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OBESITY-ASSOCIATED PHENOTYPES AND CIRCULATING LEVELS OF GHRELIN, CHOLECYSTOKININ, LOW-DENSITY LIPOPROTEIN AND ZINC: GENETIC AND OBSERVATIONAL STUDIES

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by

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OBESITY-ASSOCIATED PHENOTYPES AND CIRCULATING LEVELS OF GHRELIN, CHOLECYSTOKININ, LOW-DENSITY LIPOPROTEIN AND ZINC: GENETIC AND OBSERVATIONAL STUDIES

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Obesity is a complex problem that is believed to result from both genetic and environmental factors. This condition greatly increases the risk of developing serious health consequences, such as metabolic syndrome and cardiovascular disease. The primary goal of this research was to study the genetic influence on plasma ghrelin, cholecystokinin (CCK), and low-density lipoprotein (LDL), and their relationship with obesity. A secondary goal was to investigate the effect of weight loss on plasma zinc and other risk factors for metabolic syndrome. Aims 1 and 2 were to estimate the additive heritabilities and to localize the responsible chromosomal quantitative trait loci associated with circulating levels of the appetite regulating hormones, ghrelin and CCK, in baboons. Plasma ghrelin and CCK were higher in baboons than humans, with males exhibiting greater levels. Ghrelin was inversely linked to body weight ($r = 0.23$, $p < 0.001$), insulin

($r = -0.19$, $p < 0.05$), and leptin ($r = -0.14$, $p < 0.05$). Significant heritabilities were observed for ghrelin and CCK. A strong signal was detected for plasma CCK on chromosome 17p12 (LOD = 3.1, $p < 0.01$). Aim 3 was to detect heritability and pleiotropy between the obesity-related anthropometric phenotypes and low- density lipoproteins (LDL) in humans. Effect of genes on LDL size was evident, with substantial heritability and strong genetic correlations between LDL and obesity-related traits; small LDL and weight ($rho_G = 0.65$, $p < 0.001$), waist ($rho_G = 0.80$, $p < 0.001$), and BMI ($rho_G = 0.67$, $p < 0.001$), respectively. Aim 4 was to study the impact of weight loss on plasma zinc and risk factors for metabolic syndrome in humans. Weight reduction significantly increased the low plasma zinc observed in obese women, and improved risk factors for metabolic syndrome (HDL, BMI, waist and body fat). A negative correlation was observed between changes in zinc and body fat ($r = -0.28$, $p < 0.05$). Collectively, these results demonstrate significant influence of genetic factors on plasma ghrelin, CCK, LDL and obesity phenotypes. In addition, weight loss produced beneficial effects of weight loss on plasma zinc and risk factors for metabolic syndrome.

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CHAPTER 1

REVIEW OF LITERATURE

Obesity has become a major health problem in the world. This rapidly escalating condition is a known risk factor for numerous disorders such as type 2 diabetes, hypertension, cardiovascular disease (CVD), and metabolic syndrome [Bray, 2004], and precedes all other risk factors [Lemieux, 2001]. Obesity results from an imbalance between calorie intake and expenditure [Bray, 2004], and is influenced by environmental and genetic factors [Woods and Clegg, 2003]. Energy homeostasis is a complex mechanism involving both the gastrointestinal tract and the brain [Wynne et al., 2004]. This project will study the gut hormones, ghrelin and cholecystokinin (CCK), which play an important role in the control of food intake and development of obesity [Barsh and Schwartz, 2002]. In addition, the role of obesity as a contributor to CVD and metabolic syndrome will be investigated. The relationship of known and possible obesity-related phenotypes with risk factors of the metabolic syndrome will be explored.

SPECIFIC AIMS:

The specific aims of this project are:

1. To estimate the additive heritabilities and localize the responsible chromosomal quantitative trait loci associated with circulating levels of the appetite regulating hormones, ghrelin and CCK, in baboons.

Hypothesis: There is a significant genetic contribution of specific quantitative trait loci to phenotypes related to appetite regulation in baboons.

2. To detect heritability and pleiotropy between the obesity-related anthropometric phenotypes and low- density lipoproteins (LDL) in humans.

   Hypothesis: There is substantial genetic influence on the relationship between obesity-related phenotypes and LDL.

3. To study the impact of weight loss on the circulating levels of zinc and risk factors for metabolic syndrome in humans.

   Hypothesis: Obesity is associated with low circulating levels of zinc. Weight loss has beneficial effect on zinc status and metabolic risk factors.

The primary goal of this research is to study the genetic impact on the circulating levels of ghrelin, CCK, and LDL, and their relationship with obesity-related traits. A secondary goal is to investigate the influence of weight loss on plasma levels of zinc and other risk factors for metabolic syndrome.

INTRODUCTION:

The continuous rise in the incidence of obesity is alarming. In addition to physical discomfort, this condition increases the risk for several common diseases and, subsequently, mortality (Yang et al., 2001). The pathological outcome of obesity is due

predominantly to the growth in adipose tissue and its metabolic effects [Bray, 2004]. Excessive energy stores in the body enhance the number and size of fat cells. The regulation of body fat and weight is influenced by the interaction of hormones related to gut and brain, and this relationship is under considerable genetic influence [Woods & Clegg, 2003]. This research will focus on the effect of genes on the circulating levels of hormones related to appetite such as ghrelin and CCK, as well as other obesity-associated traits.

The association of obesity with LDL size, a significant risk factor for CVD is an additional component to be investigated. Low-density lipoprotein was selected as its small and dense particles pose a greater risk for CVD than other lipoproteins. The small and dense form is highly atherogenic and remains longer in the plasma; thereby, increasing the chances of deposition into arterial walls [Rainwater et al., 2003]. This lipoprotein has been positively associated with weight-related parameters such as waist circumference, weight, body mass index (BMI) [Rainwater et al., 1999]. Thus, the genetic influence on LDL size and obesity-related anthropometric measures also will be explored.

Finally, obesity will be examined for its role as a risk factor of the metabolic syndrome. Metabolic syndrome is a cluster of disorders that includes obesity, type 2 diabetes, insulin resistance, dyslipidemia and hypertension [Hansen, 1999]. The two main risk factors for metabolic syndrome, obesity and type 2 diabetes, also alter the metabolism of some trace minerals, such as zinc. Zinc is essential for human health, with

important roles in the structural and catalytic function of several enzymes [Tapeiro, 2003]. Furthermore, this trace element is known to have insulin-like functions [Tallman & Taylor, 2003]. Therefore, the possible association of risk factors of metabolic syndrome to plasma zinc will be analyzed in the current research.

The first aim of this project is to estimate the additive heritabilities and localize the responsible chromosomal quantitative trait loci associated with phenotypes related to appetite regulation in baboons. Control of body weight and food intake is regulated predominantly by the central nervous system centering on the hypothalamus in the brain. Three major areas in the hypothalamus that regulate or influence the food intake are the 1) arcuate nucleus (ARC); 2) paraventricular nucleus (PVN); and 3) lateral hypothalamic area [LHA] [Barsh & Schwartz, 2002]. Two sets of neurons, neuropeptide Y (NPY)/ agouti-related protein [AGRP] and proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART), are present in the arcuate nucleus. These neurons act as first order neurons to receive signals from peripheral molecules, and transmit them to the second order neurons, present in the paraventricular nucleus and the lateral hypothalamus [Schwartz et al., 2000]. Regulation of food intake takes place in these areas. Activation of paraventricular nucleus (PVN) by these neurons decreases food intake; whereas, stimulation of lateral hypothalamic area leads to an increase [Barsh & Schwartz, 2002].

The first-order neurons receive signals from peripheral molecules that influence the regulation of energy. These molecules include both long-term and short-term signals.

Leptin and insulin are two long-term, energy-regulating molecules that are produced by adipose tissue and pancreas, respectively, and circulate in proportion to body fat stores [Barsh & Schwartz, 2002]. Both of these have receptors on the first-order neurons in the arcuate nucleus and act via the stimulation of POMC/CART and the inhibition of NPY/AGRP neurons [Figure 1]. Other peripheral molecules with a role in food intake and satiety are ghrelin [Cummings et al., 2002], cholecystokinin [Moran, 2000], peptide YY $_{3\text{-}36}$ [Batterham et al., 2002], glucagon-like peptide (GLP) [Schick et al., 2003], gastrin-releasing peptide [GRP] [Fekete et al., 2002], bombesin [Yamada et al., 2002], enterostatin [Mei et al., 1993], amylin [Rushing, 2003], pancreatic polypeptide [Batterham et al., 2003], and apolipoprotein A-IV [Tso & Xu, 2003]. In this research, the first two of these hormones, ghrelin, and cholecystokinin, will be studied for their contribution towards obesity in baboons.

Ghrelin is an orexigenic (increases food intake) hormone that is produced in the stomach by the oxyntic gland [Cummings et al., 2002]. It affects energy regulation by increasing food intake and lowering the metabolic rate [Cummings et al., 2002]. This hormone is a 28 amino acid peptide that is up-regulated during starvation, cachexia, anorexia nervosa and insulin-induced hypoglycemia, and down-regulated in feeding and obesity [Shimada et al., 2003]. Ghrelin is a natural ligand for growth hormone secretagogue receptor [Ukkola et al., 2002] and is distributed in areas of brain such as the arcuate nucleus and lateral hypothalamic area [Rosicka et al., 2002]. It enters the brain through the blood brain barrier and increases food intake through the NPY/AGRP

system, rather than the growth hormone/intrinsic growth factor system [Rosicka et al., 2002].

The primary production site for ghrelin is X/A-like cells in the oxyntic mucosa of the stomach. This hormone also is widely expressed in several other tissues, such as the hypothalamus [Cowley et al., 2003], small intestine [Date et al., 2000], placenta [Gualillo et al., 2001], kidney [Mori et al., 2000], lung [Volante et al., 2002a], pancreas [Volante et al., 2002b], ovary and testis [Gaytan et al., 2003]. It has been proposed that ghrelin may regulate food intake by three pathways: 1) Circulating levels of ghrelin cross the blood-brain barrier and activate NPY/ AGRP neurons in the arcuate nucleus, thereby affecting appetite; 2) Ghrelin transmits signals to nucleus of the solitary tract (NTS) from the stomach through the vagus nerve. These signals are later relayed to the arcuate nucleus where they stimulate NPY/AGRP neurons; and 3) Hypothalamic ghrelin acts locally to activate NPY/AGRP neurons.

In humans the ghrelin gene, located on chromosome 3p25-26, consists of four exons and three introns. This hormone is expressed as a prepropeptide; subsequently, the prepropeptide is cleaved to produce the mature protein [Ambrogi et al., 2003]. The peptide sequence of ghrelin is highly conserved across the mammalian species. For example, rhesus monkey [Angeloni et al., 2004] and rodents [Rindi et al., 2004] differ from humans by just one and two amino acids, respectively. Studies have shown diminished circulating levels of ghrelin in obese humans [Tshcop et al., 2001] and rodents [Moesgaard et al., 2004]. Also, ghrelin acts opposite to insulin [Ukkola et al.,

2003] and leptin [Tschop et al., 2001], presumably, due to the activation of NPY/AGRP neurons by ghrelin. This action of ghrelin is in direct contrast to the inhibition of these neurons by insulin and leptin.

The other appetite-regulating hormone that will be investigated in this research is CCK, a major satiety hormone. It has numerous isoforms; CCK-8, CCK-33 and CCK-39 are the major ones in plasma [Konturek et al., 2004]. This hormone is secreted by the duodenum and jejunum and is believed to regulate appetite by controlling meal volume. It is also expressed in brain and other areas of gastrointestinal tract [Moran, 2000]. Two receptors of cholecystokinin have been recognized: cholecystokinin A (CCK1) and cholecystokinin B (CCK2). Inhibition of feeding is thought to be regulated primarily by the A-receptor [Fink et al., 1998]. Similar to CCK, the A-receptors also are found in the brain and the gastrointestinal tract [Geary, 2004]. The signals emitted by the interaction of cholecystokinin with its receptor A are transmitted to the nucleus of the solitary tract (NTS), and mixed with those sent from arcuate nucleus, to affect feeding behavior [Schwartz, 2000].

Other functions of CCK include contraction of gall bladder for releasing bile, control of pancreatic enzymes, gastric emptying and reduction in gastric acid secretion [Baranowska et al., 2000]. Regulation of meal volume by CCK is enhanced by leptin, another satiety hormone. Leptin is an adipose tissue derived hormone that has a major role in energy homeostasis. Injection of leptin is known to augment CCK in the

monitoring of meal volume [Barrachina et al., 1997]. In addition, the function of CCK is augmented by insulin, an adiposity-related hormone [Reidy et al., 1995].

In the proposed research, baboons from the Southwest Foundation for Biomedical Research [SFBR] in San Antonio, Texas, will be the models for studying the variation in the circulating levels of ghrelin and CCK. These animals are ideal for the study of genetics due to minimum environmental variations (same diet and housing conditions). Despite the similarity in the living standards, 10 % of these baboons develop obesity spontaneously. Baboons are highly comparable to humans with respect to genetic variations, protein and chromosomal structure. [Comuzzie et al., 2003]. Also, obesity-related traits have been shown to be heritable in baboons, just as in humans [Comuzzie et al., 2003].

The second aim of this study is to detect heritability and pleiotropy between the obesity-related phenotypes and low- density lipoproteins (LDL) in humans. Obesity is associated with impaired levels of triglycerides and HDL cholesterol [Scott, 2003]. Accumulation of fat, particularly in the abdominal region, increases the production of free fatty acids, which stimulate the synthesis of triglycerides and very low-density lipoproteins (VLDL) in the liver [Ginsberg, 2003]. Overproduction of VLDL and triglycerides diminish HDL cholesterol [Ginsberg, 2003] and low-density lipoprotein cholesterol (LDL) size [Sonnenberg et al., 2004].

Low-density lipoprotein consists of a cholesterol core and a surrounding layer of phospholipids and apolipoprotein B100 [Nabel, 2003]. In plasma, this lipoprotein transports cholesterol [Nabel, 2003], and is present in different sizes and densities [Rajman et al., 1996]. Variation in the sizes of LDL is a major determinant of cardiovascular risk [Rainwater et al., 1999]. Small and dense LDL is known to be more susceptible to oxidation, has greater permeability through the arterial wall and lower affinity for LDL receptors, and is the most atherogenic of all the sizes of the LDL. Also, obesity particularly the abdominal type is strongly related to small LDL [Rainwater et al., 1999].

Genetics also play an important role in the variation in plasma LDL. Studies have shown that LDL and its size are heritable in humans [Austin et al., 2003, Sonnenberg et al., 2004] and animals [Rainwater et al., 2003]. These lipoproteins have been mapped to chromosome 7 in families in the Take Off Pounds Sensibly (TOPS) study [Sonnenberg et al., 2004]; chromosomes 2, 3 and 19 in old order Amish [Pollin et al., 2004], and chromosomes 1, 11, 15 and 19 in the Quebec family study [Bosse et al., 2004]. Also, linkage for LDL size was found on chromosome 6 in families from the Genetic Epidemiology of Hypertriglyceridemia Study [Austin et al., 2003].

The present research on the genetic factors influencing LDLs will be conducted in Alaska natives. Historically, Alaska natives had a low incidence of CVD that was attributed to genetic predisposition, traditional diet and high physical activity. The westernization of diet and lifestyle has changed these trends, and currently, the

prevalence of CVD in this population is similar to other ethnicities in the United States (US). Although the effect of the environment is apparent, the genetic impact on the drift has not been followed closely. Thus, this unique population will be utilized to explore the environmental and genetic relations between obesity-related phenotypes and LDLs.

The third aim is to study the impact of weight loss on the circulating levels of zinc and risk factors for metabolic syndrome in humans. Metabolic syndrome is a multifactorial disease comprising of obesity, type 2 diabetes, hypertension and dyslipidemia [Lemieux, 2001] and is an intermediate step in the progression of coronary-related disorders (Ardern et al., 2003). The National Cholesterol Education Program Adult Treatment Panel III [NCEP ATP III] provides guidelines that elaborate the risk factors of metabolic syndrome [NCEP, 2002]. Any three of the following indicates the presence of this syndrome in humans: waist circumference > 88 cm for women and > 102 cm for men; fasting glucose > 100 mg/dl; triglycerides > 150 mg/dl; high density lipoprotein [HDL] cholesterol < 50 mg/dl for women and < 40 mg/dl for men; and blood pressure > 130/85 mm Hg [Ford et al., 2004].

Current estimates are that approximately 64 million adults in the US exhibit metabolic syndrome [Ford et al, 2004]. It is more prevalent in those with obesity, as more than 40% of individuals with a body mass index (BMI) > 35 kg/m^2 suffer from this condition [Hill, 2003]. Individuals with abdominal [central or visceral] obesity have greater chances of developing metabolic syndrome than those with peripherally stored fat [Hill, 2003]. Abdominal obesity is associated with increased supply of plasma free fatty

acids that are directly drained into the portal vein [Bosello & Zamboni, 2000]. Fat in this area has a high turnover rate and is metabolically active [Groop, 2000]. These fatty acids result in insulin resistance, especially in skeletal muscle and liver [Arner, 2002, Lam et al., 2003] by interfering with the activities of insulin receptor substrate-2 (IRS-2) associated phosphatidyl inositol 3 (PI 3) kinase and the glucose transporter, GLUT 4.

Visceral obesity is associated with impaired levels of triglycerides, increased very low-density lipoproteins (VLDL) and HDL cholesterol (Scott, 2003). Higher levels of VLDL diminish HDL cholesterol because of an exchange of core lipids, which is mediated by cholesterol ester transfer protein (Ginsberg, 2003). High triglyceride and low HDL cholesterol levels are associated with a greater risk of atherosclerosis (Ginsberg, 2003). In fact, all five risk factors of metabolic syndrome also are risk factors for coronary artery disease (Alexander et al., 2003).

Weight gain alters the metabolism of some trace elements, primarily zinc. Zinc is an essential trace element required for the function of numerous enzymes involved in growth and development and regulation of gene function and stability of cell membranes [Tapeiro, 2003; Cousins et al., 2003]. This mineral has an important role in type 2 diabetes and insulin resistance due to its insulin-like properties [Ilouz et al., 2002]. Zinc deficiency presents several features in common with type 2 diabetes such as insulin resistance, hyperglycemia, and impaired glucose tolerance [Tallman & Taylor, 1999]. It is believed that zinc enhances insulin signaling by increasing tyrosine kinase phosphorylation [Simon and Taylor, 2001], as well as activating sites further downstream

such as PI3 kinase and GLUT 4 [Adachi et al., 2004]. Similar to insulin, zinc is known to promote lipogenesis in adipocytes, and its supplementation improves hypoglycemic levels in diabetic mice [Ilouz et al., 2002]. In 2002, Simon and Taylor found increased glucose transport in adipocytes treated with zinc. Additionally, zinc has an important role in the synthesis and release of insulin from beta cells in pancreas [Shisheva et al., 1992]

Several studies have depicted altered levels of zinc in obese and diabetic humans [Chen et al., 2000a, Tallman and Taylor, 1999, DiSilvestro, 2000] and rodents [Tallman and Taylor, 2003, Chen et al., 200b]. Also, zinc deficiency is known to alter leptin, an adipose tissue-derived hormone, in studies conducted in humans [Mantzoros et al., 1998], rats [Ott and Shay, 2001] and mice [Tallman and Taylor, 2003]. Thus, it has been proposed that zinc has an important role in adipose metabolism. According to Tallman and Taylor [2003], mice fed a high fat diet had lower adipose zinc concentrations as compared to mice given a normal diet. Others have shown a considerable increase in adiposity in obese rats [Bock et al., 1995] and mice [Chen et al., 2000] that were fed a low zinc diet. Finally, zinc deficiency alters metabolism of essential fatty acids, primarily, linoleic acid towards oxidation and affects the distribution of fat stores in rats [Cunnane & Yang, 1995].

Several new components, such as small and dense LDL, hyperurecemia, hyperapolipoprotein B, and impaired fibinolysis activity, have been added to the standard risk factors of metabolic syndrome. [Lemieux, 2001]. C-reactive protein also has been proposed as an important component of this syndrome. All these new risk factors that

have been proposed are linked to either insulin resistance or cardiovascular etiology [Lemieux, 2001]. Since zinc plays an essential role in insulin resistance (a component of metabolic syndrome), we will investigate its relationship with metabolic risk factors and the effect of weight loss on its circulating levels.

Effect of weight loss on plasma zinc has been depicted in several studies. Di Martino et al. [1993] observed an increase in serum zinc levels with weight loss in subjects who were fed hypocaloric diets for eight weeks. At the start of the study, these individuals were obese and had low zinc levels as compared to controls. Woodhouse et al. [2004] also conducted a similar study in obese men and women, who were given high dairy, hypocaloric diets for 12 weeks. A 7 % rise in plasma zinc was seen with a 5.9 % decrease in weight. In another study in obese children and adolescents, reduction in weight after 10 weeks was concomitantly associated with enhanced plasma zinc levels [Di Toro et al., 1997].

The beneficial effect of weight loss on the risk factors for metabolic syndrome has been documented by several investigators. Han et al. [1997] showed a concurrent decline in total and LDL cholesterol with decreased waist circumference. Dattilo and Kris-Etherton [1992] had earlier reported that with every 1 kg of weight loss, lipids levels diminish by 0.015 to 0.05 mM. Similarly, Markovic et al. [1998] found that triglycerides and VLDL reduced with energy restriction. Also, reduction in weight is known to improve insulin action [Aronne and Segal, 2002], plasma glucose, and confer health

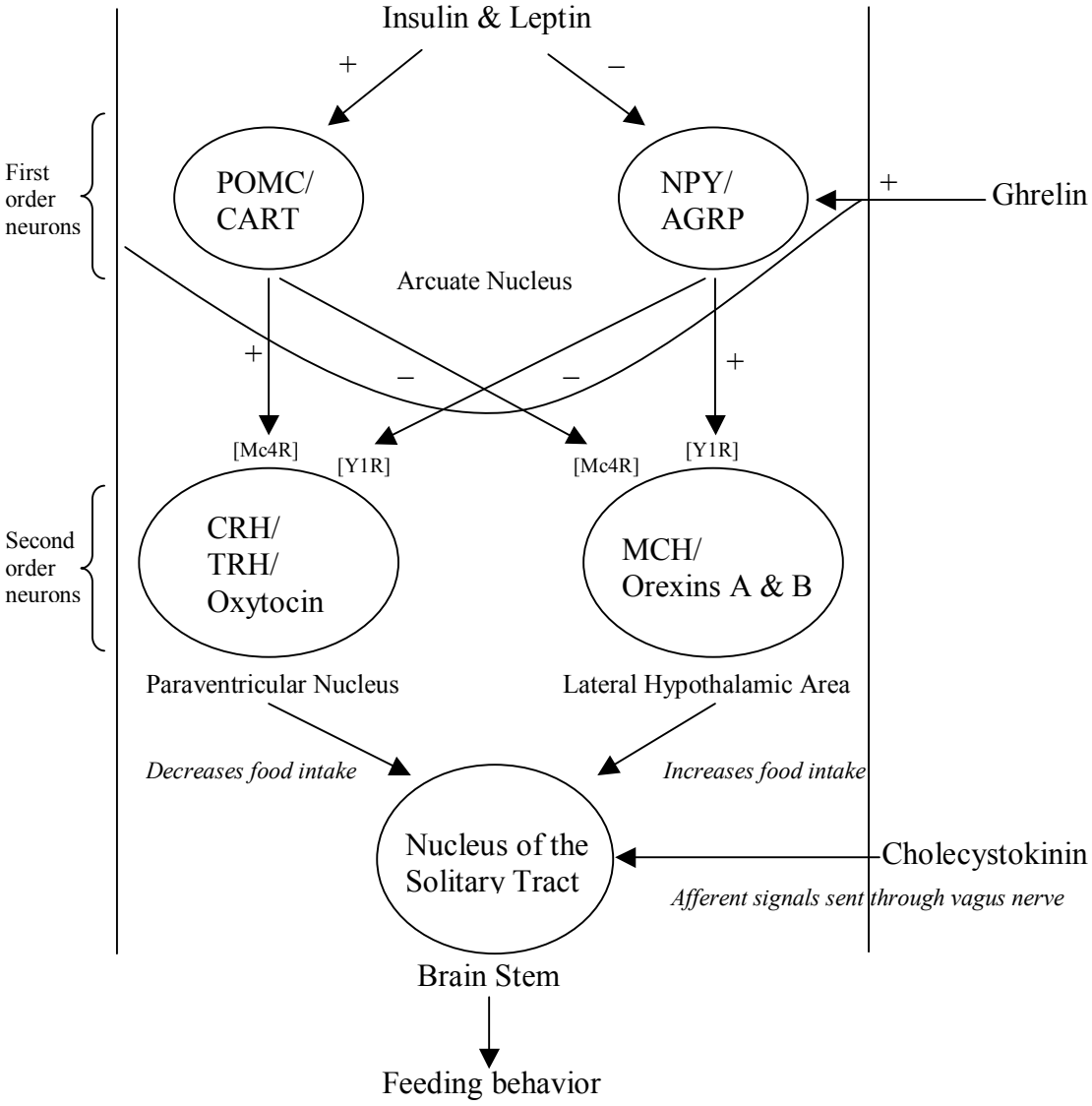
benefits [Klein et al., 2004], therefore we will investigate the effects of weight loss on metabolic risk factors and plasma zinc in low-income mothers.

The population chosen for this aim are low-income, obese/overweight women, who are Hispanic, Caucasian or African-American ethnicity. This sample is at high risk for developing obesity, as it has been demonstrated that obesity rates are high in minority [Cossrow and Falkner, 2004] and low-income women [Breitkopf and Berenson, 2004]. All these women have a youngest child 1-3 years of age and are participating in a weight loss study. The intervention program focusing on nutritious food choices and lifestyle changes will be administered in order to improve weight status and diabetic risk factors [Klohe et al., In preparation]. Other components such as behavioral modification and physical activity will be incorporated into this weight loss program. Overall, this aim stresses on understanding the influence of obesity on plasma zinc and metabolic syndrome. It also will investigate the relation of plasma zinc with risk factors for metabolic syndrome, and effect of weight loss on them.

In summary, the goals of this research include identification of the extent of genetic influence on the circulating levels of ghrelin, cholecystokinin and low-density lipoproteins, and their relationship with obesity-related phenotypes. A secondary goal deals with the effect of weight loss on plasma zinc and other risk factors for metabolic syndrome.

FIGURE 1.1 Mechanism of appetite regulation in the brain.

The peripheral molecules, leptin, insulin, ghrelin, and cholecystokinin , are that stimulate/inhibit the first order neurons which, in turn, stimulate the second order neurons. These neurons send appetite-regulating signals to the nucleus of the solitary tract [NTS] where the signals are mixed with peripheral signals such as those from cholecystokinin and finally affect the feeding behavior. [Adapted from Barsh & Schwartz, 2002]. CRH-Corticotropin releasing hormone, TRH- Thyrotropin releasing hormone, MCH- Melanocorticotropin hormone, Y1R- Y1 receptor, Mc4R-Melanocortin 4 receptor, GhsR-Ghrelin receptor, Y2R- Y2 receptor, Mc3R-Melanocortin 3 receptor, LepR-Leptin receptor, NPY-Neuropeptide Y, AGRP-Agouti-related protein, POMC-Pro-opiomelanocortin, CART-Cocaine-and amphetamine-regulated transcript.



CHAPTER 2

QUANTITATIVE GENETIC ANALYSIS AND CHARACTERIZATION OF GHRELIN IN BABOONS

2.1 INTRODUCTION

Ghrelin is an orexigenic hormone that is produced primarily in the stomach, but also is expressed in other tissues such as hypothalamus, pancreas, kidney, placenta [Kishimoto et al., 2003] and lymphocytes [Gualillo et al., 2003]. Plasma ghrelin is known to increase two-fold before a meal and decline to its lowest level within one hour of food intake [Meier & Gressner, 2004]. Continuous ghrelin administration tends to raise body weight and lower metabolic rate [Cummings & Shannon, 2003], indicating an important role for ghrelin in appetite stimulation.

In the stomach, ghrelin is secreted by A/X-like cells in the oxyntic glands. This hormone reaches the arcuate nucleus in the hypothalamus via the bloodstream, where it is believed that the stimulation of food intake is mediated through receptors situated on neuropeptide Y/Agouti-related protein (NPY/AGRP) neurons [Bagnasco et al., 2003].

In humans, the ghrelin gene is located on chromosome 3 (p25-26) and consists of four exons and three introns. The 511-nucleotide cDNA encodes for a prepro-ghrelin, from which the mature ghrelin of 28 amino acids is cleaved off [Casanueva and Dieguez, 2004]. This peptide sequence is well conserved across a number of mammalian species,

including rhesus monkey [Angeloni et al., 2004], dog, rat, mouse, pig, sheep and cattle [Rindi et al., 2004].

It is established that ghrelin levels are low in obese individuals [Tschop et al., 2001; Robertson et al., 2004; Perrault et al., 2004] and increase with weight loss [Cowley et al., 2003]. In humans, Tschop et al. [2001] demonstrated an inverse relationship between ghrelin and leptin in lean and obese individuals. Also, both glucose administration [Nakagawa et al., 2002] and hyperinsulinemic euglycemic clamp conditions [Mohlig et al., 2002] diminished circulating ghrelin levels.

Ghrelin acts as a link between the gastrointestinal system and the brain [Lee et al., 2002]. In addition to food intake, this hormone has important role in the release of growth hormone, gastric acid secretion and motility, and pancreatic activity [Gualillo et al., 2003]. Human studies have shown several weight-related phenotypes to be under genetic influence [Bouchard, 1997]. In this study, baboons were used as animal models to explore the factors governing the circulating levels of ghrelin. These animals share similar diet and housing conditions, thus having minimum environmental differences. Also, their genetic and protein constitutions are analogous to those of humans [Comuzzie et al., 2003]. The purpose of this study was to examine the structure of ghrelin gene and investigate the genetic and environmental factors affecting the variation in its plasma levels in baboons.

2.2 METHODS

Experimental Design

Body weight and fasting blood samples were collected from adult baboons under sedation. Plasma samples were analyzed for biochemical parameters such as glucose, insulin, c-peptide, adiponectin, leptin and ghrelin. Genotyping was performed according to standard procedures. Univariate and bivariate quantitative genetic analysis were conducted to estimate additive genetic and environmental components of variance in the phenotypes.

Subjects

A pedigreed population of 376 adult baboons (263 females, 113 males) from the Southwest Foundation for Biomedical Research (SFBR), San Antonio, TX, was chosen without regard to any pre-existing clinical condition. The baboons were a mixture of yellow baboons *(Papio hamadrayas cynocephalus)* and olive baboons *(Papio hamadrayas anubis)*. The animals were housed in open-air group cages with similar living conditions and were fed ad libitum on a regular low fat diet (Harlan Teklad 15% monkey diet, 8715, Indianapolis, IN).

Sample collection

A total of 11 ml blood was drawn from the antecubital vein under ketamine sedation after a 12-hour fast. Plasma was separated by centrifugation at 2000 x g for 10 minutes and stored in aliquots at -80^0C until analysis. Body weight was measured with a calibrated electronic scale (GSE, Chicago, IL).

Assays for plasma levels of weight-related phenotypes

The glucose oxidase method with an Analox spectrophotometer (Analox Instruments, Lunenburg, MA) was used to analyze plasma glucose. Luminex 100, an endocrine Multiplex Immunoassay (LINCO Research, Inc, St Charles, MO) was utilized to measure plasma insulin, leptin and c-peptide. Ghrelin levels were obtained by commercially available radioimmunoassay kits (LINCO Research, Inc, St Charles, MO). All samples were analyzed in duplicate.

Sequencing of the prepro-ghrelin and tissue distribution of baboon ghrelin

Total RNA was extracted from the fundus portion of the stomach of baboon with trizol reagent [Molecular Research Center, Inc., Gaithersburg, MD]. Quality of the RNA was studied via UV light at 230, 260 and 280 nm and its integrity was ascertained by staining with ethydium bromide on a 1.2 % agrarose gel. The coding region of baboon ghrelin cDNA was amplified from this RNA sample by a two-step RT-PCR system (Invitrogen, Carlsbad, CA). Primers for ghrelin that were derived from human ghrelin

nucleotide sequence (accession number: BC025791) were: forward primer 5'- CCATGCCCTCCCCAGGGACC-3'and reverse primer 5'-ATCACTTGTCGGCTGGGGCCTC-3' [Angeloni et al., 2004]. The cDNA fragment was sequenced on an ABI 377 automated DNA sequencer using a Big Dye Terminator kit (Applied Biosystems, Foster City, CA). Expression of ghrelin in various tissues was detected by RT-PCR, using the same primers and PCR conditions that were used for sequencing ghrelin cDNA.

Genotypes

Human short-tandem repeat loci were used to develop a baboon genetic map [Rogers et al., 2000]. Published human PCR primers were utilized to amplify these loci from baboon genomic DNA. Fluorescently labeled primers and Genescan and Genotyper software (Applied Biosystems, Foster city, CA) were used to acquire the baboon genotypes. These sequences were obtained by gel electrophoresis and analyzed on ABI 373 and ABI 377 automated sequencers using. A total of 331 markers were obtained, with an average space of 10 cM between markers. All the baboon genotypes and data related to pedigree were prepared and managed by the computer program PEDSYS [Dyke et al., 1994].

Statistical methods

Quantitative genetic analyses of weight-related phenotypes were conducted utilizing the maximum likelihood-based variance decomposition method which is

implemented by the computer program, SOLAR (Almasy & Blangero, 1998). The total phenotypic variance of these phenotypes (σ^2_P) was split into its genetic (σ^2_G) and non-genetic or environmental components (σ^2_E),

$$\sigma^2_P = \sigma^2_G + \sigma^2_E$$

with heritability $h^2 = \sigma^2_G / \sigma^2_P$ (Hopper & Mathews, 1982).

Heritabilities of the ghrelin and other weight-related phenotypes in baboons were estimated by likelihood ratio tests. According to this method, the likelihood of the model in which the parameters were estimated was compared to the likelihood of a model in which heritability is zero. Twice the difference in the logarithmic likelihoods was distributed asymptotically as a ½: ½ mixture of a χ^2 variable with one degree of freedom and a point mass at zero [Self and Liang, 1987]. Sex, sex-specific age and age squared were used as covariates to estimate residual heritability.

Linkage between markers and plasma ghrelin was determined by variance component linkage analysis This technique is based on designating the expected genetic covariances between relatives as a function of the identity by descent (IBD) relationship at the marker locus. Also, it establishes whether the genetic variation at a specific chromosomal locus can explain the variation in the phenotype.

Covariance matrix for the members from the same pedigree is expressed as:

$$\Omega = \Pi \sigma^2_q + 2\,\Phi\sigma^2_a + I\,\sigma^2_e$$

Ω = covariance matrix for the all the members of the same pedigree

Π = the matrix of the IBD sharing of the pedigree members at a particular location of the QTL, in which

σ^2_q = additive genetic effects of the specific loci

Φ = kinship matrix

σ^2_a = non genetic effects of the loci

I = identity matrix

σ^2_e = individual specific random environmental effects

A null hypothesis in which the additive genetic variance for a specific QTL equals zero was tested against an alternate hypothesis in which the additive variance was estimated [Blangero & Almasy, 1997]. Logarithm of the odds (LOD) score (difference between the two-$\log^{10}$ likelihoods) was used to estimate the significance of the test. Twice the difference in the logarithmic likelihoods was distributed asymptotically as a ½: ½ mixture of a χ^2 variable with one degree of freedom (Self and Liang, 1987).

2.3 RESULTS

A profile of the pedigreed baboons is shown in **Table 2.1.** Males were younger, but had higher body weights than females. Plasma levels of ghrelin also were greater in males; however, glucose, insulin, c-peptide, and leptin were lower than those in females.

Mean plasma levels of ghrelin were 3406 ± 1848 pg/ml, ranging from 857 to 8731 pg/ml. According to Pearson's correlations, ghrelin was negatively related to body weight ($r = -0.23$, $p < 0.001$), insulin ($r = -0.19$, $p < 0.05$), C-peptide ($r = -0.14$, $p < 0.01$), and leptin ($r = -0.14$, $p < 0.05$)

The nucleotide sequences of the prepro-ghrelin in baboons and humans are depicted in **Figure 2.1**. The baboon sequence shows a 97 % sequence identity with humans. **Table 2.2** shows the amino acid sequence of ghrelin in various mammals. Ghrelin peptide sequence was 96 % similar to humans and identical to rhesus monkey. However, ghrelin sequence in dog, rat and mouse differed from humans by two amino acids. Tissue distribution of ghrelin and 18S in various baboon tissues are depicted in **Figure 2.2.** Maximum expression of ghrelin was in the stomach, followed closely by the hypothalamus, and then small amounts in the intestine and skeletal muscle. Minimal expression also was observed in monocytes, placenta, omental adipose, kidney, colon, pancreas and liver.

Heritabilities of weight-related traits are listed in **Table 2.3**. Significant additive genetic influence (h^2) on the variations in plasma glucose ($h^2 = 0.68$, $p < 0.0001$), body weight ($h^2 = 0.62$, $p < 0.0001$) and plasma ghrelin ($h^2 = 0.25$, $p < 0.001$) were observed. A preliminary genome-wide scan showed a linkage signal for ghrelin on chromosome 9p23 (50 cM near marker D9S268, LOD = 1.65) with sex, age, age-squared and sex-specific age as covariates (**Figure 2.3**).

2.4 DISCUSSION

The present study demonstrated the unique characteristics of ghrelin in baboons and the effect of genetic factors on the variation in plasma levels. Ghrelin was first isolated as a ligand for the receptor of a secretagogue of growth hormone [Cummings and Shannon, 2003]. The regulation of food intake and energy balance by ghrelin is believed to occur through G-protein coupled receptors situated on NPY/AGRP neurons in the arcuate nucleus [Chen et al., 2003].

Fasting plasma levels of ghrelin have been reported for various mammals such as humans, rats, mice, cow, pig [Ambrogi et al., 2003] and rhesus monkeys [Angeloni et al., 2004]. These range from 395 to 1738 pg/ml, with humans being the lowest and rhesus monkey, the highest. Our study in baboons found slightly greater values. In addition, a distinct sexual dimorphism existed, with male baboons exhibiting higher concentrations of circulating ghrelin than females. This difference may be related to the greater body size and lower adiposity in male baboons, as ghrelin tends to decrease with increasing adiposity [Cummings and Shannon, 2003]. In humans, however, conflicting reports are with respect to sex-specific differences in plasma ghrelin. Tschop et al.[2001] and Purnell et al. [2003] found no clear differences in the plasma levels between sexes. In contrast, Barkan et al.[2003] and Greenman et al. [2004] observed significantly higher levels of circulating ghrelin in females.

The inverse association between body weight and plasma ghrelin in baboons observed in this study is in agreement with human research in which lower plasma ghrelin is present with obesity [Tschop et al., 2001; Cummings and Shannon, 2003; Ambrogi et al., 2003; Casanueva and Dieguez, 2002]. In addition, plasma ghrelin was negatively related with leptin and insulin, two hormones that act opposite to ghrelin in its regulation of food intake. Both leptin [Tschop et al., 2001] and insulin [Poykko et al., 2003] have been negatively related with plasma ghrelin in humans. In rhesus monkey, the relationship with leptin was not found [Angeloni et al., 2004].

Tissue distribution of ghrelin in the baboons showed maximum expression in the stomach. This finding is not surprising, as others have reported that stomach is the primary source of ghrelin in humans [Ueno et al., 2005, Kojima & Kangawa, 2002] and other animals [Ueno et al., 2005]. This hormone also is expressed in hypothalamus, where it activates NPY/AGRP neurons in the arcuate nucleus, in addition to gastric ghrelin.

Baboon preproghrelin was 97 % similar to humans. It has 354 nucleotides, consisting of a 22 amino acid signal peptide and the 28 amino acid ghrelin. Human and baboon preproghrelin differed from each other by nine nucleotides. But there was only a single amino acid difference between human and baboon ghrelin protein, and two amino acid differences from dog, rat and mice [Rindi et al., 2004].

Several weight-related phenotypes have been shown to be under genetic influence in baboons [Comuzzie et al., 2003, Cai et al., 2004, Tejero et al., 2004]. In this study, significant heritability of plasma ghrelin demonstrated considerable influence of genes on its variation. The genome wide scan for chromosomal loci affecting plasma ghrelin found a suggestive evidence of linkage on chromosome 9 at p23 (50 cM, LOD = 1.65) near marker D9S268. Previously, this chromosome has been implicated in the regulation of HDL cholesterol in a large Hutterite population [Newman et al., 2003] and Mexican-Americans [Arya et al., 2002]. It has also been linked with adiponectin [Lindsey et al., 2003] and insulin sensitivity [Hanson et al., 2001] in Pima Indians, and the onset of diabetes in Mexican-Americans [Duggirala et al., 1999].

In summary, ghrelin is a 28 amino acid peptide that is expressed primarily in stomach in baboons. The sequence of the coding region and the tissue expression of ghrelin and its relationships with other weight-related phenotypes follow the same pattern as in humans. Significant heritability and suggestive linkage indicate a substantial genetic impact on the plasma variations of ghrelin in these animals.

TABLE 2.1 Profile of baboons

Phenotype	Males*	Females*	p value
Age (yrs)	18.5 (0.31)	20.6 (0.29)	0.0001
Body weight (kg)	32.7 (0.55)	20.2 (0.26)	0.0001
Plasma			
Ghrelin (pg/ml)	4346.5 (191)	3035.0 (107)	0.0001
Glucose (mg/dl)	86.2 (2.05)	95.7 (2.08)	0.021
Insulin (uIu/ml)	16.3 (1.72)	36.8 (2.60)	0.0001
C-peptide (ng/ml)	1.9 (0.25)	3.9 (0.43)	0.002
Leptin (ng/ml)	1.5 (0.17)	2.2 (0.18)	0.015

*Mean (SEM)

TABLE 2.2 Amino acid sequence of ghrelin in mammals*

Mammal	Ghrelin amino acid sequence
Baboon	**G S S F L S P E H Q** R A Q **Q R K E S K K P P** A K **L** Q **P R**
Human	**G S S F L S P E H Q** R V Q **Q R K E S K K P P** A K **L** Q **P R****
Rh.Monkey	**G S S F L S P E H Q** R A Q **Q R K E S K K P P** A K **L** Q **P R*****
Dog	**G S S F L S P E H Q** K L Q **Q R K E S K K P P** A K **L** Q **P R****
Rat	**G S S F L S P E H Q** K A Q **Q R K E S K K P P** A K **L** Q **P R****
Mouse	**G S S F L S P E H Q** K A Q **Q R K E S K K P P** A K **L** Q **P R****
Pig	**G S S F L S P E H Q** K V Q **Q R K E S K K P A** A K **L** K **P R****
Sheep	**G S S F L S P E H Q** K L - **Q R K E S K K P S** G R **L** K **P R****
Cattle	**G S S F L S P E H Q** K L - **Q R K E S K K P S** G R **L** K **P R****

*Conserved sequences are bold and underlined
**Rindi et al., Exp Biol Med. 2004; 229:1007-1016
***Angeloni et al., Endocrinol. 2004; 145(5):2197-2205

TABLE 2.3 Heritabilities of weight-related traits

Trait	Heritability (h^2) *	p value
Glucose (mM)	0.68 (0.17)	0.0000006
Body weight (kg)	0.62 (0.15)	0.0000001
Log Ghrelin (pg/ml)	0.25 (0.11)	0.0008

*Mean (SE)

FIGURE 2.1 Nucleotide sequences of the prepro-ghrelin in baboons* and humans**

ATG CCC TCC CCA GGG ACC GTC TGC AGC CTC CTG CTC CTC GGC ATG
ATG CCC TCC CCA GGG ACC GTC TGC AGC CTC CTG CTC CTC GGC ATG
CTC TGG CTG GAC TTG GCC ATG GCA GGC TCC AGC TTC CTG AGC CCT
CTC TGG CTG GAC TTG GCC ATG GCA GGC TCC AGC TTC CTG AGC CCT
GAA CAC CAG AGA GCC CAG CAG AGA AAG GAG TCC AAG AAG CCA CCA
GAA CAC CAG AGA GTC CAG CAG AGA AAG GAG TCG AAG AAG CCA CCA
GCC AAG CTG CAG CCC CGA GCT CTA GGA GGC TGG CTC CGC CCA GAA
GCC AAG CTG CAG CCC CGA GCT CTA GCA GGC TGG CTC CGC CCG GAA
GAT GGA GAT CAG GCA GAA GGG GCG GAG GAT GAA CTG GAA ATC CAG
GAT GGA GGT CAA GCA GAA GGG GCA GAG GAT GAA CTG GAA GTC CGG
TTC AAC GCC CCC TTT GAT GTT GGA ATC AAG CTG TCA GGG GTT CAG
TTC AAC GCC CCC TTT GAT GTT GGA ATC AAG CTG TCA GGG GTT CAG
TAC CAG CAG CAC AGC CAG GCC CTG GGG AAG TTT CTT CAG GAC ATC
TAC CAG CAG CAC AGC CAG GCC CTG GGG AAG TTT CTT CAG GAC ATC
CTC TGG GAA GAG GCC AAA GAG GCC CCA GCC GAC AAG TGA
CTC TGG GAA GAG GCC AAA GAG GCC CCA GCC GAC AAG TGA

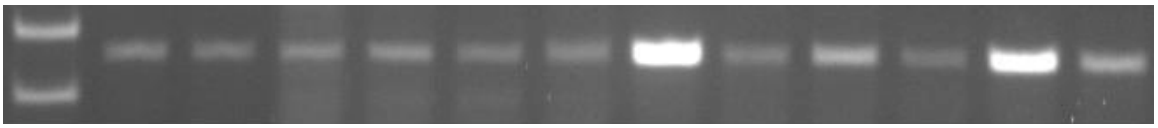
* Baboon sequences are in bold

** Differences in nucleotides between humans and baboons are underlined

FIGURE 2.2 Tissue distribution of ghrelin and 18S in baboon tissues*

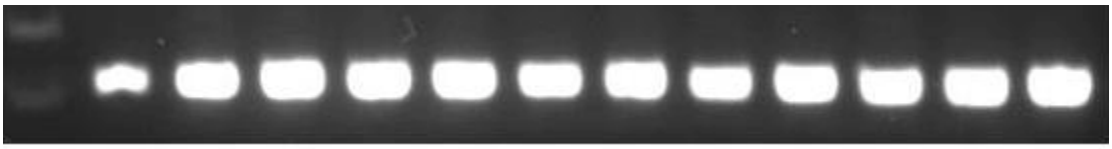
Ghrelin

1 2 3 4 5 6 7 8 9 10 11 12 13



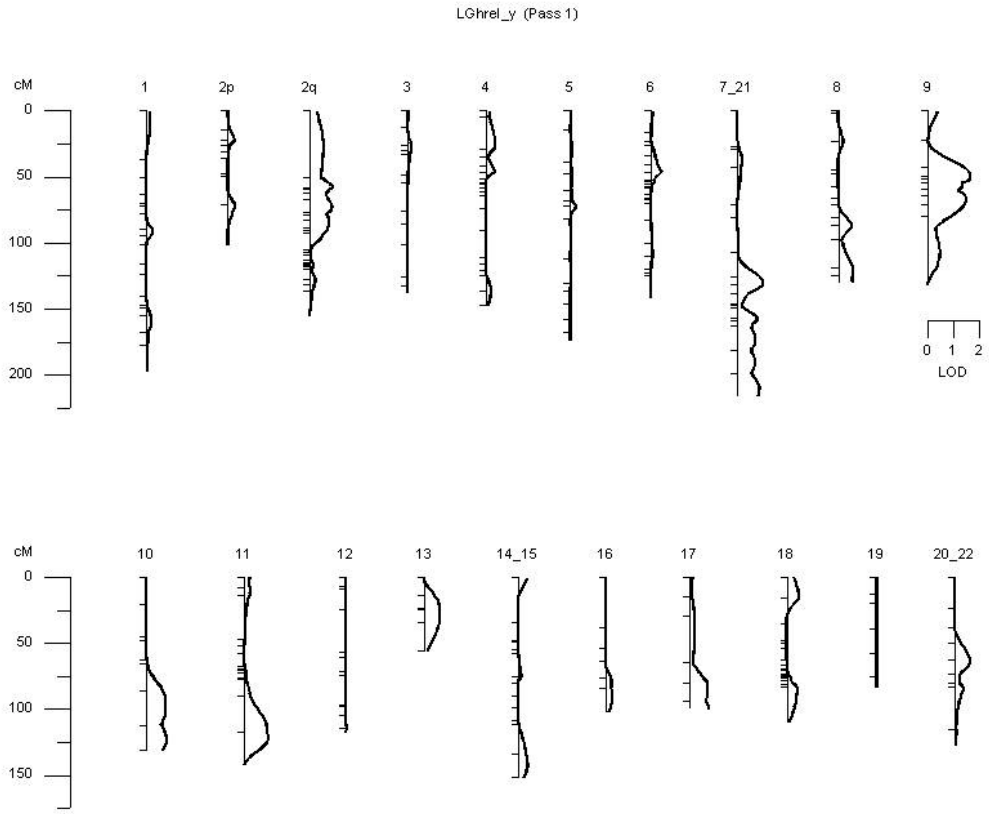
18 S

1 2 3 4 5 6 7 8 9 10 11 12 13



* 1 = Ladder, 2 = Monocytes, 3 = Placenta, 4 = Ovary, 5 = Omental adipose, 6 = Kidney, 7 = Colon, 8 = Stomach, 9 = Pancreas, 10 = Small intestine, 11 = Liver, 12 = Hypothalamus, 13 = Skeletal muscle

FIGURE 2.3 Genome-wide scan of plasma ghrelin. Chromosomal location is represented on the x-axis and LOD score is shown on the y-axis



CHAPTER 3

QUANTITATIVE GENETIC ANALYSIS SHOWS LINKAGE OF PLASMA CHOLECYSTOKININ IN BABOONS TO HUMAN CHROMOSOME 17

3.1 INTRODUCTION

Cholecystokinin (CCK) is a major satiety signal that is produced in response to the presence of food (particularly high in fat or protein) in the duodenum. It is secreted by the endocrine I-cells in the mucosal layer of the anterior small intestine [Havel, 2001] and the neurons of the central nervous system [Liddle, 1997]. The CCK hormone interacts with its locally based receptors CCK-1 (formerly CCK-A) and the resultant information is transmitted to the brainstem through the vagus nerve to inhibit food intake and control meal size [Havel, 2001].

A reduction in meal volume with CCK is compensated usually by an increase in its frequency. However, the interaction of CCK with long-term signals such as leptin prolongs this effect [Strader & Woods, 2005]. It is believed that leptin and CCK act concomitantly to regulate body weight and amount of the meal, as injection of leptin into the duodenum increases plasma CCK [Guilmeau et al., 2003]. In addition, administration of CCK enhances the leptin-induced reduction in body weight and meal quantity, indicating that this synergistic action has a direct influence on food intake [Peters et al., 2004].

Weight regulation is a complex process involving energy intake, body fat stores, brain and peripheral signals that regulate meal intake and termination [Woods & Seeley, 2000], and genetics [Bouchard, 1997]. In humans, heritability of obesity phenotypes has been shown to be greater than 50 %. Evidence from genetic studies point to a number of chromosomal loci that affect variations in obesity traits. Similar to humans, obesity related phenotypes in baboons are heritable. A study conducted by Comuzzie et al. [2003] showed considerable additive genetic effects for body weight ($h^2 = 0.62$, $p < 0.0001$), fat mass ($h^2 = 0.41$, $p < 0.00001$), fat free mass ($h^2 = 0.32$, $p < 0.000001$), and leptin ($h^2 = 0.21$, $p < 0.05$).

Baboons are ideal models for genetic studies, especially obesity, since the environmental variations are minimal due to the same diet and housing conditions. These animals also have a high degree of similarity with humans with respect to genetic variations, and protein and chromosomal structure [Comuzzie et al., 2003]. In our pedigreed population of baboons, 10% of the baboons become obese spontaneously. This weight gain occurs despite the similarity in their diets and dwellings. Thus, these primates are valuable resources for studying the genetics of body weight.

The objective of this study was to explore the genetic influences on phenotypes related to body weight, particularly the satiety hormone CCK. In the process, heritability of plasma CCK was estimated and specific chromosomal loci affecting the circulating levels of CCK were identified.

3.2 METHODS

Experimental Design

Adult baboons were measured for body weight and length and blood samples were collected when these animals were sedated for a routine medical examination. Plasma was separated and used to analyze biochemical parameters such as glucose, insulin, c-peptide, leptin and CCK. For the linkage analysis, genotyping was conducted in 376 baboons in accordance with the standard protocols. Heritabilities and quantitative trait loci influencing these phenotypes were estimated utilizing univariate quantitative genetic analysis.

Subjects

The baboons for this study were obtained from a pedigreed population of 376 adults (263 females, 113 males) from the Southwest Foundation for Biomedical Research (SFBR) at San Antonio, TX. The species of the baboons is a mixture of yellow baboons *(Papio hamadrayas cynocephalus)* and olive baboons *(Papio hamadrayas anubis)*. The group of genotyped baboons spans 2 - 4 non-inbred generations, ranging from 6 to 213 members [Comuzzie et al., 2003]. The relative pairs used for this study are listed in **Table 3.1**. All these animals shared the same living conditions, were housed in open-air group cages and fed ad libitum on a standard low fat diet (Harlan Teklad 15% monkey diet, 8715, Indianapolis, IN).

Sample collection

Blood was drawn from the antecubital vein under ketamine sedation after an overnight fast (~ 12 hours). Total volume of blood drawn was 11 ml; 4 ml was collected in sodium fluoride tubes for analysis of glucose and 7 ml in EDTA tubes for analyzing insulin, leptin, c-peptide and cholecystokinin. Body weight was estimated with a calibrated electronic scale (GSE, Chicago, IL). Plasma was separated by centrifugation at 2000 x g for 10 minutes and stored in aliquots at -80^0C until analysis.

Assays for glucose, insulin, leptin, c-peptide and cholecystokinin

Glucose was analyzed by the glucose oxidase method with an Analox spectrophotometer (Analox Instruments, Lunenburg, MA). Plasma insulin, leptin and c-peptide were measured by quimiluminiscence in a Luminex 100, utilizing the Endocrine Multiplex Immunoassay (LINCO Research, Inc, St Charles, MO). Commercially available radioimmunoassay kits (LINCO Research, Inc, St Charles, MO) were used to obtain cholecystokinin. All samples were analyzed in duplicate.

Genotypes

A baboon genetic map has been developed using human short-tandem repeat loci [Rogers et al., 2000]. Homologous microsatellite loci from baboon genomic DNA were amplified using published human PCR primers. Baboon genotypes were obtained by gel electrophoresis and analyzed on ABI 373 and ABI 377 automated sequencers using

fluorescently labeled primers and Genescan and Genotyper software (Applied Biosystems, Foster city, CA). A total of 331 markers were obtained, with an average space of 10 cM between markers. The computer program PEDSYS managed and prepared the baboon genotypes and pedigree data [Dyke et al., 1994].

Statistical methods

To locate the specific QTL affecting plasma CCK, the residual heritability of CCK was estimated using age, sex, sex-specific age and age squared as covariates. The total phenotypic variance (σ^2_P) was divided into its additive genetic (σ^2_G) and environmental (σ^2_E) components. The heritability of a phenotype refers to the contribution of additive genetic effects to the total phenotypic variance and is denoted by $h^2 = \sigma^2_G / \sigma^2_P$ (Hopper and Mathews, 1982). Likelihood ratio tests were used to obtain the p-values for heritabilities, where the null hypothesis, in which the additive genetic variance for a specific QTL equals zero, was tested against an alternate hypothesis in which the additive variance has been estimated. Twice the difference in the logarithmic likelihoods was distributed asymptotically as a ½: ½ mixture of a χ^2 variable with one degree of freedom and a point mass at zero (Self and Liang, 1987).

A variance component linkage analysis was employed to detect the specific linkage between the markers and plasma CCK. This technique is implemented in the software program SOLAR [Almasy and Blangero, 1998]. It is based on denoting the expected genetic covariances between relatives as a function of the identity by descent

(IBD) relationship at the marker locus, and ascertains whether the phenotypic variation can be explained by the genetic variation at a specific chromosomal locus.

Covariance matrix for the members from the same pedigree is expressed as:

$$\Omega = \Pi \sigma^2_q + 2\,\Phi\sigma^2_a + I\,\sigma^2_e$$

where Ω = covariance matrix for the all the members of the same pedigree

Π = the matrix of the IBD sharing of the pedigree members at a particular location of the QTL, in which

σ^2_q = additive genetic effects of the specific loci

Φ = kinship matrix

σ^2_a = non genetic effects of the loci

I = identity matrix

σ^2_e = individual specific random environmental effects

The null hypothesis that the additive genetic variance for a specific QTL equals zero (no linkage) was tested by comparison with an alternate hypothesis in which the additive variance was estimated [Blangero and Almasy, 1997]. Significance of the test was determined by the logarithm of the odds (LOD) score, which is calculated as the difference between the two-$\log^{10}$ likelihoods. Twice the difference in the logarithmic likelihoods was distributed asymptotically as a ½: ½ mixture of a χ^2 variable with one degree of freedom (Self and Liang, 1987).

3.3 RESULTS

The analyzed phenotypes and the relative differences between males and female baboons are depicted in **Table 3.2**. Females were older, but had lower body weights (22 – 54 kg) than males (11- 35 kg). Plasma levels of glucose, insulin, C-peptide and leptin were higher in female baboons. In contrast, circulating levels of CCK were elevated in males.

Mean heritability of CCK was 0.14 ± 0.1 ($p = 0.028$), suggesting that additive genetic effects might be influencing a considerable part of the variations in plasma levels. In addition, body weight ($h^2 = 0.53 \pm 0.15$, $p = 0.0000013$) and glucose ($h^2 = 0.68 \pm 0.17$, $p = 0.0000006$), were heritable.

A summary of the univariate linkage analysis by each chromosome is presented in **Figure 3.1**. The strongest signal was detected on chromosome 17 (LOD = 3.1, $p < 0.01$) at approximately 29 cM pter near marker D17S804 **(Figure 3.2).** Suggestive linkages were observed on chromosome 4 (LOD = 2.26, $p < 0.05$) at 136 cM qter near marker D4S2374 **(Figure .33)** and chromosome 7_21 (LOD = 1.8, $p < 0.05$)

Obesity-related phenotypes that have been previously reported on chromosome 17 are shown in **Table 3.3**. Leptin, acylation stimulating protein (ASP), BMI, insulin, glucose transporter 4 (GLUT 4), % fat, adiponectin and glucose have all been mapped to this chromosome. Of these phenotypes, the loci detected for leptin [Kissebah et al.2000],

acylation-stimulating protein (ASP) [Martin et al.2004], body mass index (BMI) [Mitchell et al.1999] and adiponectin [Comuzzie et al.2001] were within 10cM of CCK.

3.4 DISCUSSION

The most important finding of this study was the location of a strong signal for CCK on a baboon homolog of human chromosome 17p12-13. Cholecystokinin is a major satiety hormone that regulates food intake and meal size [Woods et al., 2000]. It also controls gastric motility, pancreatic and gastric acid secretion, and gall bladder contraction [Moran, 2004].

Identifying QTL for CCK in this region is of considerable importance as the area has been previously mapped to several loci related to obesity phenotypes. Kissebah et al. [2000] reported a linkage with leptin in families from Take Off Pounds Sensibly study (TOPS) near marker D17S947 (LOD = 5.0 at 38 cM) on 17p12. Leptin is an adipocyte-derived hormone with a significant role in energy homeostasis [Peters et al., 2004], and is known to enhance the action of CCK in controlling the meal volume [Peters et al., 2004].

Moreover, CCK also is augmented by insulin, a hormone associated with satiety [Riedy et al., 1995]. Two studies, one in African-Americans and Hispanic [Rich et al., 2005], and the other in Caucasians [Panhuysen et al., 2003], have found loci for fasting insulin on this gene.

The adipose-derived hormones, ASP and adiponectin, were mapped to the same section as for CCK. The acylation stimulating protein has an important role in obesity in the regulation of fat storage and enhancement of glucose transport [Martin et al., 2004]. A study conducted in Mexican-Americans revealed a QTL for ASP on chromosome 17 near marker D17S1303 at 32cM [Martin et al., 2004]. Adiponectin is a hormone implicated in insulin function that is low in obese individuals. In northern Europeans, Comuzzie et al. [2001] found a signal for serum adiponectin on 17, but with a smaller LOD score of 1.7. Also, two suggestive linkages for BMI on the same chromosome were found by Mitchell et al. [1999] in the San Antonio Family Heart Study (SAFHS). One was near marker D17S1293 at 64 cM pter (LOD = 2.33) and the other close to D17S786 at 21 cM pter (LOD = 1.34). In Pima Indians, % fat was located on an adjacent marker D17S785 [Norman et al., 1998]. Collectively, these studies indicate the importance of this section of chromosome 17, with reference to body weight and adiposity traits.

In addition, candidate genes connected to adiposity GLUT4 [Bell et al., 1989] and gClq (a putative adiponectin binding protein) [Comuzzie et al., 2001] have been mapped to chromosome 17. Other hormones linked to this chromosome that are involved in energy homeostasis are PYY [Hort et al., 1995] and orexin [Taheri & Bloom, 2001].

A weaker signal for CCK was observed at chromosome 4q34-35 with a LOD score of 2.2. This chromosome 4 also has been associated with weight-related traits. For example, Arya et al. [2004] reported a LOD score of 4.5 for BMI in Mexican-Americans on the p-terminal. In a study by Chen et al. [2005], BMI was mapped to chromosome 4

(LOD= 1.68) at q23-26 in West Africans. Other obesity positional candidate genes such as CCK-A receptor and peroxisome proliferator-activated receptor gamma coactivator1 (PPARGC1) have been localized to this chromosome (Arya et al., 2004], suggesting a contribution towards the regulation of food intake.

In summary, there was a significant contribution of additive genetic effects to the variations of plasma levels of CCK. The identification of a QTL for plasma CCK on chromosome 17 is important, in view of the fact that several weight-related traits have already been mapped to this region.

TABLE 3.1 Relative pairs of baboons

Relationship	Number of pairs
Unrelated	3449
Parent-offspring	50
Siblings	173
Avuncular	40
Half-siblings	2360
Half-avuncular	413
Half first cousins	29
Half- first cousins, 1rem	10
Half-siblings & first cousins	3
Half-siblings & half first cousins	34
Half-siblings & half avuncular	5
Total	6566

TABLE 3.2 Descriptive statistics of baboons*

Phenotype	Males	Females	p value
Age (yrs)	18.5 (0.31)	20.6 (0.29)	0.0001
Body weight (kg)	32.7 (0.55)	20.2 (0.26)	0.0001
Glucose (mg/dl)	86.2 (2.05)	95.7 (2.08)	0.021
Insulin (uIU/ml)	16.3 (1.72)	36.8 (2.60)	0.0001
C-peptide (ng/ml)	1.9 (0.25)	3.9 (0.43)	0.002
Leptin (ng/ml)	1.5 (0.17)	2.2 (0.18)	0.015
CCK (pmol/l)	13.8 (0.57)	12.5 (0.34)	0.031

*Mean (SEM)

TABLE 3.3 Obesity-related QTLs, previously reported on chromosome 17

Phenotype	Location (cM)	LOD	Reference
Leptin	38	5.0	Kissebah et al., 2000
ASP[1] & BMI[2]	28	4.7	Martin et al., 2004
Insulin	54	3.3	Rich et al., 2005
BMI	90.6	3.16	Bell et al., 2004
GLUT 4[3]	p13	3.0	Bell et al., 1989
ASP	32	2.7	Martin et al., 2004
% fat	123.7	2.4	Norman et al., 1998
BMI	64	2.33	Mitchell et al., 1999
HOMA-IR[4]	60	2.0	Panhuysen et al., 2003
Insulin	60	1.8	Panhuysen et al., 2003
Adiponectin	38	1.7	Comuzzie et al., 2001
Glucose	58	1.44	Rich et al., 2005
BMI	21	1.34	Mitchell et al., 1999

[1]Acylation-Stimulating Protein
[2]Body Mass Index
[3]Glucose Transporter 4
[4]Homeostatic Model Assessment-Insulin Resistance (Mathews et al. 1985)

FIGURE 3.1 Summary of the univariate linkage analysis by each chromosome. Chromosomal location is represented on the x-axis and the LOD score shown on the y-axis

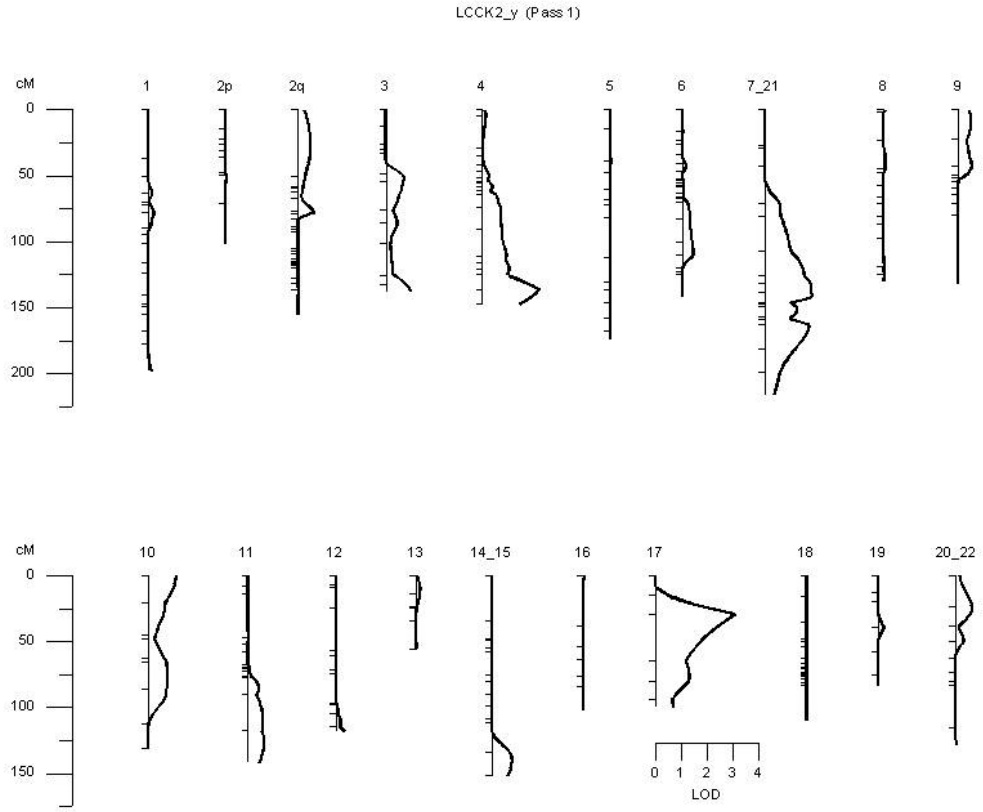


FIGURE 3.2 Map depicting LOD scores and marker distances for plasma CCK on chromosome 17.

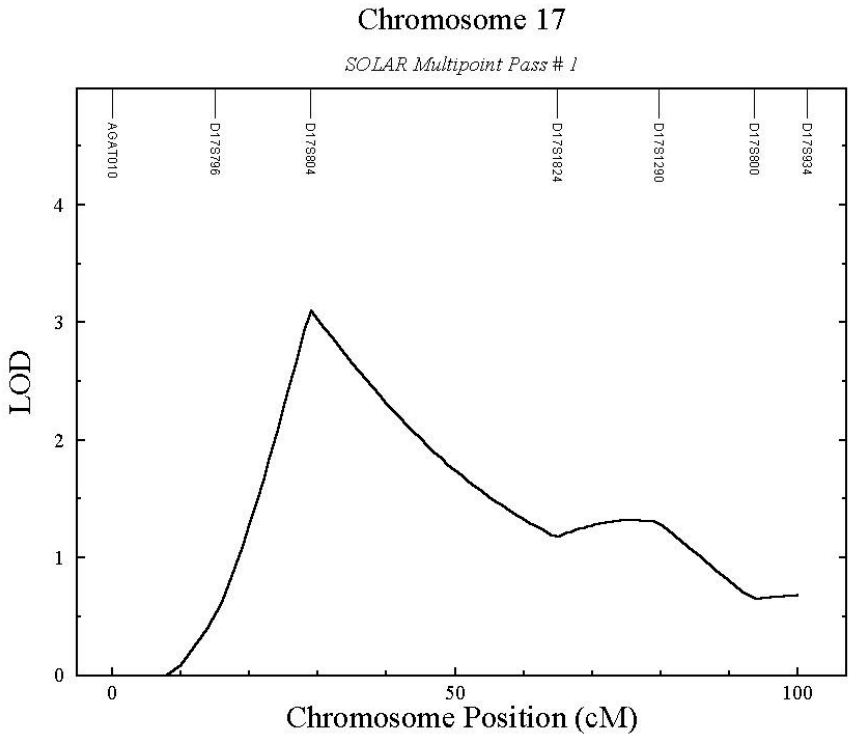
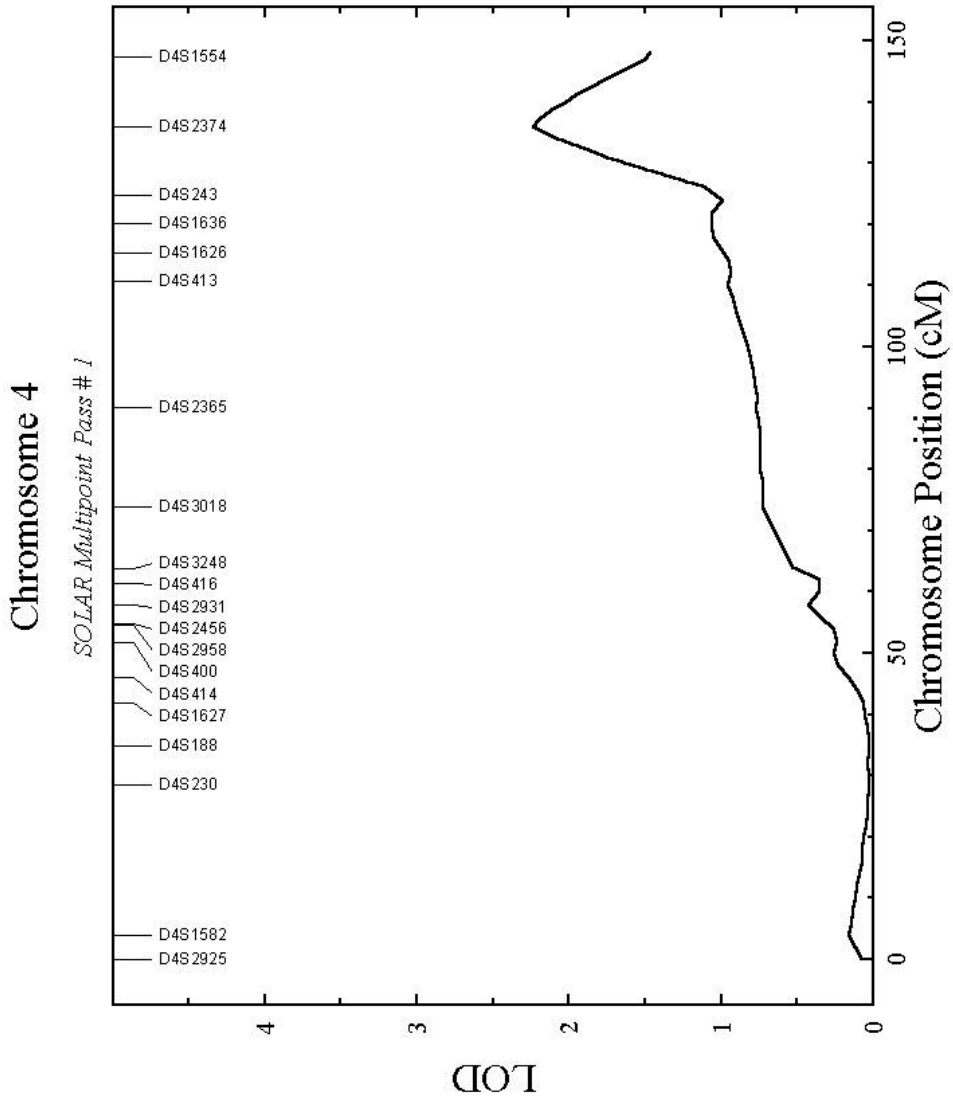


FIGURE 3.3 Map depicting LOD scores and marker distances for plasma CCK on chromosome 4.



CHAPTER 4

A COMMON SET OF GENES REGULATES LDL SIZE AND OBESITY-RELATED FACTORS IN ALASKA NATIVES

4.1 INTRODUCTION

Mortality rates due to cardiovascular disease (CVD) in Alaska Natives have been historically low. This lower incidence has been attributed to greater dietary intake of fish oils, which are excellent sources of omega-3 fatty acids [Kromhout et al., 1985]. In the past decade, this trend has changed with the westernization of diet and lifestyle. Heart disease is now the third leading cause of death in Alaska Natives, behind accidental injuries and cancer [Day and Lanier, 2003].

Obesity, particularly abdominal is one of the major risk factors for CVD [Rexrode et al., 1998], as an obese person is two to three times more likely to exhibit CVD than a non-obese person [Stein and Colditz, 2004]. More than one-third (37.2 %) of Alaska Natives are either overweight or obese, with more women (47.2 %) being overweight than men (26.2 %). Also, women from this region have equivalent or higher visceral adiposity as compared to the world's highest reported averages [Risica et al., 2000]. Abdominal fat deposition is crucial because visceral adipose tissue enhances the flow of free fatty acids into liver via the portal vein increasing the production of triglycerides. This occurs with simultaneous suppression of high-density lipoprotein (HDL) production

and low-density lipoprotein (LDL) particle size. These liver-derived LDLs transport triglycerides and cholesterol esters in the plasma [Nabel, 2003].

Low-density lipoproteins are composed of well defined and different sized subclasses. These particles are differentiated into LDL 1 through LDL 3 based on their size, with LDL1 being the smallest. Small, dense LDLs are established CVD risk factors and are associated strongly with other lipid and obesity-related phenotypes. The significance of smaller LDL particles is that they have lesser affinity for their receptors [Herron et al., 2004], and remain longer in the plasma, providing a greater chance of being deposited on arterial walls [Rainwater et al., 2003]. Furthermore, small LDLs are more susceptible to oxidation than other forms. Oxidized LDL demonstrates greater atherogenicity because of its increased uptake by macrophages [Herron et al., 2004].

The plasma level of LDL is used as one of the primary criteria for the diagnosis of CVD. However, two individuals may have same levels of LDL, but differ in their susceptibility to atherosclerosis, due to the different concentrations of small and large LDLs. Thus, LDL size may be a better tool for measuring individual CVD risk than total LDL [Johnson et al., 2004]. The size of LDL is under substantial genetic influence. Heritability studies have shown that genetic factors are responsible for 30% to 60 % of the variation of LDL size [Bosse et al., 2004]. On the other hand, effects of other phenotypes such as weight, body fat, triglycerides, and HDL cholesterol, on LDL size cannot be ignored [Kang et al., 2002]. Thus, the purpose of this study was to investigate genetic and environmental factors that affect LDL and its subclasses in Alaska Natives.

4.2 METHODS

Experimental Design

The Genetics of Coronary Artery Disease in Alaska Natives study (GOCADAN) involves populations from villages in the Norton Sound region on the northwestern coast of Alaska. Two GOCADAN investigators visited each village, conducted interviews and explained the nature of the study to the household members. Subjects (n = 1214), over 18 years of age, were recruited and demographics collected during the first visit. Participants attended clinics for medical examination and blood draw after a 12-hour fast. Also, information regarding medical history, dietary intake, current physical activity and anthropometrics were obtained. Blood was drawn by venipuncture and samples were stored in aliquots at -80 C for future analyses. This study was approved by Institutional Review Boards from all participating institutions and informed consent was taken from the participants.

Study population

Participants belonged to the villages of Teller, Golovin, Elim, Koyuk, Shaktoolik, Unalakleet, White Mountain, Brevig Mission and Nome. Subjects were primarily Inupiat Eskimo. For these analyses, data were available for 954 individuals (420 men, 534 women) ranging in age 17-92, with an average age of 43 ± 16 years.

Demographic and phenotypic data

Demographic and genealogical data collected during the surveys included names, genders, dates, and places of birth, current home of proband and his/her spouse and first degree relatives of all household members. This information was sent to the Southwest Foundation for Biomedical Research (SFBR), San Antonio, TX, and entered into the PEDSYS pedigree database system (Dyke et al., 1994).

Anthropometric measurements included height, weight, and waist and hip circumference. Height was estimated to the nearest quarter inch, using a Frankfort horizontal plane in an erect position; weight was determined to the nearest tenth pound, using a balance scale (Detecto, model 683-P). Waist circumference was obtained by measuring at the level of the umbilicus with the subject in a supine position.

Skin-fold measurements (subscapular and triceps) were measured to the nearest millimeter with a Lange caliper. The subscapular measurement was taken one-centimeter to the inferior angle of the right scapula while the subject was standing with shoulders relaxed and arms hanging loosely at the side. The triceps skin fold was determined directly over the right triceps muscle, halfway between the acromial and olecranon processes, with the arm hanging comfortably at the subject's side.

Total cholesterol, HDL cholesterol, LDL cholesterol, VLDL cholesterol and triglycerides were measured by an auto analyzer (Hitachi 717, Amposta, Spain). Lipoprotein subclasses type, size, and concentrations were measured by nuclear magnetic

resonance (NMR) spectroscopy [Otvos et al., 1992]. This method is based on the NMR signal emitted by the terminal methyl groups on the lipids contained in the particle core and the shell.

Statistical genetic methods

Quantitative genetic analyses were performed utilizing the maximum likelihood-based variance decomposition method that is implemented by the computer program, SOLAR (Almasy and Blangero, 1998). According to classical quantitative genetics principle, the total phenotypic variance (σ^2_P) can be split into its genetic (σ^2_G) and non-genetic or environmental components (σ^2_E),

$$\sigma^2_P = \sigma^2_G + \sigma^2_E$$

Heritability of a phenotype refers to the ratio of the variance contributed by additive genetic effects to the total phenotypic variance and is denoted by $h^2 = \sigma^2_G / \sigma^2_P$ [Hopper and Mathews, 1982]. Likelihood ratio tests were used to obtain the p-values for heritabilities, where the null hypothesis, in which the additive genetic variance (σ^2_G) equals zero, was tested against an alternate hypothesis in which the additive genetic variance is estimated. Twice the difference in the logarithmic likelihoods was distributed asymptotically as a ½: ½ mixture of a χ^2 variable with one degree of freedom and a point mass at zero (Self and Liang, 1987).

Univariate quantitative genetic analysis was used to estimate residual heritability using sex, sex-specific age and age squared as covariates. Bivariate genetic analysis [Lange and Boehnke, 1983] was conducted to examine the genetic correlations between LDL, its subclasses and obesity-related factors.

The phenotypic correlation between the phenotypes can be expressed in terms of core genetic and environmental correlations:

$$\rho_P = \rho_G [\sqrt{h_1^2}\sqrt{h_2^2}] + \rho_E [\sqrt{(1-h_1^2)}\sqrt{(1-h_2^2)}];$$

h_1^2 and h_2^2 are the heritabilities of the two phenotypes being studied, and ρ_G and ρ_E are the additive genetic and environmental correlations between the traits, respectively.

A model where all the parameters are estimated is compared with a model in which the genetic correlation is constrained to zero. If the result of this statistical test is significant, then the traits shared effects of a common set of genes. The extent of the shared genetic effect is verified by a second statistical test that compares the model in which all the parameters are estimated with one in which the genetic correlation is constrained to one. The basic premise of this model is that the genes controlling expressions of the two traits completely overlap with each other. The alternative hypothesis is that some genes influencing trait one do not affect trait two and vice versa.

In this study, sex, sex-specific age and age squared were used as covariates and twice the difference in the logarithmic likelihoods was distributed asymptotically as a ½: ½ mixture of a χ^2 variable with one degree of freedom and a point mass at zero [16].

An independent student t-test was applied to study the comparisons between men and women, using SPSS version 10.0.

4.3 RESULTS

Descriptive statistics of anthropometrics and lipids are depicted in **Table 4.1**. Sex-specific comparisons showed that men weighed more but had lower BMI, waist circumference and skinfolds than women. Lipoprotein measurements displayed higher circulating small and medium LDL in men as compared to women. Women, on the other hand, had greater plasma concentrations of large LDL, IDL and HDL cholesterol than men. Plasma triglyceride levels were slightly higher in women than men, although the difference was not statistically significant. In the univariate analysis, significant heritabilities were exhibited by all obesity (47 % to 64 %) and LDL related phenotypes (20 % to 36 %) (**Table 4.2**).

Genetic correlations between low-density lipoproteins (LDL), its subclasses and obesity-related anthropometric measures are shown in **Table 4.3**. Significant and positive genetic correlations were observed between small to medium LDL and obesity-related factors. Large LDL was not genetically correlated with any of the obesity-related factors. **Table 4.4** depicts genetic correlations between LDL subclasses and other lipids. Small

LDL displayed negative correlations with HDL. Medium LDL had significant associations with HDL (negative) and triglycerides (positive). Large LDL was correlated negatively with triglycerides and positively with HDL. All the tests for complete pleiotropy are significantly different than one, suggesting that no two traits tested were governed by completely overlapping genes.

4.4 DISCUSSION

This study demonstrated significant genetic correlations between small to medium sized LDL particles and obesity-related factors indicating that a common set of genes might be regulating these phenotypes in Alaska Natives. Obesity, its related factors and LDL are strong cardiovascular risk factors. However, the mode of action is complex. Weisberg et al. [2003] suggest that macrophage infiltration into the adipose tissue might be the basis for the development of several obesity-related pathologies. According to their study, increasing adiposity tends to escalate the number of macrophages in the adipose tissue [Weisberg et al., 2003]. These bone marrow-derived cells induce a rise in pro-inflammatory markers and fat accumulation in blood vessels [Lehrke and Lazar, 2004] thereby, increasing the risk of CVD. In addition, visceral adiposity results in a greater flow of free fatty acids to the liver altering lipid levels, such as increased triglycerides, lowered HDL levels and reduction in the size of LDL [Sonnenberg et al., 2004].

The size of LDL is a major determinant in the risk for CVD and varies with age, race and sex. Small, dense LDL is considered the most crucial as it is more atherogenic than other forms [Austin et al., 2003]. Freedman et al. [2004] observed that men had lower levels of large LDL and higher concentrations of small to medium LDL than women, indicating an increased risk of CVD. These same results were echoed in our study, with men exhibiting elevated levels of smaller LDLs despite lower BMIs.

Small LDL had strong genetic correlations with weight and BMI, suggesting that 42 % ($\rho^2_G = (0.65)^2 = 0.42$) to 47 % ($\rho^2_G = (0.67)^2 = 0.47$) of additive genetic variance in small LDL is shared with weight and BMI. Abdominal obesity poses a greater CVD risk than other types, irrespective of ethnicity and gender. Individuals with large waist circumferences have a greater risk of developing CVD than those with normal or smaller waist [Vega, 2002]. In the present study, the genetic correlations between small LDL and waist circumference was 0.80 indicating that 64 % ($\rho^2_G = (0.80)^2 = 0.64$) of the additive genetic contribution to small LDL is shared with waist circumference. Other studies also have found that abdominal adiposity, as measured by waist circumference, was positively associated with CVD despite controlling for BMI [Vega, 2002]. Similar results were presented by James et al. [2004] in a group of Caucasian men and women. Their study observed positive correlations between small LDL and abdominal adiposity, as measured by waist-hip ratio. Medium LDL showed modest genetic correlations with weight, BMI and waist circumference, yet ~ 25 % of the additive genetic variance in these phenotypes can be attributable to shared genes. However, large LDL showed negative, but nonsignificant, genetic and environmental correlations with obesity related factors.

Rainwater et al. [1999] in the San Antonio Family Heart Study have also reported the negative relationship of large LDL with obesity-related phenotypes.

Obesity is associated with high triglycerides and low HDL [Friedlander et al., 2000]. These altered lipid levels coupled with decreased LDL size are thought to result from the overproduction of very low-density lipoproteins (VLDL) in the liver, which is regulated by the flow of free fatty acids from visceral adipose tissue. The negative genetic correlation between small to medium LDL and HDL in this study may indicate substantial genetic influence on the altered lipid levels.

LDL size also is influenced, to a large extent, by plasma triglycerides [Wallace et al., 2000]. In the present study, positive genetic correlations between triglycerides and medium LDL ($\rho^2_G = (0.88)^2 = 0.77$) were observed, suggesting that 77% of the additive genetic variance in medium LDL is shared with triglycerides. The relationship between triglycerides and LDL size is further corroborated by the fact that quantitative trait loci (QTLs) for both of these phenotypes were linked to the same region on human chromosome 7q35-q36 [Sonnenberg et al., 2004] and that the same set of genes might be affecting their variations.

Significant genetic correlations of LDL subclasses with obesity-related factors and other lipids demonstrate considerable genetic influence on these phenotypes. Understanding the core of these genetic relationships and the identification of

chromosomal regions containing genes that control variation in these phenotypes will help gain insight into the complex nature of cardiovascular disease.

TABLE 4.1 Descriptive statistics of anthropometrics and lipids in Alaska Natives

Phenotype	Men*	Women*	P value
Obesity			
Weight (kg)	76.3 (0.73)	71.1 (0.69)	0.001
BMI** (kg/m2)	26.4 (0.23)	28.5 (0.26)	0.001
Waist circumference (cm)	87.4 (0.59)	88.6 (0.58)	NS
Skinfold (mm)			
Subscapular	14.6 (0.36)	20.8 (0.40)	0.001
Triceps	12.8 (0.30)	21.7 (0.33)	0.001
LDL† concentration (mg/dl)			
Small	10.5 (0.69)	6.6 (0.51)	0.001
Medium	22.0 (1.23)	17.7 (1.13)	0.011
Large	65.4 (2.16)	76.3 (2.00)	0.001
Mean	117.2 (1.74)	114.7 (1.52)	NS
Other lipids (mg/dl)			
Chylomicrons	0.16 (0.02)	0.13 (0.02)	NS
IDL‡	2.9 (0.25)	3.8 (0.25)	0.015
HDL#	54.7 (0.79)	64.6 (0.75)	0.001
Triglycerides	121.5 (2.93)	123.0 (2.54)	NS

*Mean (SEM)
**BMI – Body mass index
†LDL- Low-density lipoprotein
‡IDL – Intermediate-density lipoprotein
#HDL – High-density lipoprotein

TABLE 4.2 Heritabilities (h^2) of anthropometric and lipid related measurements:

Phenotype	h^2	SEM	p value	Effects of covariates
Obesity				
Weight (lb)	0.64	0.06	1.71E-25	0.044
BMI (kg/m^2)	0.57	0.07	7.62E-22	0.037
Waist circumference (in)	0.55	0.07	1.76E-19	0.030
Skinfold (mm)				
Subscapular	0.53	0.07	3.66E-21	0.124
Triceps	0.47	0.07	2.90E-17	0.269
LDL concentration (mg/dl)				
Small	0.20	0.06	2.07E-04	0.039
Medium	0.31	0.08	5.00E-06	0.026
Large	0.30	0.07	5.00E-07	0.058
Mean	0.36	0.07	1.44E-10	0.108
Other lipids (mg/dl)				
HDL	0.51	0.07	5.23E-18	0.117
Triglycerides	0.31	0.08	1.10E-06	0.020

TABLE 4.3 Genetic (rho_G) correlations between plasma concentrations of LDL subclasses and obesity-related measures

LDL concentration (mg/dl)	Phenotype	rho_G*	p value
Small	Weight	0.65 (0.27)	0.01
	BMI	0.67 (0.25)	0.009
	Waist	0.80 (0.22)	0.001
	Subscapular skin fold	0.93 (0.27)	0.001
	Triceps skin fold	0.78 (0.22)	0.002
Medium	Weight	0.46 (0.22)	0.048
	BMI	0.52 (0.22)	0.028
	Waist	0.46 (0.23)	0.060
	Subscapular skin fold	0.44 (0.22)	NS
	Triceps skin fold	0.60 (0.19)	0.01
Large	Weight	- 0.29 (0.18)	NS
	BMI	- 0.21 (0.19)	NS
	Waist	- 0.13 (0.19)	NS
	Subscapular skin fold	- 0.21 (0.20)	NS
	Triceps skin fold	- 0.27 (0.20)	NS
Mean	Weight	0.22 (0.16)	NS
	BMI	0.34 (0.16)	0.042
	Waist	0.49 (0.16)	0.004
	Subscapular skin fold	0.23 (0.17)	NS
	Triceps skin fold	0.07 (0.18)	NS

*Mean (SEM)

TABLE 4.4 Genetic (rho_G) correlations between plasma concentrations of LDL subclasses and other lipids

LDL concentration (mg/dl)	Other lipids (mg/dl)	rho_G*	p value
Small	HDL	- 0.49 (0.24)	0.06
	Triglycerides	0.24 (0.34)	NS
Medium	HDL	- 0.65 (0.20)	0.005
	Triglycerides	0.88 (0.19)	0.003
Large	HDL	0.58 (0.16)	0.002
	Triglycerides	- 0.35 (0.23)	NS
Mean	HDL	- 0.07 (0.18)	NS
	Triglycerides	0.18 (0.25)	NS

*Mean (SEM)

CHAPTER 5

WEIGHT LOSS IN LOW-INCOME WOMEN IMPROVES PLASMA ZINC AND METABOLIC RISK FACTORS

4.1 INTRODUCTION

Metabolic syndrome is a group of disorders involving obesity, insulin resistance, dyslipidemia and hypertension [Hansen, 1999]. Currently, it affects about 64 million adults in the United States (US), which is an increase of 14 million since 1994 [Ford et al, 2004]. The escalation in the incidence is particularly alarming for women, as they have shown a greater upsurge (23.5 %) than men (2.2 %) [Ford et al, 2004].

According to the National Cholesterol Education Program - Adult Treatment Panel (NCEP ATP III), metabolic syndrome is characterized by the presence of at least three of the following risk factors; waist circumference > 88 cm, fasting glucose > 100 mg/dl, fasting triglycerides > 150 mg/dl, HDL cholesterol < 50 mg/dl and blood pressure > 130/85 mm Hg [Ford et al., 2004]. Although metabolic syndrome is considered to be the combined effect of all these, each individual component independently augments the risk of associated diseases such as cardiovascular disorders and type 2 diabetes [Bosello & Zamboni, 2000].

Obesity is the most crucial risk factor of metabolic syndrome, because it is known to precede the others [Lemieux, 2001]. The prevalence of this disease has escalated from

about 23 % to 31 % during 1988- 2000. Also, it is more widespread in women, predominantly in those who are low-income [Breitkopf & Berenson, 2004] and minority [Cossrow & Falkner, 2004].

In addition to metabolic syndrome, obesity is associated with disturbances in the metabolism of trace minerals such as zinc [Tallman & Taylor, 1999]. Plasma zinc has been shown to be low in obese children and adolescents [Di Toro et al.1997, Marriero et al., 2004], adult men and women [Di Martino et al., 1993, Woodhouse et al., 2004], and mice [Chen et al., 2000]. Zinc status may be linked to body fat stores, as obese mice fed zinc deficient diets had a higher body fat content in comparison to lean animals [Chen et al., 1996].

Type 2 diabetes, another component of metabolic syndrome, alters zinc metabolism in humans [Blonstein-Fujii et al., 1997] and rodent models [Simon & Taylor, 2001]. Zinc deficiency has several features in common with type 2 diabetes including insulin resistance, hyperglycemia, and impaired glucose tolerance [Tallman & Taylor, 1999]. Influence of zinc on glucose metabolism may be related to its insulin-like properties. For example, this mineral promotes lipogenesis and glucose transport in rat adipocytes [Ilouz et al., 2002] and glucose uptake in mouse skeletal muscle [Miranda & Dey, 2004]. Thus, it is conceivable that zinc may play a role in the metabolic syndrome.

Weight loss is an effective treatment for the metabolic syndrome [Bray, 2004] because it leads to improvements in blood pressure and plasma concentrations of glucose,

insulin and lipids [Aronne & Segal, 2002]. Plasma zinc also is enhanced with weight loss, as levels returned to normal in obese patients who were fed hypocaloric diets [Woodhouse et al., 2004]. The overall purpose of this study was to investigate the effects of short-term weight loss on metabolic syndrome risk factors and plasma zinc. The population selected were low-income, triethnic women who are at high risk for this condition.

5.2 METHODS

Design

An 8-week weight loss intervention study was conducted with 90 overweight/obese, [body mass index (BMI) $\geq$ 25 kg/m^2] low-income mothers. All the participants were administered a 33-item demographic questionnaire at week 0. Anthropometric variables such as body weight, waist circumference, percent body fat, BMI and blood pressure were obtained at week 0 and 8. Plasma levels of zinc, glucose, insulin, leptin, triglycerides, total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol were measured and compared at week 0 and 8 of the weight loss program.

Subjects

Selection criteria included: ages 18-45 years; youngest child, 1-3 years; Hispanic, Caucasian or African-American ethnicity; literacy in English; eligibility for WIC or

Medicaid; and income less than 200% federal poverty index. The ethnicity of the participants was 63 % Hispanic, 19 % African-American and 18 % Caucasian. The nature of the risks and benefits of the study were explained and informed consent was obtained. The Institutional Review Board of The University of Texas at Austin approved this study.

Anthropometric measurements

Height was measured in inches using a stadiometer (Perspective Enterprises, Portage, MI) and weight was determined with an electronic weighing scale (Model HS-100-A, Fairbanks Scales, St. Johnsbury, VT). Waist circumference was obtained by positioning a measuring tape around the abdomen at the highest lateral border on the right ilial crest, as recommended by NHANES III (National Heart Lung and Blood Institute 1998). Percent body fat was measured by bioelectric impedance (Model TBF-300A, Tanita Corporation, Arlington Heights, IL). Body mass index was calculated by dividing body weight in kilograms by height in meters squared. Systolic and diastolic blood pressures were estimated with sphygmamometer (Model Marshall Medical, Placerville, CA).

Biochemical measurements

Plasma zinc was determined by a flame atomic absorption spectrophotometer (5100PC, Perkin Elmer Corporation, Shelton, CT). Plasma glucose was analyzed by a glucometer (Glucometer Elite, Bayer corporation, Elkhart, IN). Plasma insulin and leptin were measured using commercial enzyme-linked immunoabsorbent assay kits (ALPCO,

Windham, NH). Lipids (triglycerides, total and HDL cholesterol) were obtained enzymatically (Sigma Diagnostics Inc., St. Louis, MO). Freidwald formula was used to estimate LDL cholesterol [Cantin et al., 2003]. Insulin sensitivity was calculated by the Quicki method [Katz et al., 2000] and insulin resistance was computed by the homoeostatic assessment method [Mathews et al., 1985].

Statistical analyses

The computerized program SPSS (version 10.0; SPSS Inc, Chicago, USA) was used for all statistical analyses. Descriptives (means, standard deviation and range) were computed for both anthropometric and biochemical parameters. Relationships were tested using Pearson's correlation between anthropometric and biochemical measurements. Analysis of variance (ANOVA) with post hoc Scheffe analyses was used to determine the significance of differences between three or more subject groups. All differences were considered to be significant at $p< 0.05$.

5.3 RESULTS

The pre and post study characteristics of overweight/obese mothers are presented in **Table 5.1**. At pre study, all mothers were premenopausal and their ages ranged from 18 to 42 years. Based on BMI, 22 % were overweight (BMI 25-29.99 kg/m^2), 56 % obese (BMI 30-39.99 kg/m^2) and 22 %, morbidly obese (BMI $\geq$ 40 kg/m^2). The BMI, % body fat ($p < 0.001$), and total and LDL cholesterol ($p < 0.05$) decreased significantly by post study.

At pre study, plasma zinc was low in 39 % (< 0.8 mg/l) of obese/overweight mothers and within normal values in 46 % (0.8 – 1.2 mg/l). Plasma zinc rose by 22 % during the intervention; by post study, only 5 % of the mothers continued to exhibit low plasma zinc. For metabolic risk factors, 88 % had elevated waist circumference; 67 %, high fasting glucose; 29 %, enhanced triglycerides; and 88 %, low HDL cholesterol. At the post study, waist circumference, HDL cholesterol, and diastolic blood pressure ($p < 0.05$) showed significant improvements.

Relationships between changes in % body fat with weight, plasma zinc and HDL cholesterol are depicted in **Figure 5.1.** Change in % body fat was related positively with changes in weight ($r = 0.57$, $p < 0.001$) and negatively with changes in plasma zinc ($r = -0.28$, $p < 0.05$) and HDL cholesterol ($r = -0.27$, $p < 0.05$).

Figure 5.2 depicts the pre and post study values of plasma zinc and metabolic risk factors in mothers with low and normal plasma zinc. Plasma zinc increased by a greater margin (67 %) in women with low zinc, as compared to those with normal zinc (18 %). There was a comparable decrease of weight in both the groups. Waist circumference and plasma glucose declined significantly in women with low plasma zinc. However, considerable changes in HDL cholesterol and % body fat were seen in both the categories.

5.4 DISCUSSION

A modest reduction in weight and metabolic risk factors is known to dramatically enhance well-being and improve health status [Klein et al., 2004]. In the present study, small weight loss normalized plasma zinc and diminished risk factors for metabolic syndrome. This response is encouraging, as many individuals find it difficult to lose substantial amounts of weight.

We observed low plasma levels of zinc in 39 % of the overweight/obese women. Ninety percent of these levels returned to normal with weight loss. Woodhouse et al. [2004] also observed a rise in plasma zinc (7 %), with a 5.9 % decrease in weight, of men and women fed a high dairy, hypocaloric diet for 12 weeks. In the present study, plasma zinc was elevated approximately 9 % for every 1 % of body weight lost. Similarly, Di Martino et al. [1993] reported increases of 16 % for in men and 33 % in women who had a 15 % decline in BMI after weight reduction. Obese children and adolescents fed a very low calorie diet responded in the same manner, with plasma zinc escalating by 12 % after 10 weeks of treatment [DiToro et al., 1997]. This overall response of zinc to weight reduction may be due to tissue redistribution or catabolism of tissues. Whether or not plasma zinc levels return to low baseline concentrations with time merits further investigation.

In animal studies, a link has been proposed between zinc and body fat. In obese rodents, low zinc diets resulted in a significant elevation in body fat as compared to those

who were fed normal zinc [Bock et al., 1995, Chen et al., 2000]. Also, obese mice with genetically-induced obesity (C57BL/6J) had low concentrations of zinc in adipose tissue in response to a high-fat diet [Tallman & Taylor, 2003]. In normal weight humans, zinc deficiency was observed in conjunction with low concentrations of leptin, a hormone produced in adipose tissue. In obese men (n = 5), Chen et al. [2000] reported an inverse relationship between plasma levels of zinc and leptin. In our study of 90 women, increased plasma zinc was associated with a decrease in % body fat; however, no link was observed between leptin and zinc. The conflicting results may be due to differences in sample size, age, sex or type of adiposity.

In addition, we observed that women with low zinc had higher body weight, waist circumference, plasma glucose and % body fat than those with normal zinc. This is notable since all these traits are directly or indirectly related to metabolic syndrome. Improvements in these metabolic risk factors as well as plasma zinc were more pronounced in women with low zinc as compared to those with normal zinc, despite similar reductions in weight. This outcome suggests that the elevation in plasma zinc seen in women with low zinc may be dependent on gender-related factors.

Metabolic syndrome is associated with altered lipid levels particularly, high triglycerides and low HDL cholesterol [Ginsberg, 2003]. Also, metabolic syndrome is an intermediate step in the progression of coronary artery disease [Ardern et al., 2003]. In this study, for every 1 kg of weight lost, we observed a decrease of 2.8 %, 2.1 % and 6.7 % in triglycerides, total and LDL cholesterol, respectively; and an increase of 14.4 % in

HDL cholesterol. A meta analysis of 70 studies [Dattilo and Kris-Etherton, 1992] found that the reduction in 1 kg of weight was effective in lowering the circulating levels of triglycerides, total and LDL cholesterol by 1.93 %, 0.99 % and 0.68 %, respectively. A study by Nieman et al. [2002] showed similar findings in women who lost weight either by diet alone or by diet and exercise. However, both these investigations reported results that were contradictory to ours with respect to changes in HDL cholesterol in women. Dattilo and Kris-Etherton [1992] showed no change, and Nieman [2002] showed a decrease in these lipoprotein levels with weight loss.

In summary, 53 % of the overweight /obese low-income women exhibited risk factors for metabolic syndrome and 39 % low plasma zinc at the start of this study. The circulating levels of zinc, as well as the metabolic syndrome components, showed significant improvements in overweight/obese low-income women after weight loss.

TABLE 5.1 Pre and post study characteristics of mothers

Characteristics	Pre study	Post study	p value
Age (y)	27.6 ± 5.6		
Parity	2.3 ± 1.3		
BMI[b] (kg/m^2)	35.4 ± 7[a]	34.6 ± 7	< 0.001
Body fat (%)	43.2 ± 6	42.2 ± 7	< 0.001
Leptin (ng/ml)	18.3 ± 10	19.0 ± 10	NS
Insulin (IU/l)	13.0 ± 9	14.7 ± 12	NS
HOMA[c] IR	4.0 ± 5	3.6 ± 3	NS
Insulin sensitivity[d]	0.3 ± 0.03	0.3 ± 0.03	NS
Cholesterol (mg/dl)			
Total	166 ± 38	158 ± 30	< 0.05
LDL[e]	103 ± 33	87 ± 36	< 0.05
Zinc (mg/l)	0.85 ± 0.3	1.04 ± 0.2	< 0.001
Metabolic syndrome risk factors			
Waist circumference (cm)	107 ± 19	105 ± 18	< 0.01
Glucose (mg/dl)	112 ± 39	107 ± 40	NS
Triglycerides (mg/dl)	124 ± 62	116 ± 56	NS
HDL[f] cholesterol (mg/dl)	39 ± 10	52 ± 30	< 0.001
Blood pressure (mm Hg)			
Systolic	118 ± 13	116 ± 13	NS
Diastolic	78 ± 10	76 ± 9	< 0.01

[a]Mean ± SD

[b]Body mass index

[c]Homeostatic model assessment method (Mathews et al. Diabetologia. 1985;28:412-419)

[d]Quicki method (Katz et al. J Clin Endocrinol Metab. 2000; 85(7):2402-2410)

[e]Low-density lipoprotein

[e]High-density lipoprotein

FIGURE 5.1 Relationship of changes in % body fat with weight, plasma zinc and HDL cholesterol

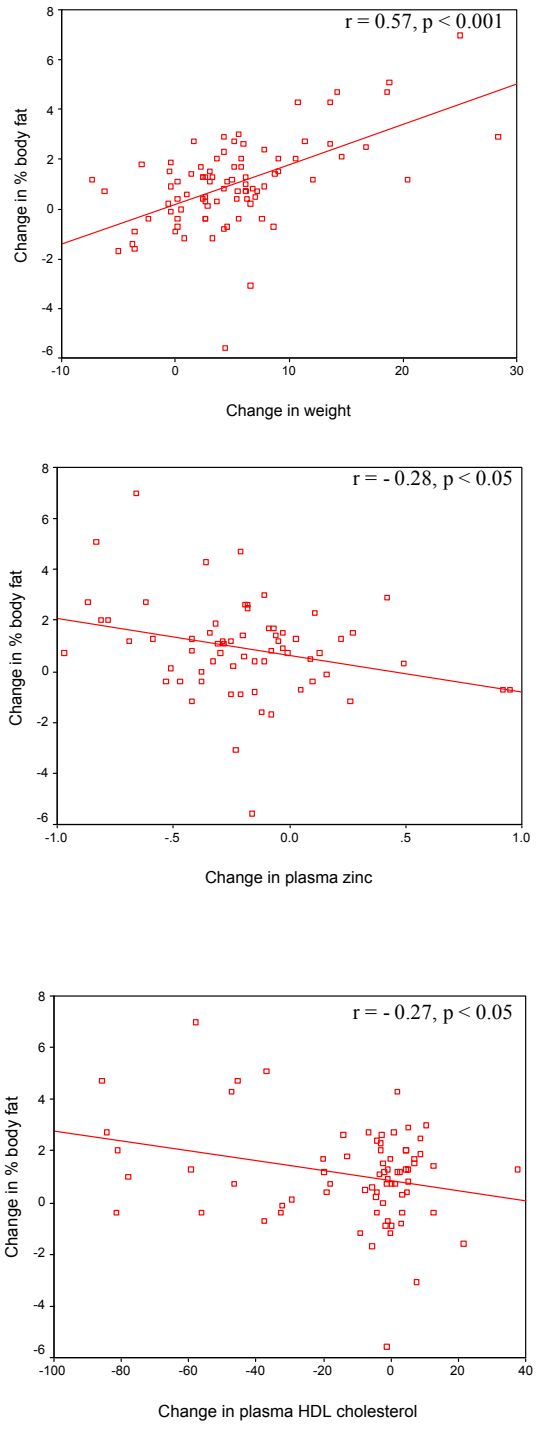
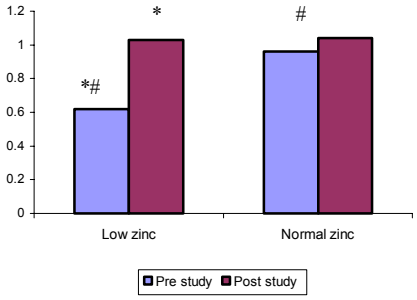
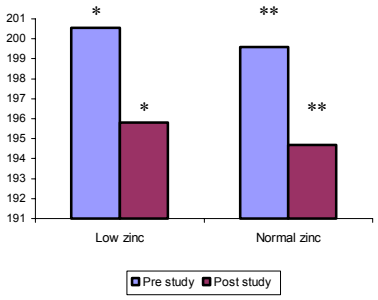


FIGURE 5.2 Pre and post study values of plasma zinc and metabolic risk factors in mothers with low and normal plasma zinc

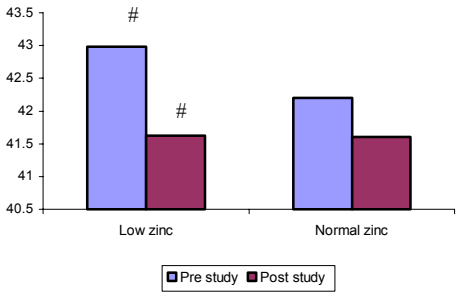
a) Plasma zinc



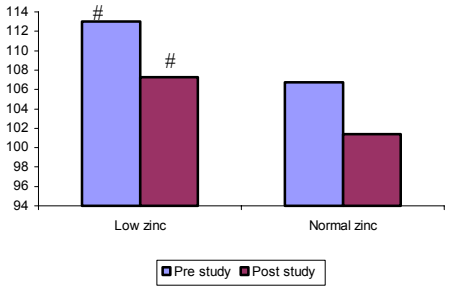
b) Body weight



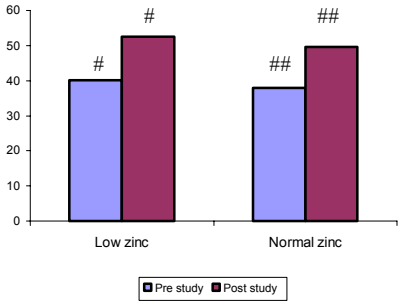
b) Waist circumference

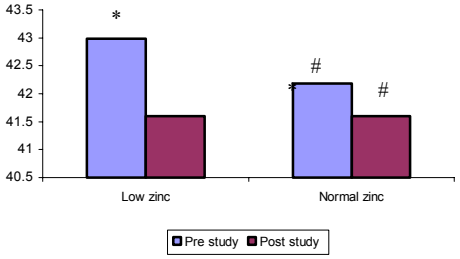


d) Plasma glucose



e) HDL cholesterol f) Percent body fat





*, ** $p < 0.001$
#, ## $p < 0.05$

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The results of these studies show strong genetic influences on the circulating levels of appetite regulating hormones, low-density lipoprotein and significant relationships between obesity traits, plasma zinc, and risk factors related to metabolic syndrome. Baboons were used as animal models in the first aim because these animals are highly comparable to humans in their genetic constitution and expression.

Ghrelin is a hormone that stimulates food intake. Its peptide sequence is considered to be highly conserved across a number of animal species [Cummings and Shannon, 2003]. Results from chapter 2 depicted high sequence identity among baboon and human ghrelin, the protein and the coding region were 96 % and 97 % similar respectively. The tissue distribution of ghrelin in baboons followed the same pattern as humans. Also, circulating levels of ghrelin were inversely related to body weight, plasma insulin, c- peptide and leptin.

Obesity usually results when energy intake exceeds expenditure, and is influenced by genes to a great extent [Bouchard, 1997, Comuzzie et al., 2001]. Significant heritability and suggestive linkage on chromosome 9 for the circulating levels of ghrelin showed evidence of a substantial impact of genes. This result is notable, as other phenotypes with a role in obesity have been mapped to this same chromosome such as

HDL cholesterol [Newman et al., 2003], adiponectin [Lindsey et al., 2003] and insulin sensitivity [Hanson et al., 2001]. However, in the present study, LOD score was low (1.65). Thus, it is recommended that sample size be increased to enhance the power of linkage. In addition, further studies should be directed towards sequencing the whole ghrelin gene and identifying the single nucleotide polymorphisms (SNPs) and their effects on obesity in baboons.

Cholecystokinin (CCK) was another appetite-regulating hormone that was investigated. It is a major satiety hormone that controls meal volume by acting in conjunction with leptin [Peters et al., 2004] and insulin [Riedy et al., 1995]. Results from chapter 3 show that the fasting plasma levels of CCK are slightly higher than humans [MacIntosh et al., 1999], and depict substantial heritabilities for body weight, plasma glucose and CCK. A strong signal (LOD score = 3.1) was located on chromosome 17 at p12 for plasma CCK. Identification of linkage in this region is significant because other satiety hormones such as leptin [Kissebah et al. 2000] and insulin [Rich et al., 2005] have been localized to the same region on this chromosome. Also, positional candidate genes glucose transporter 4 (GLUT4) [Bell et al., 1989] and gClq (a putative adiponectin binding protein) [Comuzzie et al., 2001] have been linked to this region. Linkage of CCK on chromosome 17p12 increases the chances of this region to be recognized as a potential positional candidate gene in the study of obesity-related phenotypes. However, the small sample size of 371 baboons may be a limitation of this study. Increasing the sample would not only increase the power of linkage but will also help in identifying the effect of genes on the relationships with other obesity phenotypes.

Chapters 4 and 5 examined the relationship of obesity-related phenotypes with low-density lipoproteins and other risk factors of metabolic syndrome in humans. Low-density lipoproteins (LDL), especially small sized LDLs, are major risk factors of cardiovascular disease (CVD) and metabolic syndrome. Small LDLs are prone to oxidation and increase the risk of atherogenicity by remaining longer in the plasma, thus increasing the chances of being deposited on arterial walls [Rainwater et al., 2003]. Considerable heritabilities for weight associated traits and lipoproteins were found. Strong genetic correlations between obesity-related traits and LDL sizes also were observed. The important findings were the positive associations of small and medium LDLs with obesity traits such as body weight, BMI, waist circumference, skinfolds and triglycerides. These relationships suggest that same set of genes might be regulating these phenotypes. These results are particularly important for Alaska Natives, because there has been an increase in the incidence of obesity and cardiovascular disease in this population in recent years. This change is believed to be due to the advent of industrialization and modernization that altered diets and lifestyles dramatically. The effect of environment is highly evident, but the results of this study show substantial genetic impact on obesity and cardiovascular risk factor LDL. Since the genetic influence on LDL has been documented in this study, further investigations should concentrate on the location of genes affecting the variation in plasma LDL and its different sizes.

Metabolic syndrome is a cluster of disorders including obesity, insulin resistance, glucose intolerance, and hypertension. Obesity is known to precede all other metabolic risk factors [Lemieux, 2001]. In chapter 5, we demonstrated that even a small weight loss

is effective in improving plasma zinc and risk factors for metabolic syndrome in overweight/obese mothers. In this study, for every 1 kg of weight lost, we observed a decrease of 2.8 %, 2.1 % and 6.7 % in triglycerides, total and LDL cholesterol, respectively; and an increase of 14.4 % in HDL cholesterol. These results were similar to those reported by Dattilo and Kris-Etherton [1992] and Nieman et al. [2002].

Plasma zinc also was investigated for its role in metabolic syndrome. Zinc acts similar to insulin in the metabolism of glucose and is known to be altered in obesity, insulin resistance and type 2 diabetes [Tallman and Taylor, 2003]. We found that the plasma zinc was low in these mothers and weight reduction increased these values, and this increase in zinc was associated with decreased body fat. Also, mothers with low plasma zinc showed greater body weight, waist circumference, plasma glucose and lower HDL cholesterol (all risk factors for metabolic syndrome) than those with normal zinc. This shows the relevance of studying the role of zinc in obesity and the progression of metabolic syndrome.

One limitation of this study was its short duration. It is recommended that an increase in the weight loss intervention period will help achieve a greater weight loss. However, it is encouraging to observe an improvement in metabolic risk factors and plasma zinc with small weight loss, and it is consistent with reports of benefits on health with loss of as little as 5 % of weight.

Collectively, these results display the importance of the baboon as a model for investigating the effects of genes on appetite-regulating hormones ghrelin and CCK. They also emphasize the extent of genetic influence on LDL and obesity-associated traits. In addition, the effectiveness of weight loss in the improvement of plasma zinc and risk factors for metabolic syndrome was demonstrated.

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