MELANOCORTIN-4 RECEPTOR (MC4R) VARIANTS AND MEASURES OF ADIPOSITY IN THE GENERAL POPULATION

by

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Background: As the prevalence of obesity has steadily increased, it has rapidly emerged as a major public health concern due to a high risk of morbidity and mortality. Rare missense and nonsense mutations in the melanocortin-4 receptor (MC4R) gene are a cause of genetic forms of severe obesity, and targeted disruption of the mouse MC4R leads to obesity. The role of variation at the MC4R locus in influencing interindividual variation in body size and composition in the general population is controversial.

Objective: To test the hypothesis that polymorphic variation at the MC4R locus is significantly associated with measures of adiposity in the general population.

Methods: Two single nucleotide polymorphisms, -4599 T>G and -4850 T>C, in the 5'-flanking region of MC4R were verified by resequencing in 16 individuals and genotyped in the larger sample by fluorescence polarization. 1,099 healthy, non-Hispanic white volunteers, age 30-54 years, were recruited from the Pittsburgh community. A medical and demographic history was collected and anthropomorphic measures were determined. ANOVA was used to assess the relationship between genotype and metabolic parameters.

Results: BMI was greater in participants having a -4599 G allele (GG + TG genotypes) than among TT homozygotes ($p < 0.03$), and this association was of similar magnitude in both men (BMI 28.2 vs. 27.4) and women (BMI 26.5 vs. 25.9). Nominally defined overweight (BMI ≥ 27) also varied significantly (Chi-square = 6.874, $p < 0.04$) across -4599 genotypes (GG: 52.4%; TG: 46.8%, TT: 40.6%). A similar relationship was seen for the -4850 T>C SNP (CC: 53.2%; TC: 48.0%; TT: 41.4%; Chi-square = 6.256, $p < 0.05$). Finally, subjects with any -4599 G allele had significantly higher weight (177.0 vs. 172.8; $p < 0.05$) and percent of body fat (29.8% vs. 28.8%; $p < 0.04$). Greater waist circumference was also significantly associated with both the -4599 G allele (36.3 vs. 35.6; $p < 0.04$) and the -4850 C allele (36.4 vs. 35.6; $p < 0.05$).

Conclusion: Common variation in the 5'-flanking region of the MC4R gene is significantly associated with measures of adiposity in men and women in the general population.

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PREFACE

I want to express my sincere appreciation to the many people who have helped me accomplish this project, especially Dr. Ferrell and Dr. Manuck as they have offered continual support, guidance, and assistance. I am also thankful to Dr. Barmada for his willingness to be on my committees – even at the last minute! And finally, I would like to thank everyone in the Ferrell Lab for the friendship and advice during my years here at Pitt.

1.0 INTRODUCTION

1.1 OVERWEIGHT AND OBESITY - BACKGROUND

Obesity is a complex, multi-factorial chronic disease that is characterized by an excess accumulation of body fat. While everyone needs a certain amount of body fat for stored energy, heat insulation, shock absorption, and other functions, obesity increases the risk of illness from about 30 serious medical conditions. Frequently, obesity is defined by body mass index (BMI), which is thought to generally correlate to measurements of body fat. BMI is a calculation based on both height and weight. Traditionally, a BMI of 30 kg/m^2 or higher is indicative of obesity, and a BMI of 40 kg/m^2 or greater is classified as morbid obesity. "Overweight" is a less severe condition that corresponds to a BMI between 25 and 29.9 kg/m^2.

Overweight and obesity result from an energy imbalance when more calories are consumed from a person's diet than are expended through physical activity. The cause of the imbalance may differ from one person to another; Environmental (social and cultural), genetic, physiological, metabolic, behavioral, and psychological components all play a role. Simply put, obesity has a strong familial component and is promoted by an environment of decreased physical activity and high-calorie, low-cost foods.

The prevalence of overweight and obesity is increasing rapidly in both developing and developed countries, worldwide.[72] In the United States, the number of overweight and obese Americans has increased relentlessly since 1960[60,61] (see Table 1). The latest data indicate that

64.5% of adult Americans (approximately 127 million people) are categorized as being overweight or obese. About 60 million of those are classified as obese, constituting 1/3 of the adult American population. Another 9 million Americans are considered morbidly obese.

Table 1. Increase in Prevalence of Overweight, Obesity, and Morbid Obesity among U.S. Adults

	Overweight (BMI ≥ 25)	**Obesity (BMI ≥ 30)**	**Severe Obesity (BMI ≥ 40)**
1999 to 2000	64.5%	30.5%	4.7%
1988 to 1994	56.0%	23.0%	2.9%
1976 to 1980	46.0%	14.4%	No Data

Source: CDC, National Center for Health Statistics, National Health and Nutrition Examination Survey. Health, United States, 2002. Flegal et. al. JAMA. 2002;288:1723-7. NIH, National Heart, Lung, and Blood Institute, Clinical Guidelines on the Identification, Evaluation and Treatment of Overweight and Obesity in Adults, 1998.

As the prevalence of obesity has steadily increased, it has rapidly emerged as a major public health concern[62,63] due to significantly increased mortality[2] and a high risk of morbidity.[63] Because obesity disrupts lipid and glucose homeostasis, it is frequently associated with dyslipidemia, hypertension, diabetes mellitus, atherosclerosis, and cardiovascular disease[47,55,63,82,85] and often leads to a diagnosis of the metabolic syndrome.[70] In 1999, Colditz estimated that obesity-related morbidity may account for 9.4% of U.S. healthcare costs,[17] with approximately $100 billion total cost in 1995 and $52 billion in direct medical costs.[88] Each year, obesity causes at least 300, 000 excess deaths in the U.S.,[2] making it the second leading cause of unnecessary deaths. Even more alarming is the dramatic increase in childhood obesity and comorbidities (particularly type 2 diabetes) in industrialized countries.[21] In the U.S., the percentage of young people who are overweight has more than tripled since 1980; Currently 16% of children and teens aged 6-19 years are considered overweight.

Many treatments for obesity are available, including dietary therapy, physical activity, behavior therapy, drug therapy, combined therapy, and surgery. Surgical treatments have

dramatically increased in popularity and are used to modify the stomach and/or intestines to decrease the amount of food that can be eaten and sometimes absorbed. However, obesity has proven difficult to treat, as indicated by the continued rise in prevalence. It is important to identify the pathophysiologic mechanisms underlying this disease as a means of identifying new prevention strategies[34] and treatment targets.[21]

1.2 GENETICS OF OBESITY

Although obesity was once thought to be simply a behavioral disorder, recent research has shown that several genetic factors play a role in regulating energy balance via the neuroendocrine system.[48,57,76] Twin studies have estimated the heritable component of obesity between 30 – 70% with the typical estimate at 50%.[53] Some studies have shown that heritability rates for obesity may equal that of height and possibly surpass that of almost all other major diseases that have been studied.[3,9,27,71,81]

The neuroendocrine system regulates energy balance by controlling appetite and food intake and utilization.[10,57,76] Once adipose tissue accumulates, this system prevents it from diminishing,[48] which proved useful for defending against body weight loss when food was scarce. With the current abundance of food in industrialized nations, humans appear to have only weak physiological mechanisms to defend against body weight gain.[34] Neurons in the hypothalamus regulate appetite and basal metabolic activity by mediating signaling of the adipostatic hormone leptin.[12,22,23,91]

Ravussin and Bogardus concluded that among different populations, the prevalence of obesity is largely determined by environmental factors, but that among individuals from the same

population living in a given environment, the variability in body size and composition is mostly related to genetically determined response to that environment.[72]

Linkage studies have shown major obesity susceptibility loci to be located on chromosomes 2, 4, 5, 10, 11, and 20.[30,50,72,80] Possible candidate genes include leptin, leptin receptor, melanocortin-4 receptor (MC4R), prohormone convertase 1 (PCSK1), and proopiomelanocortin (POMC).[6,9] Common obesity is likely to be a polygenic disorder, but monogenic causes have been described.[6,48] To date, MC4R mutations cause the most common form of monogenic obesity seen in humans.[26,31,68,70]

1.3 MELANOCORTIN-4 RECEPTOR (MC4R) - BACKGROUND

Melanocortins are a group of ligands derived from proopiomelanocortin (POMC) by enzymatic processing.[14] The four major ligands are α-melanocyte-stimulating-hormone (α-MSH), β-MSH, γ-MSH, and adrenocorticotropic hormone (ACTH).[12,14,41,68] The melanocortin system is also composed of five melanocortin receptors and two antagonists, agouti and agouti-related protein (AGRP). [12,14,41]

The melanocortin system has multiple functions, including roles in memory and learning, thermoregulation, analgesia, stress response, inflammation, pigmentation, and feeding behavior.[14] Leptin, a protein hormone that plays an important role in regulating body weight, metabolism, and reproductive function, is responsible for linking peripheral adipostatic signals to central melanocortinergic neurons.[59,68,75] Leptin is released from adipose tissue and crosses the blood-brain barrier to both inhibit the melanocortin receptor antagonist AGRP and to activate

POMC expression.[19,38] POMC is then cleaved to generate α-MSH, β-MSH, γ-MSH, and ACTH which induce satiety and an elevated energy expenditure[32] by binding to melanocortin receptors.

The melanocortin receptors (MC1R, MC2R, MC3R, MC4R, and MC5R) belong to the G-protein coupled receptor (GPCR) superfamily class A.[14] When the melanocortin ligands bind to the receptors, stimulatory G-proteins are released which activate adenylate cyclase.[12,14] This results in an increase in cellular cyclic AMP (cAMP).[12] The central melanocortin system receives several inputs from both central and peripheral appetite regulating pathways that can modulate the effects of the melanocortin system on food intake and energy expenditure.[32,41]

In 1993, Gantz et al. reported the cloning, expression, and gene localization of the melanocortin-4 receptor (MC4R).[28] MC4R is a 332-amino acid protein which is primarily expressed in the brain.[82] MC4R is located on chromosome 18q22 and is encoded by a single exon.[49] Of the five receptors, MC4R is the most conserved with ~94% homology among mammals.[56] Although it is found throughout the central nervous system,[51,56,82] the highest level of MC4R expression is observed in the hypothalamus,[56,68,78] which is a primary brain region for regulating food intake and body weight.[22,23,46,91] High levels of melanocortin ligands are also seen in the arcuate nucleus of the hypothalamus.[68]

1.4 MC4R - ROLES

MC4R functions include regulating body weight, food behaviors, sexual function, HPA axis activity, emotional states, pain, drug addiction, and stress response.[14] In 1997, Huszar et al. was the first to establish that MC4R is a critical component of the homeostatic circuit that regulates energy balance in mammals.[40] Activation of MC4R has been shown to inhibit appetite

and increase basal metabolic rate (BMR) while antagonism leads to hyperphagia and decreased metabolic activity.[12,51,78]

ACTH and α-MSH are the melanocortin ligands that have the highest binding affinity to MC4R.[14] In the fed state, levels of these peptides increase due to activation of POMC resulting from high concentrations of circulating leptin.[68] Upon binding to MC4R, ACTH and α-MSH both have an inhibitory effect on feeding and possibly cause increased energy expenditure and weight loss.[8,32,41]

AGRP is normally suppressed by leptin in the fed state.[87] In the starved state, circulating levels of leptin decrease, leading to decreased POMC activity and increased AGRP activity. Together, these events lead to a powerful inhibition of MC4R signaling and therefore promote food intake.[1,59,68,87] AGRP has little intrinsic signaling activity and primarily acts by preventing melanocortins from binding to MC4R.[5,67,77,89]

1.5 MC4R - STUDIES

It was first established in 1986 that administration of ACTH and α-MSH markedly inhibited spontaneous feeding in rats.[41,69] Studies have also shown that central administration of MC4R-specific antagonists causes an increase in feeding and obesity.[16,44] When an antagonist and agonist were given together to mice, it was noted that the inhibition of food intake was reversed.[45]

In 1997, it was first noted that MC4R knock out mice on an ad libitum diet are obese and hyperphagic and also have increased length and hyperinsulinemia compared to wild type mice.[40] Interestingly, heterozygotes have a body weight intermediate between wild type and MC4R-null

mice,[15,32,40,79] suggesting that MC4R is quantitatively important in the regulation of energy balance.[68] Whether MC4R is required solely for feeding response or also plays a role in metabolic and activity responses remains somewhat controversial.[11,14,15,19,20,40,51,79,68] One study showed that MC4R knock out mice show a predisposition to store calories as adipose tissue.[79]

In humans, MC4R deficiency has been associated with hyperphagia,[25,26,49,55,68] obesity (including increased fat[32,74] and lean body mass[49]),[12,20,25,35,52,78,84] increased bone mineral density,[49] accelerated linear growth,[26] and hyperinsulinemia.[74] Although MC4R knock out mice demonstrate reduced energy expenditure, impairments in basal metabolic rate do not seem to be associated with MC4R mutations in humans.[26,55] One study did show, however, that a functional MC4R is needed for acute regulation of activity-based energy expenditure in response to changes in diet.[51] A phenotypic assessment of individuals with haploinsufficiency MC4R mutations done by Sina et al. showed no evidence of diabetes or hypertention, a gynoid pattern of fat distribution, and a low incidence of binge eating disorder.[78]

Linkage studies have provided suggestive evidence of linkage between BMI[66] and body fat[13,65] on chromosome 18q21, flanking the MC4R region.

Mutations in the MC4R gene are considered to be the most common form of monogenic obesity in humans,[25,26,36,70,78,85] accounting for ~5% of severe obesity.[70] More than 70 variants have been reported in various patient cohorts.[83] At least 34 are functionally relevant frameshift, nonsense, and missense mutations, most of which result in partial or total loss of function of the MC4R gene.[35,37,51,52,64,90] However, all mutations are rare, with heterozygosity rates <1% for any single mutation[12,35] and combined mutation frequencies of 2-6% in extremely obese individuals.[12,25,26,33,35,36,37,55,85] Due to the small population frequency of mutation carriers, MC4R mutations appear to have a low epidemiological but high individual relevance.[35]

Some studies have found that obesity that is associated with MC4R mutations tends to be limited to early-onset cases,[7,43,55,78,85] and that the phenotype becomes less prominent with age.[25,55]

Functional studies have shown that the regulation of body weight in humans is sensitive to variations in the amount of functional MC4R,[25,26,40,84] suggesting a codominant pattern of inheritance.[25,47] Although most of the MC4R mutations identified to date lead to a loss of function resulting in obesity,[35,37,51,52,64,90] at least one variant (Val103Ile) has been shown to cause a moderate gain of function and reduced body weight.[29,33,35,73]

Although MC4R mutations are generally considered a "monogenic" form of obesity, multiple studies have identified mutation carriers who are only moderately overweight or even lean.[20,25,26,64,78,83,85] The limited penetrance and variable expression of the MC4R mutations may be due to a partial reduction in receptor function resulting from haploinsufficiency[18,78] or a dominant negative mechanism.[85] It is clear that obesity is a complex trait and that other genetic an environmental factors are operative.[18,20,21,35,64,78]

MC4R mutations are considered causative factors in a significant proportion of morbid obesity cases,[43] however their role in common obesity and normal weight regulation is thought to be negligible due to the low frequency of mutations in the general population.[12] It remains possible that common genetic variants in the regulatory regions of the MC4R gene could play a role in influencing interindividual variation in body size and composition in the general population.[52,68,78]

1.6 PURPOSE AND SIGNIFICANCE

Because obesity has proven difficult to treat, it is important to identify the pathophysiologic mechanisms underlying this disease as a means of identifying new treatment targets[21] and prevention strategies. Much has been learned from studies on monogenic obesity, however the role of variation at the MC4R locus in influencing interindividual variation in body size and composition in the general population remains controversial.[52,68,78] To date, MC4R mutations have proven to carry a large individual relevance but a small epidemiological relevance.[35] Therefore, further research involving common polymorphic variation and measures of adiposity in the general population would help to elucidate the role of MC4R with regard to public health.

2.0 MATERIALS AND METHODS

2.1 SUBJECTS

Subjects were obtained as part of the Adult Health and Behavior (AHAB) study as a community sample of nonpatient volunteers in the Pittsburgh area. There was no history of myocardial infarction, stroke, revascularization procedure (i.e. bypass or angioplasty), angina, chronic kidney or liver disease, or psychosis. Subjects were not on any psychotropic or glucocordicoid medications and were not on insulin. Analyses were based on non-Hispanic whites only; 1,099 subjects met these criteria with >97% of subjects successfully genotyped for each site. Approval was obtained from the Institutional Review Board, and all subjects signed written informed consent.

2.2 DNA EXTRACTION

DNA was extracted from whole blood using a salting-out protocol.[58] Briefly, the red blood cells and nuclei were lysed and then digested overnight with protease K. After digestion, protein was removed from the DNA by a salting out procedure with NaCl. The DNA was then precipitated with ethanol and suspended in TE buffer for use.

2.3 MC4R GENE

There are 20 validated single nucleotide polymorphisms (SNPs) within approximately 5kb of the MC4R gene (see figure 1). Of these, only seven have a minimum allele frequency >0.1 (see table 2).

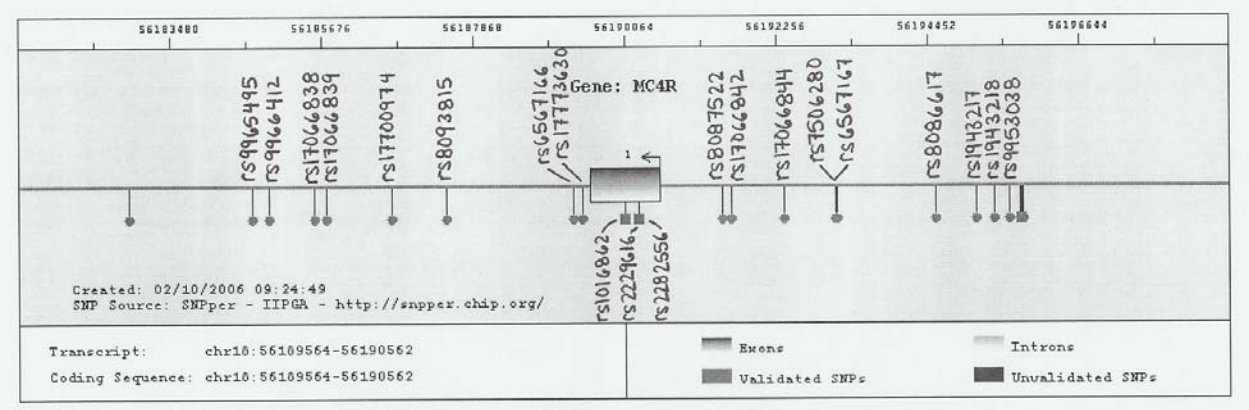


Figure 1. Validated SNPs within 5kb of the MC4R Gene

Table 2. Validated SNPs within 5kb of the MC4R Gene

rs#	Relative Location	Minimum Allele Frequency	Role
rs9965495	5907	0.411	3' UTR
rs9966412	5648	0.321	3' UTR
rs17066836	4992	0.029	3' UTR
rs17066839	4815	0.028	3' UTR
rs17700974	3872	0.007	3' UTR
rs8093815	3080	0.417	3' UTR
rs6567166	1251	0.014	3' UTR
rs17773630	1123	0.007	3' UTR
rs1016862	506	0.000	I169S
rs2229616	307	0.026	V103I
rs2282556	292	0.000	G98R
rs8087522	-896	0.242	5' Flanking
rs17066842	-1042	0.067	5' Flanking
rs17066844	-1810	0.007	5' Flanking
rs7506280	-2543	---	5' Flanking
rs6567167	-2576	---	5' Flanking
rs8086617	-4008	0.300	5' Flanking
rs1943217	-4599	0.300	5' Flanking
rs1943218	-4850	0.265	5' Flanking
rs9953038	-5081	0.034	5' Flanking

2.4 VERIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS

Resequencing was performed on 16 individuals to screen for SNPs in the MC4R gene and regulatory regions. PCR primers were designed (see Table 3) and obtained from the DNA Synthesis Facility at the University of Pittsburgh, and PCR was carried out on an MJ thermocycler using the following conditions:

	1 Reaction
10X PCR Buffer (Invitrogen™)	2.5 µL
1.25mM dNTP (Invitrogen™)	4.0 µL
50mM $MgCl_2$ (Invitrogen™)	0.75 µL
20µM Forward Primer	0.375 µL
20µM Reverse Primer	0.375 µL
Taq DNA Polymerase (Invitrogen™)	0.1 µL
dH_2O	14.4 µL
DNA	2.5 µL
Total:	**25.0 µL**

Conditions:

1. 95˚C for 5 minutes
2. 35 Cycles
   a. 95˚C for 30 seconds
   b. XX˚C for 15 seconds
   c. 72˚C for 30 seconds
3. 72˚C for 5 minutes
4. 10˚C hold forever

Table 3. MC4R Sequencing Primers and Annealing Temperatures for Verification of SNPs

	Name	Sequence (5' to 3')	Annealing Temperature
Coding Region	MC4RSeqF MC4RSeqR	ATGGAGGGTGCTACGAGCAA ATTGGCACCTTGGCGGATG	50˚C
Promoter Region	MC4RPrF MC4RPrR	CTCTGGTAACGGGAATGGAG TTCTTCTGGCTCGTAGAG	47˚C
3' UTR Region	MC4RUTR_F MC4RUTR_R	TTCCGGTCTCCTTCCACTC CTGAGCTGTAGCACATGGC	47˚C

PCR cleanup was performed using Shrimp Alkaline Phosphatase (SAP) and Exonuclease I (Exo I) on an MJ thermocycler using the following conditions:

	1 Reaction
10X SAP Buffer (USB Corp.)	0.5 µL
SAP (USB Corp.)	0.5 µL
Exonuclease (USB Corp.)	0.05 µL
dH_2O	6.45 µL
PCR	2.5 µL
Total:	**10.0 µL**

Conditions:

1. 37˚C for 35 minutes
2. 88˚C for 15 minutes
3. 10˚C hold forever

The sequencing reaction was then performed using dRhodamine Dye Terminator and an MJ thermocycler under the following conditions:

	1 Reaction
0.8µM Primer	4.0 µL
5X SeqSaver (Applied Biosystems)	3.0 µL
dRhodamine (Applied Biosystems)	1.0 µL
Cleaned PCR	4.0 µL
Total:	**12.0 µL**

Conditions:

1. 25 Cycles
   a. 96˚C for 10 seconds
   b. 50˚C for 5 seconds
   c. 60˚C for 4 minutes
2. 10˚C hold forever

Each fragment was sequenced in both the forward and reverse direction.

The dye terminators were removed from the samples using CleanSEQ beads (Agenecourt Bioscience Corp.), ethanol, and a magnetic SPRIPlate.

The capillary electrophoresis was completed by the Genomics and Proteomics Core Laboratories at the University of Pittsburgh using an ABI 3730 DNA Analyzer.

Sequencing analysis was done using Sequencher software (version 4.2.1, Gene Codes Corp.). Two SNPs in the promoter region were identified: -4599 T>G (allele frequency T=0.70, G=0.30; rs#1943217) and -4850 T>C (allele frequency T=0.74, C=0.26; rs#1943218). No other SNPs were seen in the 16 individuals and were therefore presumed to be occurring only at a low frequency.

2.5 GENOTYPE ANALYSIS

Genotyping was performed for the -4599 T>G and -4850 T>C using fluorescence polarization (FP).[39] PCR primers were designed (see Table 4) and ordered from Invitrogen. PCR conditions for -4599 T>G were as follows:

	1 Reaction
10X PCR Buffer (Invitrogen™)	1.0 µL
1.25mM dNTP (Invitrogen™)	1.6 µL
50mM $MgCl_2$ (Invitrogen™)	0.4 µL
20µM Forward Primer	0.15 µL
20µM Reverse Primer	0.15 µL
Taq DNA Polymerase (Invitrogen™)	0.04 µL
dH_2O	5.66 µL
DNA	1.0 µL
Total:	**10.0 µL**

Conditions:

1. 95˚C for 5 minutes
2. 35 Cycles
   a. 95˚C for 30 seconds
   b. 44˚C for 15 seconds
   c. 72˚C for 30 seconds
3. 72˚C for 5 minutes
4. 10˚C hold forever

For the -4850 T>C SNP, PCR conditions were as follows:

	1 Reaction
10X PCR Buffer (Invitrogen™)	1.0 µL
1.25mM dNTP (Invitrogen™)	1.6 µL
50mM $MgCl_2$ (Invitrogen™)	0.3 µL
20µM Forward Primer	0.15 µL
20µM Reverse Primer	0.15 µL
Taq DNA Polymerase (Invitrogen™)	0.04 µL
dH_2O	5.76 µL
DNA	1.0 µL
Total:	**10.0 µL**

Conditions:

1. 95˚C for 5 minutes
2. 35 Cycles
   a. 95˚C for 30 seconds
   b. 44˚C for 15 seconds
   c. 72˚C for 30 seconds
3. 72˚C for 5 minutes
4. 10˚C hold forever

Table 4. MC4R SNP Primers for Fluorescence Polarization

MC4R SNP Primers for Fluorescence Polarization			
	Name	**Sequence (5' to 3')**	**Annealing Temp.**
-4599	MC4R-4599F	AGCTACTGCAGAGCTGGTG	44˚C
	MC4R-4599R	GAGCACTTGCAAAGCTTGTC	
T>G	MC4R-4599Rdet	TTAACTAGACATATTTTCCCATTCC	
-4850	MC4R-4850F	CCACTCTCATCTTCCATC	44˚C
	MC4R-4850R	CTGGGACTTCAGTGTGATCC	
T>C	MC4R-4850Rdet	ATTGCCATAGATGAATCTAATATAATTTA	

SAP/Exonuclease was used for PCR cleanup:

	1 Reaction
10X SAP Buffer (USB Corp.)	1.0 µL
SAP (USB Corp.)	1.0 µL
Exonuclease (USB Corp.)	0.1 µL
dH_2O	7.9 µL
PCR	10.0 µL
Total:	**20.0 µL**

Conditions:

1. 37˚C for 90 minutes
2. 95˚C for 15 minutes
3. 10˚C hold forever

Finally, a single base extension step using fluorescently labeled dyes was performed for each SNP:

MC4R-4599 T>G:

	1 Reaction
10X Thermosequenase Buffer (USB Corp.)	1.0 µL
10µM Detection Primer	1.0 µL
Thermosequenase (USB Corp.)	0.1 µL
1:16 A/C Dye (Perkin Elmer Life Science)	0.05 µL
r110 C	
tamra A	
dH_2O	7.85 µL
Cleaned PCR	20.0 µL
Total:	**30.0 µL**

Conditions:

1. 94˚C for 1 minute
2. 30 Cycles
   a. 94˚C for 10 seconds
   b. 50˚C for 30 seconds
3. 10˚C hold forever

MC4R-4850 T>C:

	1 Reaction
10X Thermosequenase Buffer (USB Corp.)	1.0 µL
10µM Detection Primer	1.0 µL
Thermosequenase (USB Corp.)	0.1 µL
1:16 A/G Dye (Perkin Elmer Life Science)	0.1 µL
r110 A	
tamra G	
dH_2O	7.8 µL
Cleaned PCR	20.0 µL
Total:	**30.0 µL**

Conditions:

1. 94˚C for 1 minute
2. 30 Cycles
   a. 94˚C for 10 seconds
   b. 50˚C for 30 seconds
3. 10˚C hold forever

The samples were read on an Analyst HT plate reader (Molecular Devices) and analyzed with Allele Caller™ software (Molecular Devices, version 1.0). Genotypes were then entered into a database.

2.6 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 12.0 software (SPSS Inc.). Descriptive and frequency statistics were used to characterize the population, and analysis of variance (ANOVA) was used to check for between group differences for age, family income, and education by genotype.

BMI, weight, height, percent body fat, and fat free mass were averaged over two occasions of measurement and analyzed by ANOVA for genotype, sex, and genotype by sex associations. Waist circumference (measured once) was also analyzed. Three-group (all 3 genotypes) and 2-group (heterozygous and rare homozygous genotypes combined) analyses were done for both SNPs.

BMI was further evaluated with Chi-square tests by categorizing subjects as normal weight (BMI < 27) or overweight (BMI ≥ 27) based on a sample mean BMI of 27.04. Again, 3-group and 2-group analyses were done for both SNPs.

Finally, haplotypes were analyzed for group differences in BMI, weight, height, percent body fat, fat free mass, and waist circumference.

3.0 RESULTS

Genotyping at the -4599 T>G locus was successfully completed for 1,082 (98.5%) of the subjects. The G allele was present at a frequency of 30%. For the -4850 T>C site, 1,070 (97.4%) subjects were successfully genotyped. The C allele was seen at a frequency of 24%. All genotypes were in Hardy Weinberg equilibrium.

Subjects in this study were between the ages of 30 and 54 years, with a mean age of 44.7 years. On average they had obtained 16 years of education, and the median family income was in the range of $50,000 - $64,999 annually (see Table 5 and Appendix A). Age, family income, and education were not significantly associated with sex or genotype for either SNP, and no gene by sex interactions were seen (see Table 6). When genotypes were collapsed into 2 groups (TT vs. TG+GG for -4599 T>G and TT vs. TC+CC for -4850 T>C), age was associated with -4850 T>C status at the $p<0.05$ level.

Subjects had a mean BMI of 27.0 kg/m^2 and an average weight of 175 pounds (see Table 5 and Appendix A). All measures except BMI were distributed normally (see Appendix B). BMI is not typically transformed in the literature, and although using the natural log did normalize the data, it did not affect the outcome of the results. No significant statistical associations were seen for BMI, weight, height, percent body fat, fat free mass, or waist circumference for either SNP (see Table 6). BMI and percent body fat approached significance for the -4599 T>G site with p-values of 0.075 and 0.084, respectively. For the -4850 T>C site,

waist circumference neared significance with a p-value of 0.077. A sex effect was present for all anthropometric measures, but no gene by sex interactions were seen.

Table 5. AHAB Subject Characteristics

	N	Mean	St. Dev.	Median	Minimum	Maximum
Age (years)	1099	44.71	6.77	46	30	54
Males	542	44.6	6.88	46	30	54
Females	557	44.8	6.67	46	30	54
Family Income	1094			$50,000-64,999		
Males	541			$50,000-64,999		
Females	553			$50,000-64,999		
Individual Income	843			$25,000-34,999		
Males	431			$35,000-49,999		
Females	412			$15,000-24,999		
Years in School	1099			16	6	24
Males	542			16	10	24
Females	557			16	6	24
Education	1099			B.S./B.A.		
Males	542			B.S./B.A.		
Females	557			B.S./B.A.		
BMI (kg/m^2)	1098	27.04	5.31	26.20	16.9	51.4
Males	541	27.87	4.67	27.15	18.0	51.4
Females	557	26.27	5.77	25.05	16.9	50.3
Weight (pounds)	1099	174.94	40.79	172.00	83.3	375.0
Males	542	195.01	35.97	189.25	118.8	375.0
Females	557	155.41	35.39	148.25	83.3	291.0
Height (inches)	1099	67.30	3.82	67.5	53.5	79.0
Males	542	70.10	2.63	70.0	60.25	79.0
Females	557	64.52	2.58	64.5	53.5	72.5
% Body Fat	1097	29.33	9.01	28.3	4.6	54.5
Males	540	24.56	6.92	24.2	4.6	47.5
Females	557	33.95	8.38	34.2	9.0	54.5
Fat Free Mass (pounds)	1097	122.16	26.35	120.1	75.7	204.1
Males	540	144.98	16.12	143.5	95.1	204.1
Females	557	100.05	10.99	98.2	75.7	141.4
Waist Circumference (inches)	1098	35.90	6.13	35.5	24.0	68.0
Males	542	38.97	5.27	38.5	27.0	68.0
Females	556	32.99	5.44	32.0	24.0	52.0

Table 6. ANOVA Results

	3-Group ANOVA		2-Group ANOVA	
	vs. -4599 T>G	**vs. -4850 T>C**	**vs. -4599 T>G**	**vs. -4850 T>C**
Age (years)	p = 0.493	p = 0.124	p = 0.300	p = 0.045*
Family Income	p = 0.468	p = 0.513	p = 0.924	p = 0.234
Individual Income	N/A	N/A	N/A	N/A
Years in School	N/A	N/A	N/A	N/A
Education	p = 0.745	p = 0.475	p = 0.439	p = 0.240
BMI (kg/m*m)	p = 0.075	p = 0.115	p = 0.023*	p = 0.063
Weight (pounds)	p = 0.134	p = 0.130	p = 0.046*	p = 0.080
Height (inches)	p = 0.413	p = 0.503	p = 0.239	p = 0.297
% Body Fat	p = 0.084	p = 0.115	p = 0.039*	p = 0.078
Fat Free Mass (pounds)	p = 0.845	p = 0.485	p = 0.580	p = 0.511
Waist Circumference (inches)	p = 0.100	p = 0.077	p = 0.037*	p = 0.043*

* $p < 0.05$

When genotypes were collapsed into 2 groups for the -4599 T>G site, subjects with a G allele (TG + GG genotypes) had significantly higher BMI (Figure 2), weight (Figure 3), percent body fat (Figure 4), and waist circumference (Figure 5) at the $p<0.05$ level. A gene by sex interaction was also seen for height ($p = 0.026$). For the -4850 T>C site, the C allele (TC + CC genotypes) was associated with increased waist circumference (Figure 6). All associations were of similar magnitude in both men and women.

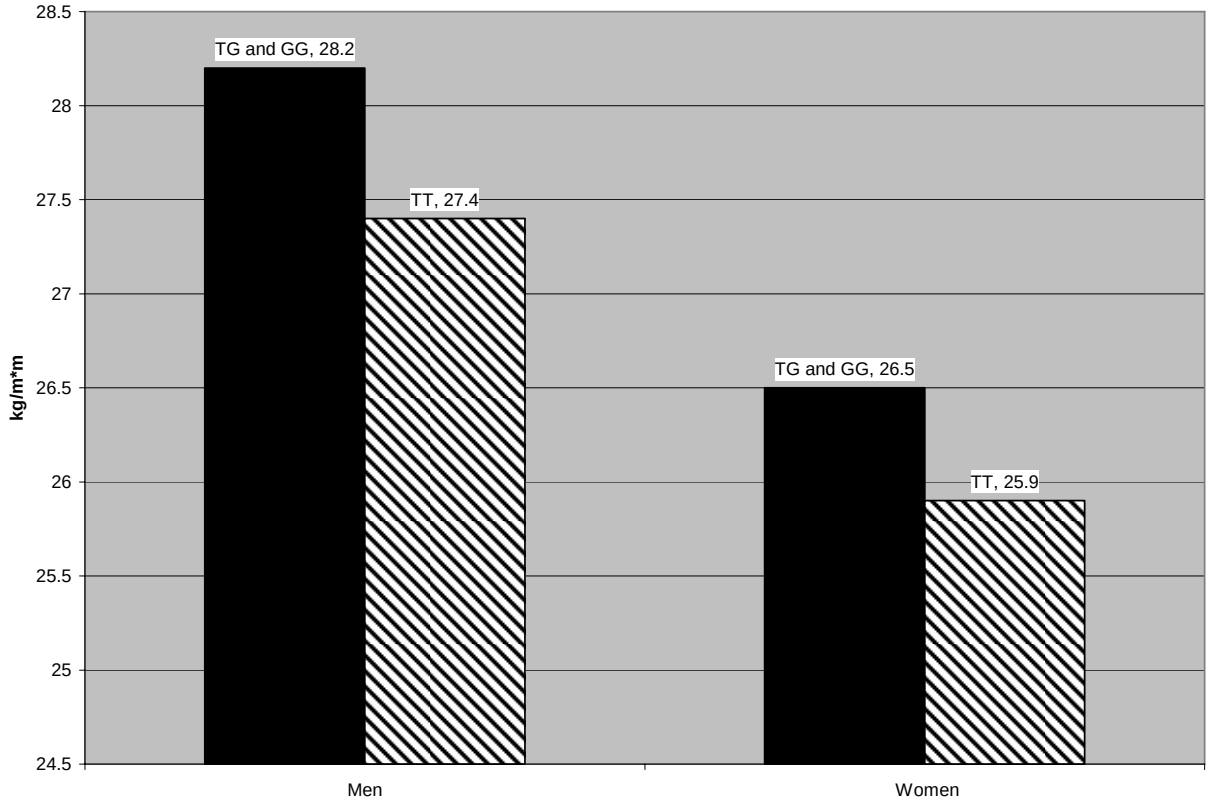


Figure 2. Mean BMI by -4599 T>G Genotype

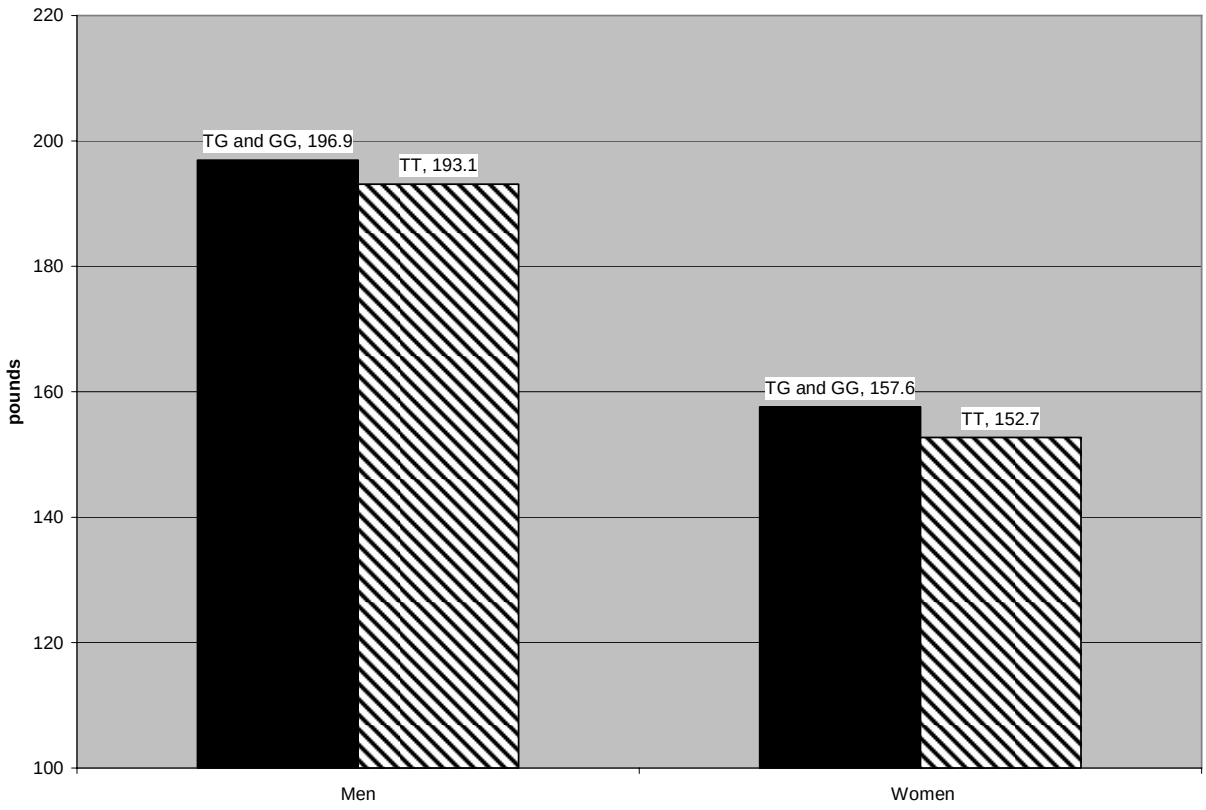


Figure 3. Mean Weight by -4599 T>G Genotype

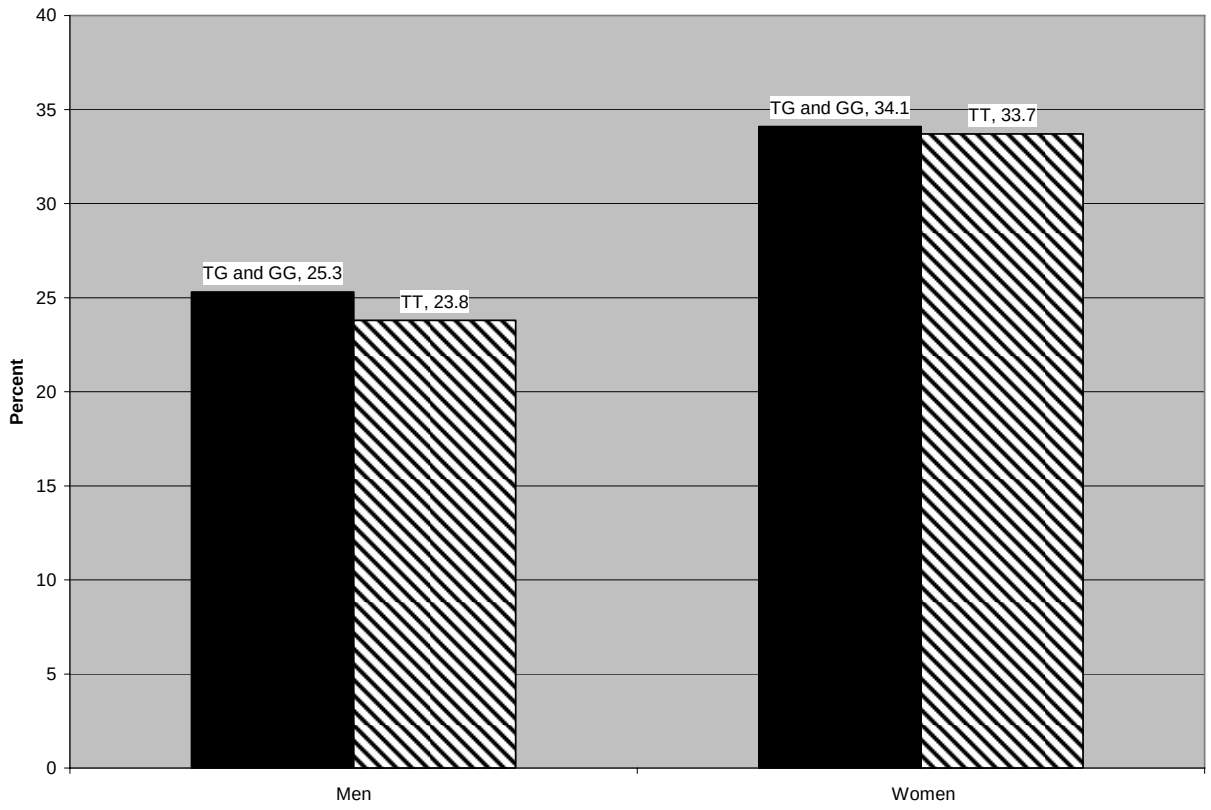


Figure 4. Mean % Body Fat by -4599 T>G Genotype

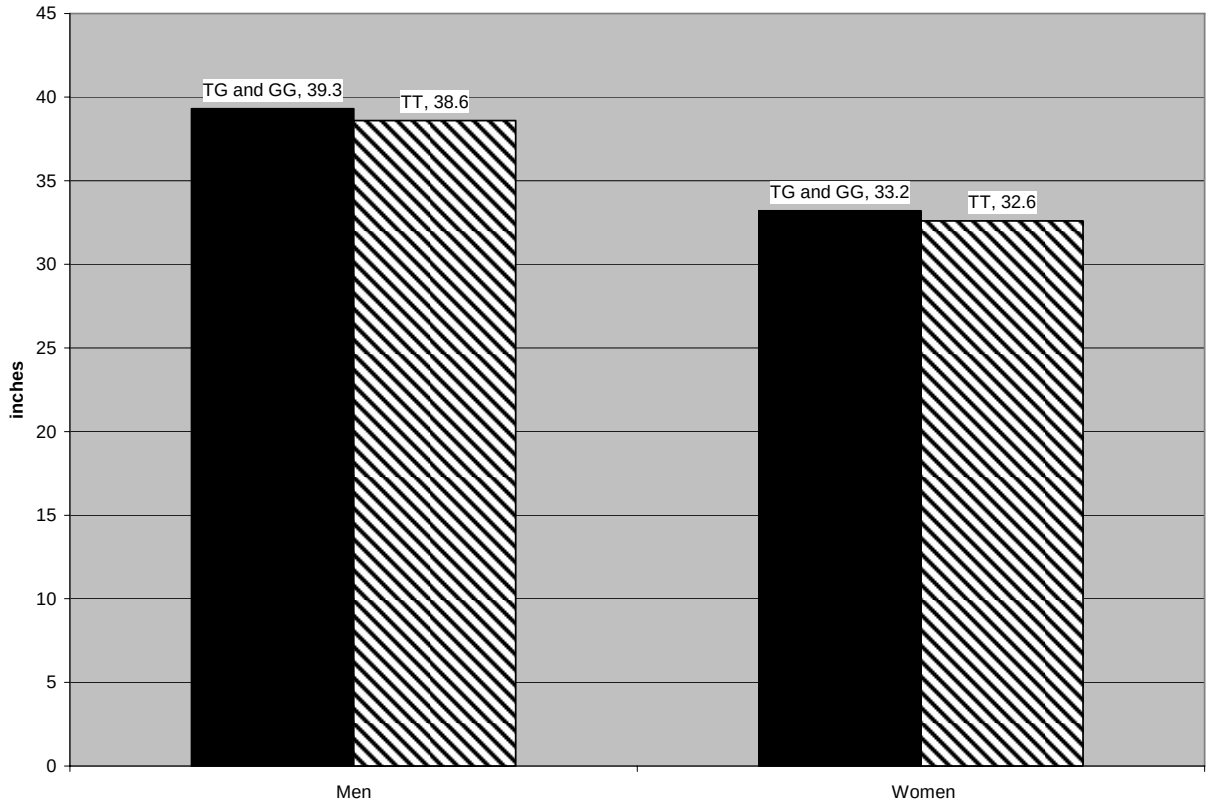


Figure 5. Mean Waist Circumference by -4599 T>G Genotype

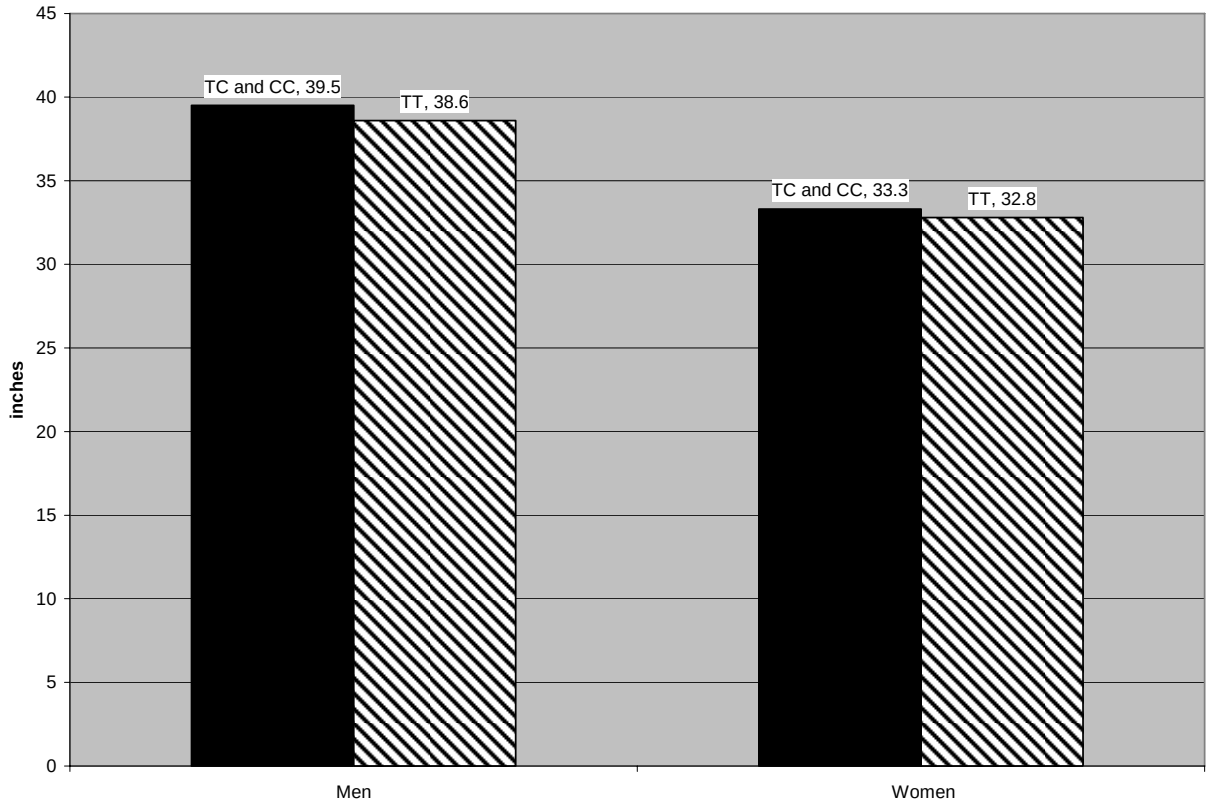


Figure 6. Mean Waist Circumference by -4850 T>C Genotype

Because BMI is a calculation based on both height and weight, it is possible that one measurement may confound the other. While BMI and weight showed a significant association with the -4599 G allele, height did not ($p = 0.239$; see Figure 7).

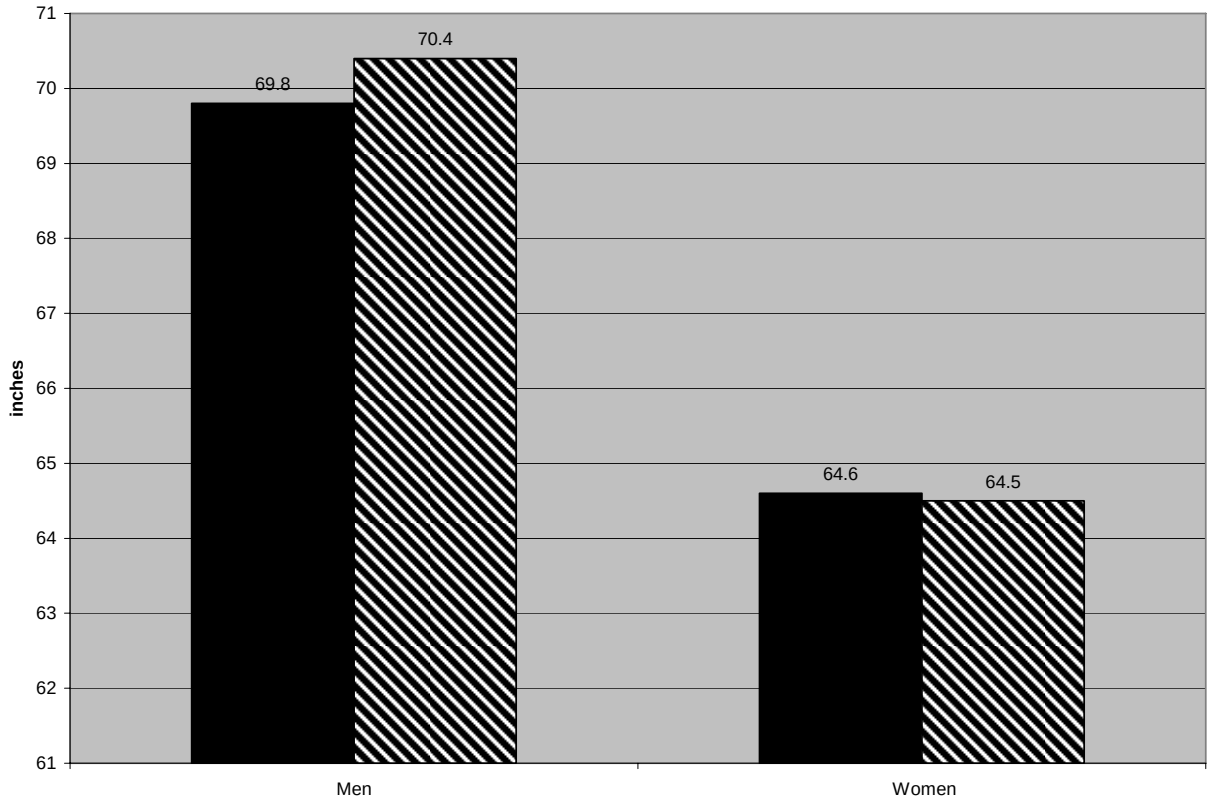


Figure 7. Mean Height by -4599 T>G Genotype

A high degree of linkage disequilibrium was noted between the two SNPs (D' = 0.98; 83% correlation), however no significant associations were seen.

Because BMI was the measure of greatest interest, further analysis was done with Chi-square tests by nominally defining "overweight" as BMI $\geq$ 27 kg/m^2. For -4599 T>G, 52.4% of GG subjects, 46.8% of TG subjects, and 40.6% of TT subjects are classified as overweight (Chi-square = 6.874, 2 df, p = 0.032; see Figure 8 and Table 7). A similar relationship is seen for the -4850 T>C SNP: 53.2% of CC subjects, 48.0% of TC subjects, and 41.4% of TT subjects are classified as overweight (Chi-square = 6.256, 2 df, p = 0.044; see Figure 9 and Table 7). When the rare homozygous genotypes were combined with the heterozygotes, the associations became stronger for both SNPs (-4599 T>G: Chi-square = 5.800, 1 df, p = 0.016; -4850 T>C: Chi-square = 5.671, 1 df, p = 0.017), suggesting a dominant effect.

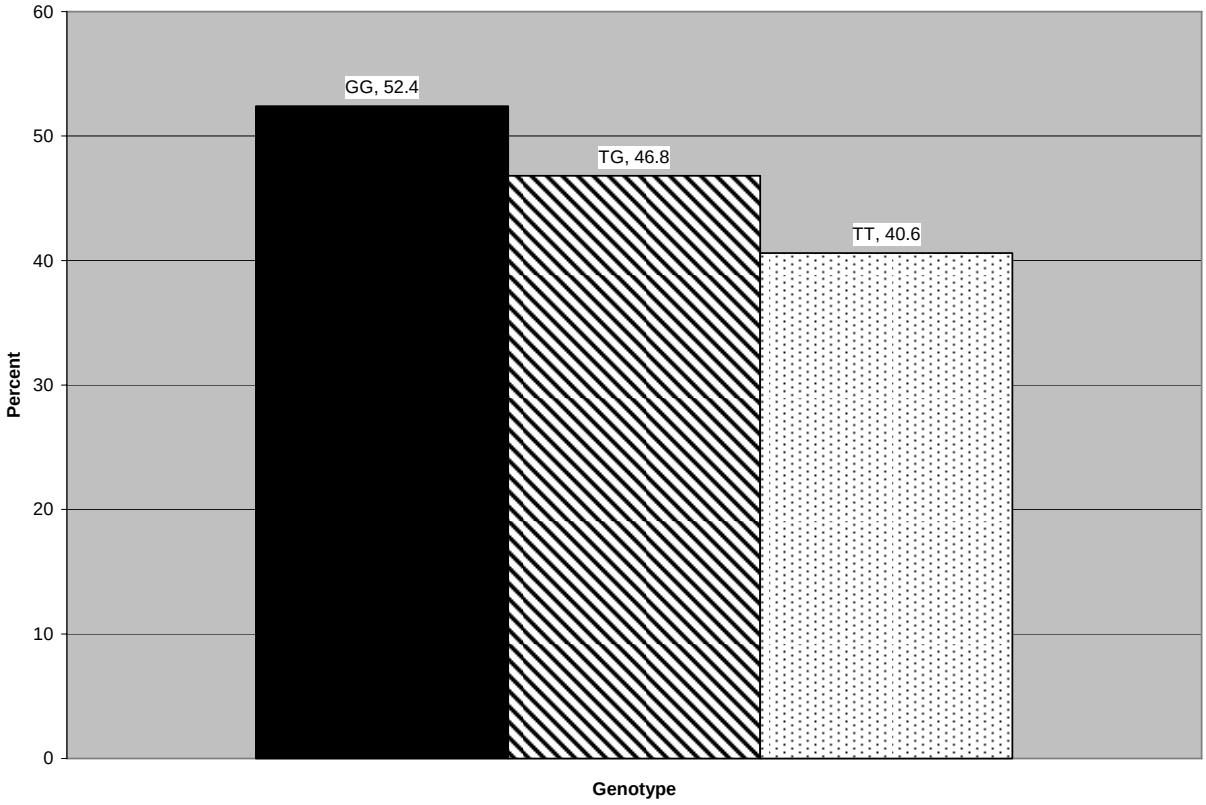


Figure 8. Percent of Overweight (BMI ≥ 27) Subjects by -4599 T>G Genotype

Table 7. Chi-Square Results; Overweight = BMI ≥ 27

		% overweight				% overweight
-4599 T>G	**GG**	52.4%	**GG vs. GT**	NS (p = 0.303)	**GG/GT**	47.9%
	GT	46.8%	**GT vs. TT**	NS (p = 0.052)		
	TT	40.6%	**GG vs. TT**	**p = 0.026**	**TT**	40.6%
		p = 0.032				**p = 0.016**
-4850 T>C	**CC**	53.2%	**CC vs. CT**	NS (p = 0.447)	**CC/CT**	48.8%
	CT	48.0%	**CT vs. TT**	**p = 0.040**		
	TT	41.4%	**CC vs. TT**	NS (p = 0.072)	**TT**	41.1%
		p = 0.044				**p = 0.017**

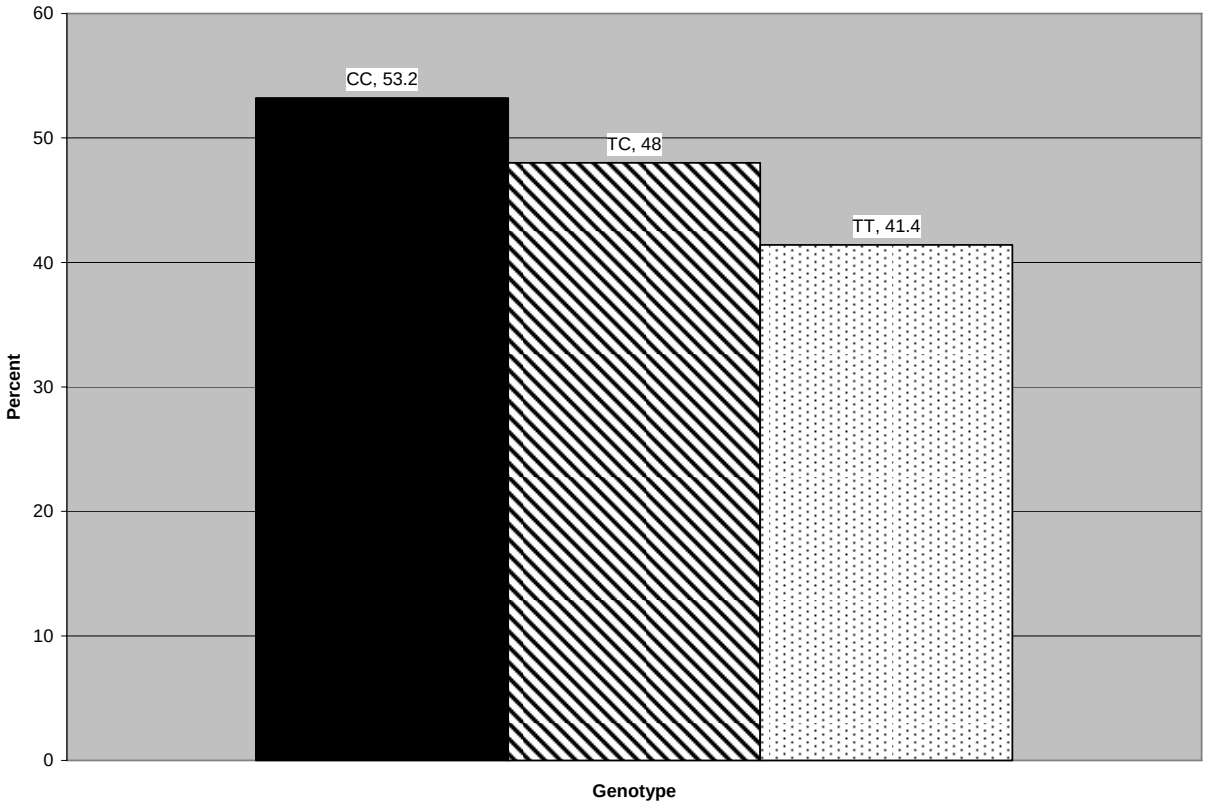


Figure 9. Percent of Overweight (BMI ≥ 27) Subjects by -4850 T>C Genotype

4.0 DISCUSSION

The melanocortin system is important in the regulation of energy balance.[8,24] In particular, a functioning MC4R has been shown to be necessary to prevent adiposity.[6] Mutations in the MC4R gene are considered to be the most common form of monogenic obesity in humans.[25,26,36,70,78,85] Common obesity is thought to be a polygenic disorder[48] with no firm evidence implicating MC4R loci to date.[68] Most MC4R variants are found at very low frequencies and have only been identified in obese patients,[12] although a few variants have been seen in similar frequencies in patients and controls.[20] Only two studies have looked at variation outside the coding region of MC4R: One study screened 80 base pairs of the promoter,[52] and the other study was done in Pima Indians.[54] It remains possible that genetic polymorphisms in the regulatory regions of the MC4R gene may contribute to the risk of developing obesity in the general population.[52,68,78]

This is the first study to show an association between variation in the MC4R gene with measures of adiposity in the general population. The main finding was a relationship between the -4599 G-allele and increased BMI. Interestingly, an additive effect was seen when subjects were categorized as normal (BMI < 27) or overweight (BMI ≥ 27), with the heterozygous group having an intermediate percentage of overweight subjects (see Figure 8). A similar effect was seen for the -4850 T>C SNP (see Figure 9).

The -4599 G-allele was also associated with elevated weight, percent body fat, and waist circumference in this study population, supporting the relationship of MC4R to increases in adiposity. Additionally, waist circumference was significantly associated with the -4850 C-allele.

Further research is needed to determine whether the SNPs evaluated in this study have functional implications for the MC4R gene. It is possible that they are in linkage disequilibrium with other variants within MC4R or another gene.

Obesity continues to emerge as a major public health problem. New information about the pathophysiology of obesity is necessary to develop improved strategies and programs for weight maintenance and weight reduction.[21,62] Mackenzie (2005) states that several pharmaceutical companies have programs to develop selective MC4R agonists for the treatment of obesity,[4] and that it is likely that MC4R agonist drugs will be successfully developed in the near future to provide effective anti-obesity treatment.[55]

Tailored approaches to prevention will also be enabled with increased knowledge about the biological causes of obesity. Barsh et al. (2000) concludes that if some environmental variables manifest themselves only on certain genotypes, efforts to prevent obesity at a public health level can be focused on recognition and counseling of susceptible individuals.[55] It has already been shown that the interaction of diet with MC4R not only involves the regulation of appetite, but extends to the regulation of metabolism and physical activity.[11] Another study found that voluntary exercise, beginning at early ages, may bypass or compensate for the central pathway involving MC4R.[42] Once the role of MC4R and other genetic factors has been more clearly defined, improved strategies for prevention can be developed.

Finally, surgical treatments for obesity are becoming commonplace. Potoczna et al. (2004) reported that MC4R variant carriers experienced 5 times more gastric complications per patient and treatment year after surgery than did noncarriers, including band problems, decreased weight loss, increased need for reoperation and conversion to gastric bypass, and vomiting.[70] Understanding the role of MC4R and its effect on surgical treatment outcome will help doctors and patients make educated decisions about treatment options and will hopefully lead to healthcare cost savings.

5.0 SUMMARY AND CONCLUSION

As the prevalence of overweight and obesity increase rapidly in both developing and developed countries worldwide, the need for greater understanding of their biological origin intensifies. Recent research has shown that several genetic factors play a role in regulating energy balance via the neuroendocrine system, and several candidate genes have been identified. MC4R mutations are considered to be the most common form of monogenic obesity in humans. However, the role of MC4R variation regarding energy homeostasis in the general population is controversial.

This study was among the first to evaluate common polymorphisms in the promoter region of MC4R with measures of adiposity in the general population, and the first to show statistical associations. The -4599 G-allele was significantly associated with increased BMI, weight, percent body fat, waist circumference, and overall probability of being overweight. The -4850 C-allele was also associated with increased waist circumference and overall probability of being overweight.

If MC4R variation were to be implicated in common obesity, new drug treatments and prevention strategies could be identified and utilized. Reversal of the obesity epidemic and related comorbidities would lead to billions of dollars in health care cost savings as well as an improved quality of life for the public.

APPENDIX A

SUBJECT CHARACTERISTICS BY GENOTYPE

Subject Characteristics by MC4R-4599 T>G Genotype

	TT		TG		GG	
	N	Mean (S.D.)	N	Mean (S.D.)	N	Mean (S.D.)
Age (years)	542	44.90 (6.83)	437	44.38 (6.86)	103	44.83 (6.21)
Males	269	44.42 (7.06)	213	44.69 (6.73)	54	44.70 (6.59)
Females	273	45.38 (6.57)	224	44.10 (6.99)	49	44.98 (5.82)
BMI (kg/m^2)	542	26.65 (6.83)	436	27.35 (5.81)	103	27.47 (4.64)
Males	269	27.43 (4.65)	212	28.21 (4.69)	54	28.29 (4.72)
Females	273	25.88 (5.17)	224	26.54 (6.61)	49	26.56 (4.43)
Weight (pounds)	542	172.75 (38.34)	437	176.91 (44.08)	103	177.48 (39.54)
Males	269	193.06 (33.26)	213	197.07 (39.02)	54	196.34 (37.96)
Females	273	152.74 (31.99)	224	157.73 (39.93)	49	156.71 (29.85)
Height (inches)	542	67.39 (3.94)	437	67.19 (3.69)	103	67.21 (3.74)
Males	269	70.38 (2.54)	213	69.86 (2.67)	54	69.75 (2.79)
Females	273	64.45 (2.65)	224	64.66 (2.57)	49	64.41 (2.41)
% Body Fat	541	28.79 (8.85)	436	29.71 (9.21)	103	30.01 (8.86)
Males	268	23.78 (6.77)	212	25.37 (7.01)	54	25.23 (7.20)
Females	273	33.69 (7.85)	224	33.81 (9.17)	49	35.27 (7.44)
Fat Free Mass (pounds)	541	122.01 (26.36)	436	122.14 (26.28)	103	123.51 (26.99)
Males	268	145.27 (14.83)	212	144.41 (17.46)	54	145.32 (17.40)
Females	273	99.18 (10.37)	224	101.06 (11.84)	49	99.48 (9.47)
Waist Circumference (inches)	541	35.60 (5.79)	437	36.16 (6.61)	103	36.66 (5.75)
Males	269	38.61 (5.03)	213	39.33 (5.52)	54	39.37 (5.49)
Females	272	32.62 (4.88)	224	33.15 (6.14)	49	33.66 (4.43)

Subject Characteristics by MC4R-4599 T>G 2-Group Genotype

	TT		TG + GG	
	N	Mean (S.D.)	N	Mean (S.D.)
Age (years)	542	44.90 (6.83)	540	44.47 (6.74)
Males	269	44.42 (7.06)	267	44.69 (6.69)
Females	273	45.38 (6.57)	273	44.26 (6.79)
BMI (kg/m^2)	542	26.65 (6.83)	539	27.37 (5.60)
Males	269	27.43 (4.65)	266	28.23 (4.69)
Females	273	25.88 (5.17)	273	26.54 (6.26)
Weight (pounds)	542	172.75 (38.34)	540	177.02 (43.22)
Males	269	193.06 (33.26)	267	196.92 (38.74)
Females	273	152.74 (31.99)	273	157.55 (38.27)
Height (inches)	542	67.39 (3.94)	540	67.20 (3.69)
Males	269	70.38 (2.54)	267	69.84 (2.69)
Females	273	64.45 (2.65)	273	64.62 (2.54)
% Body Fat	541	28.79 (8.85)	539	29.76 (9.13)
Males	268	23.78 (6.77)	266	25.34 (7.04)
Females	273	33.69 (7.85)	273	34.07 (8.89)
Fat Free Mass (pounds)	541	122.01 (26.36)	539	122.40 (26.40)
Males	268	145.27 (14.83)	266	144.60 (17.42)
Females	273	99.18 (10.37)	273	100.77 (11.46)
Waist Circumference (inches)	541	35.60 (5.79)	540	36.26 (6.45)
Males	269	38.61 (5.03)	267	39.34 (5.51)
Females	272	32.62 (4.88)	273	33.24 (5.87)

Subject Characteristics by MC4R-4850 T>C Genotype

	TT		TC		CC	
	N	Mean (S.D.)	N	Mean (S.D.)	N	Mean (S.D.)
Age (years)	628	45.04 (6.87)	380	44.17 (6.78)	62	44.29 (5.99)
Males	308	44.63 (7.09)	193	44.59 (6.67)	28	43.46 (6.39)
Females	320	45.43 (6.65)	187	44.73 (6.89)	34	44.97 (5.65)
BMI (kg/m^2)	628	26.78 (5.20)	379	27.32 (5.63)	62	27.89 (4.96)
Males	308	27.45 (4.71)	192	28.25 (4.51)	28	28.95 (5.12)
Females	320	26.13 (5.55)	187	26.36 (6.45)	34	27.02 (4.72)
Weight (pounds)	628	173.19 (39.08)	380	177.12 (43.80)	62	179.65 (41.52)
Males	308	192.76 (34.27)	193	197.00 (38.05)	28	203.36 (41.59)
Females	320	154.36 (33.86)	187	156.60 (39.78)	34	160.13 (30.04)
Height (inches)	628	67.33 (3.90)	380	67.23 (3.68)	62	67.13 (3.76)
Males	308	70.28 (2.59)	193	69.77 (2.68)	28	70.19 (2.74)
Females	320	64.49 (2.65)	187	64.61 (2.56)	34	64.60 (2.33)
% Body Fat	627	28.99 (9.08)	379	29.49 (8.99)	62	31.24 (9.12)
Males	307	23.81 (6.86)	192	25.48 (6.80)	28	25.86 (8.18)
Females	320	33.96 (8.12)	187	33.62 (9.11)	34	35.68 (7.35)
Fat Free Mass (pounds)	627	121.78 (26.21)	379	122.86 (26.61)	62	122.20 (26.76)
Males	307	144.90 (15.12)	192	144.49 (17.79)	28	147.91 (16.60)
Females	320	99.60 (10.99)	187	100.65 (11.63)	34	101.03 (8.67)
Waist Circumference (inches)	627	35.64 (5.86)	380	36.30 (6.58)	62	36.78 (6.29)
Males	308	38.56 (5.09)	193	39.41 (5.41)	28	39.68 (6.38)
Females	319	32.82 (5.12)	187	33.08 (6.14)	34	34.40 (5.18)

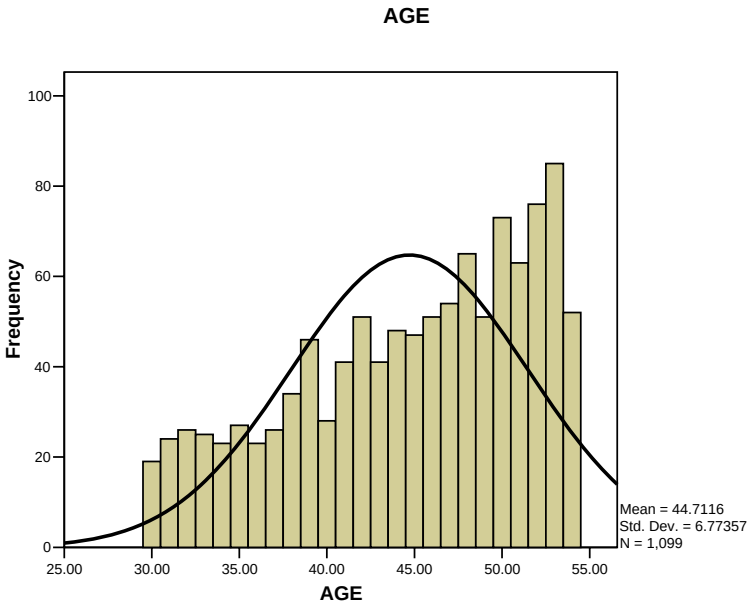
Subject Characteristics by MC4R-4850 T>C Genotype

	TT		TC + CC	
	N	**Mean (S.D.)**	**N**	**Mean (S.D.)**
Age (years)	628	45.04 (6.87)	442	44.18 (6.67)
Males	308	44.63 (7.09)	221	44.44 (6.63)
Females	320	45.43 (6.65)	221	43.92 (6.72)
BMI (kg/m^2)	628	26.78 (5.20)	441	27.40 (5.54)
Males	308	27.45 (4.71)	220	28.34 (4.59)
Females	320	26.13 (5.55)	221	26.46 (6.21)
Weight (pounds)	628	173.19 (39.08)	442	177.47 (43.45)
Males	308	192.76 (34.27)	221	197.80 (38.47)
Females	320	154.36 (33.86)	221	157.15 (38.40)
Height (inches)	628	67.33 (3.90)	442	67.22 (3.69)
Males	308	70.28 (2.59)	221	69.82 (2.69)
Females	320	64.49 (2.65)	221	64.61 (2.52)
% Body Fat	627	28.99 (9.08)	441	29.74 (9.01)
Males	307	23.81 (6.86)	220	25.53 (6.97)
Females	320	33.96 (8.12)	221	33.93 (8.88)
Fat Free Mass (pounds)	627	121.78 (26.21)	441	122.77 (26.60)
Males	307	144.90 (15.12)	220	144.93 (17.64)
Females	320	99.60 (10.99)	221	100.71 (11.21)
Waist Circumference (inches)	627	35.64 (5.86)	442	36.37 (6.54)
Males	308	38.56 (5.09)	221	39.45 (5.53)
Females	319	32.82 (5.12)	221	33.28 (6.01)

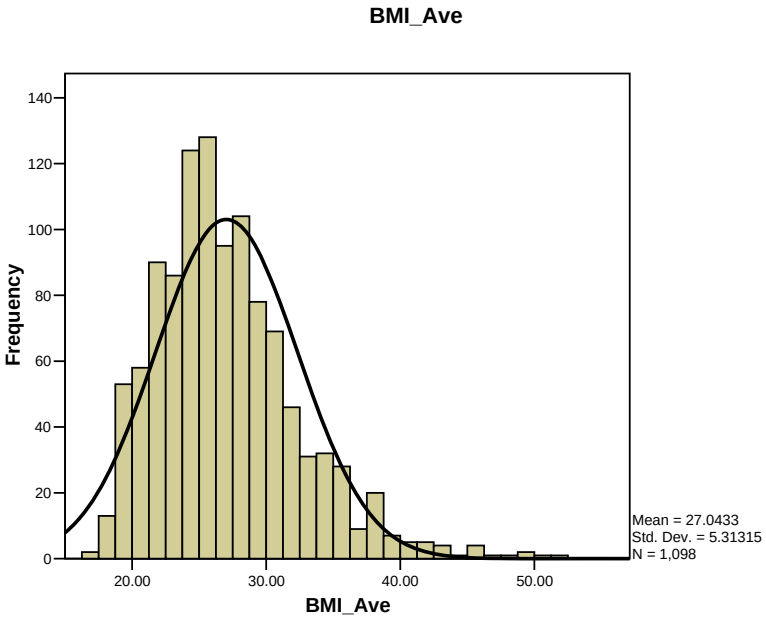
APPENDIX B

HISTOGRAMS

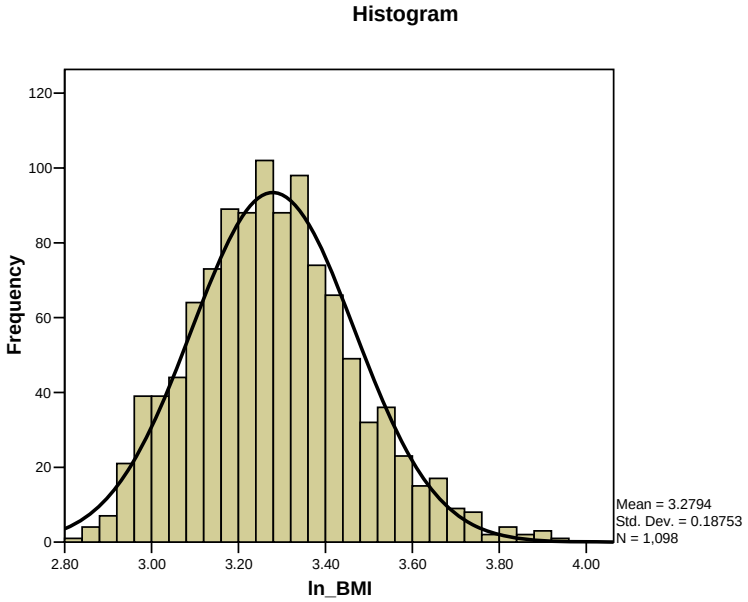
Age (Skewness: -0.494; Kurtosis: -0.841)



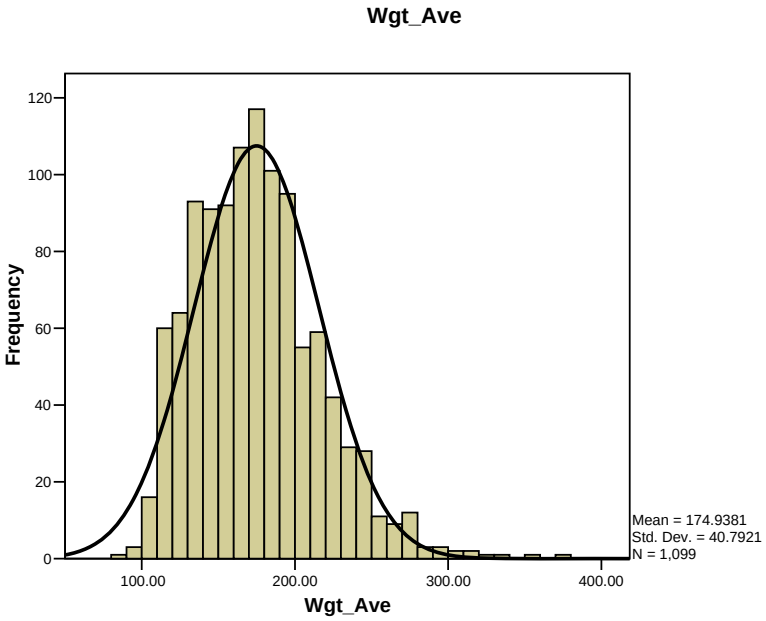
BMI (Skewness: 1.012; Kurtosis: 1.649)



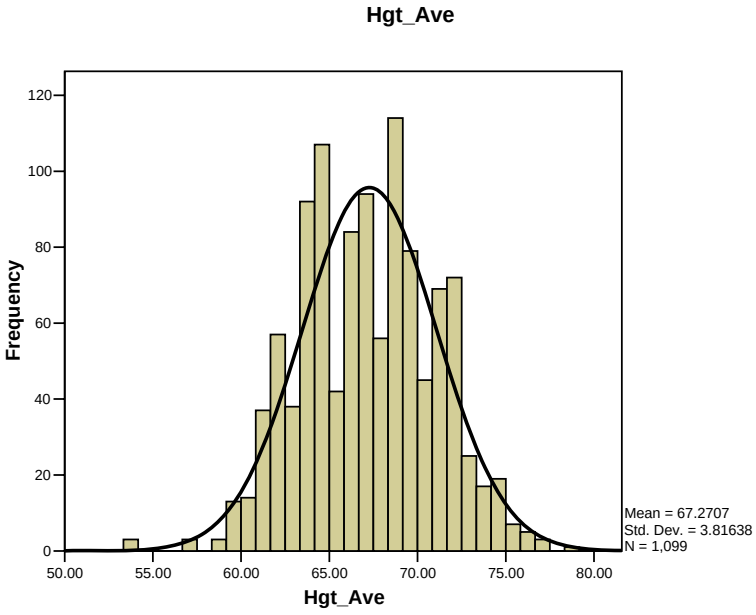
LN BMI (Skewness: 0.397; Kurtosis: 0.153)



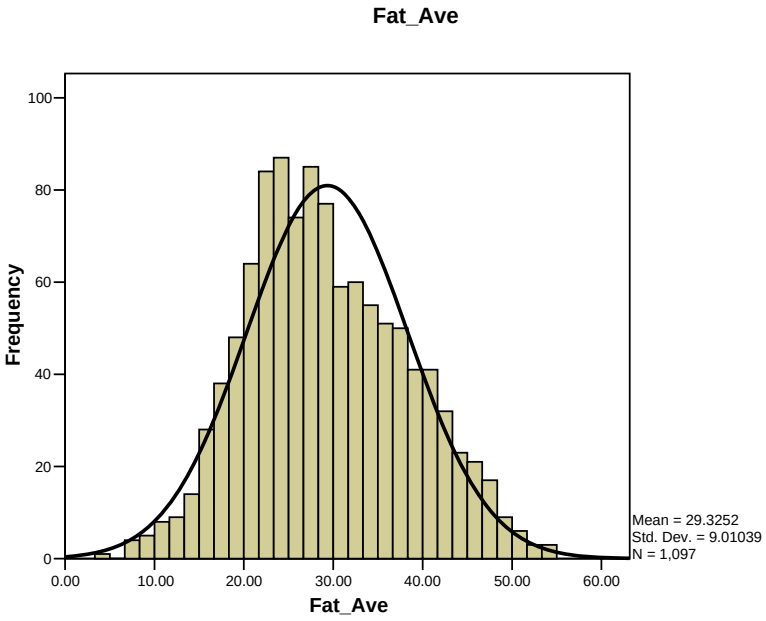
Weight (Skewness: 0.723; Kurtosis: 0.990)



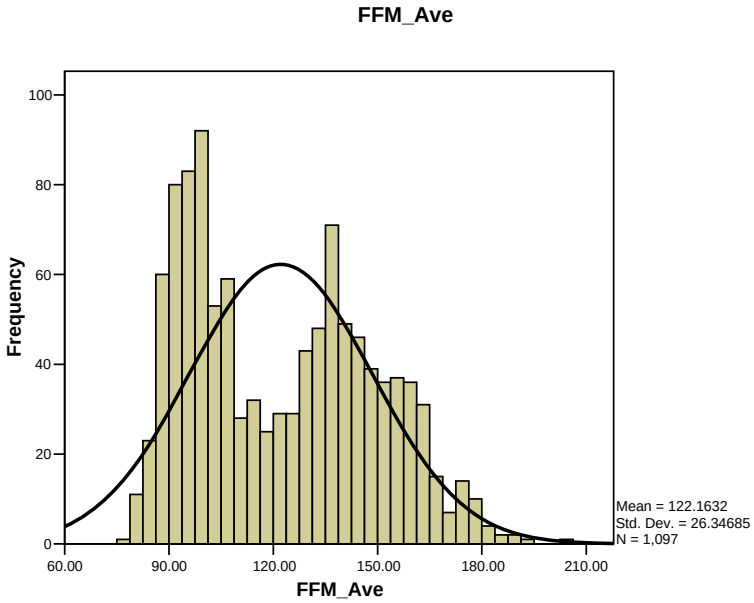
Height (Skewness: -0.024; Kurtosis: -0.265)



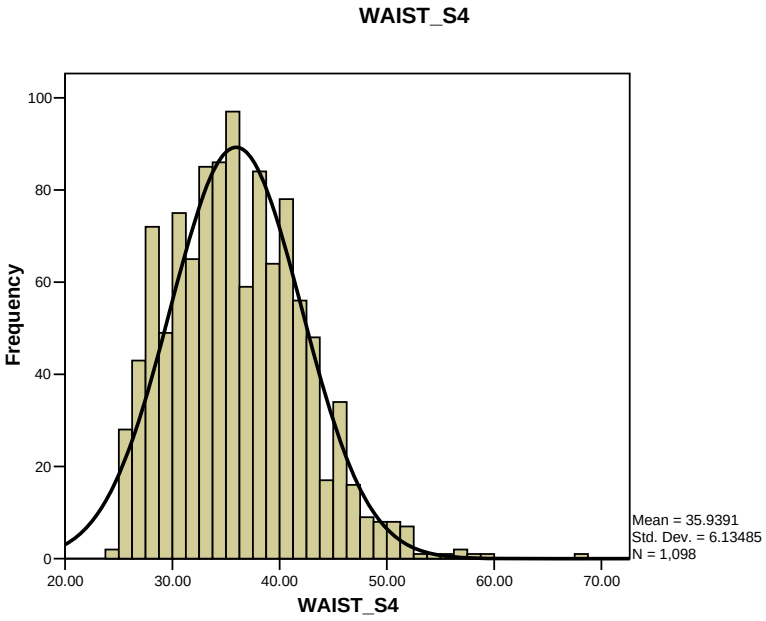
% Body Fat (Skewness: 0.262; Kurtosis: -0.391)



Fat Free Mass (Skewness: 0.336; Kurtosis: -0.998)



Waist Circumference (Skewness: 0.559; Kurtosis: 0.148)



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