deficiency play a role in the evolution of atherosclerosis.

Chapter 6 : Conclusions.

In this thesis I began by describing a small colony of cats that seemed to be suffering from lipoprotein lipase deficiency. I then defined the molecular defect underlying this affliction which then led to a well characterized animal model for LPL deficiency. This model will be useful for exploring the role of LPL in the progression of atherosclerosis and will provide an opportunity to evaluate gene therapy approaches for LPL deficiency. This may help us undertstand the best methods of gene delivery as a general therapeutic approach. Furthermore, these studies have identified manifestations of LPL deficiency not seen in the human condition.

Initially it was not clear whether or not these chylomicronemic cats did indeed have a molecular defect that resulted in LPL deficiency. It was possible that they might have a mutation in the ApoCII gene or an unknown gene may be responsible for the chylomicronemia and neurological disorders that affected these cats. The phenotypes of the human and cat with complete LPL deficiency are very similar with respect to some of the most obvious symptoms, such as fasting chylomicronemia, lipemia retinalis and cutaneous xanthomas. However, I have observed additional phenotypic markers of LPL deficiency in the cat that have not been observed in humans. These include; the decreased growth curves of homozygotes, increased stillbirth rates in homozygote queens and decreased body fat content in homozygotes. Moreover, I observed that homozygote cats have a significant reduction in percent body fat. This reduced body fat content seems more pronounced in homozygous offspring of homozygous queens.

With the availability of a partial feline LPL cDNA it was possible to initiate the testing of the primary hypothesis; that a mutation in the LPL gene was the underlying cause of chylomicronemia in this colony of cats. Isolation and sequencing of the full length feline LPL cDNA was completed, for both the normal and affected cats. A nucleotide base change at amino acid residue 412 was identifying in the affected feline LPL cDNA. This mutation lies in a region that shows a high level evolutionary conservation. The glycine residue at the codon equivalent to 409 of the human LPL gene (412 in the cat) is conserved in at least 11 other species, and highlights the importance of the amino acid glycine in this particular region of the LPL protein. Interestingly, this region of the LPL gene, including residues 390-421, are equally conserved, suggesting that this particular region plays an important role in at least one of the many functions of LPL. There has been a report of a patient that suffers from familial hypertriglyceridemia, who has a mutation that changes one of these conserved amino-acids (residue 410) (Perviata et al., 1993). The exact mechanism by which this region, when disrupted, alters the lipolytic activity of the LPL protein is yet to be determined. However, it is interesting to speculate that this region may play a role in maintaining a dimeric form of the protein, a form that is essential for maintaining catalytic activity (Brunzell, 1995).

Studies conducted in our laboratory in collaboration with Ulrika Beisiegel's laboratory in Hamburg Germany, helped to define the region of the C-terminal domain of the LPL protein that is involved in the binding and uptake of chylomicrons into cells via the low density related receptor (LRP), a receptor in the LDL receptor family (Krapp et al., 1995). In earlier experiments, Dr. Beisiegel had demonstrated that the presence of the LPL protein is

important for chylomicrons to bind and become incorporated into HepG2 cells specifically using a LRP mediated pathway (Beisiegel et al., 1989). By developing a method in which I was able to metabolically label the LPL protein that was transiently expressed in COS cells, Annette Krapp was able to perform cross-linking studies in which the S35 labled LPL protein way cross-linked to the LRP protein which helped narrow down the specific region of the LPL protein that was involved in this binding (Krapp et al., 1995). Interestingly enough, this critical region contains the region of the mutation in the cat. However, due to difficulties of detecting the "409" mutant protein using a 5D2 monoclonal antibody ELISA assay, we were not able to determine if this particular residue is essential to the binding to LRP. Other mutant proteins generated by Hanfang Zhang in our laboratory did, however, help us to delineate the region of LRP binding to include residues 390-421. This was similar to findings by Chappell et al., (1994) who also demonstrated that this region is critical for the binding to LRP.

Using site directed mutagenesis it was possible for me to determine that this single base change, when introduced into the human LPL cDNA expression construct, did indeed disrupt the lipase activity of the human LPL gene, when expressed in mammalian cells. Similarly, when the affected cat LPL cDNA was cloned using a RT-PCR strategy, and expressed in cells by Sarah Jones, it too was incapable of generating any appreciable lipase activity. Once confirmed, this mutation validated the use of these cats as a model for human LPL deficiency.

The next phase of this thesis was the characterization of serum lipids as it relates to normal and LPL deficient cats as well as to human lipid metabolism. The detailed lipoprotein analysis in normal and LPL deficient cats described in

chapter 2, indicates that the LPL deficient cat is quite similar to the human, even though the cat is naturally CETP deficient. Once the role of CETP is taken into account the differences between the human and the cat become clear. In hypertrigyceridemic patients that have increased HDL-values, it is correlated with lower levels of CETP. Furthermore, when CETP was blocked in rabbits (Whitlock et al., 1989 and Abbey et al., 1989) or in CETP deficient patients (Inazu et al., 1990) there was also an increase in HDL-cholesterol levels. Other than the differences due to the absence of CETP, the lipid profile of the LPL deficient cat is very similar to the human LPL deficient patient. The LPL deficient cat has a profound elevation of serum triglycerides. They also have an elevation of VLDL-triglycerides, VLDL-cholesterol and a decrease in LDL-cholesterol. Particle composition analysis indicates that the cat has a similar lipid particle composition to that of humans with respect to VLDL and LDL. HDL however, is different in that the triglyceride content is at the lower limit of detection, ~1%. Furthermore, the effect of LPL genotype on particle composition was most evident in LDL particles, which are enriched with triglycerides at the expense of cholesterol and is similar to that of LPL deficient humans. Contrary to humans, HDL particles from LPL homozygote cats tended to be relatively enriched with cholesterol. The main gender difference on particle composition is seen in HDL particles, which were enriched with cholesterol in females of all genotypes.

I also assessed the ability of the LPL deficient cats to tolerate an oral fat load in which the homozygotes displayed a marked reduction in the clearance of plasma triglycerides and the heterozygotes had a less dramatic yet significant reduction triglyceride clearance, thus illustrating the differential post-prandial responses of normal and LPL heterozygote cats. The ramifications of LPL

deficiency in the cat also include an altered breast milk fatty acid profile, which is also seen to a comparable degree in Type I patients (Steiner at al., 1986). Breast milk analysis of normal and LPL deficient cats demonstrates a reduced amount of total fat in the milk with the greatest reduction in the longer chained fatty acids which are normally transported via chylomicrons.

I have also described a significant alteration of body composition in homozygote cats. This most likely is exacerbated *In utero* of homozygote queens, such that the delivery or lack there of, of fatty acids impedes the ability of the developing fetus to provide the necessary signals for the proper maturation of pre-adipocytes. Future studies might include the morphological analysis of the adipose tissue from homozygotes, on what little that can be found, to determine if the appearance of a immature morphology of adipocytes can be observed. Also, ongoing studies in Dr., Quinton Rogers laboratory will provide enough animals, in all of the possible genotypic combination of gender and body composition, in order to provide a more rigorous statistical analysis of the hypotheses that I have proposed in this thesis with respect to the development of adipose tissue in the LPL deficient cat.

The diet induced atherosclerosis studies that I have conducted in normal cats are a prelude to assess the potentially atherogenic post-prandial phenotype of the LPL heterozygotes. It is clearly evident that the cat is suitable for studies of investigating diet induced atherosclerosis. The ability to assess the potential atherogenic risk of partial LPL deficiency in this model will also afford us the possibility of assessing whether the pro-atherogenic risk of delayed post-prandial clearance of triglyceride rich lipoproteins is enough to off-set the anti-atherogenic potential resulting from the CETP deficiency in this model. In fact,

these studies have already been conducted in cats heterozygous for LPL deficiency which were similarly subjected to diet induced atherosclerosis as per the normal cats in Chapter 5. These samples are awaiting a comprehensive pathological examination by an experienced pathologist.

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