

DETERMINANTS OF PLASMA LEPTIN AND AGE AT MENARCHE IN
ADOLESCENT GIRLS IN HAWAII

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By

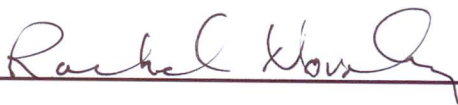
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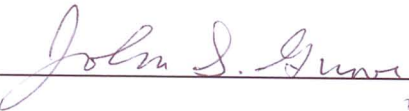
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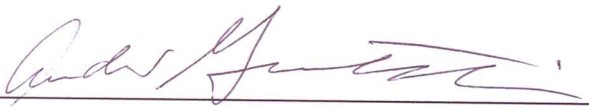
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ABSTRACT

This study investigates the association of the Leptin (*LEP*) and Leptin receptor (*LEPR*) polymorphisms *LEP* A19G, *LEP* G-2548A and *LEPR* Q223R with plasma leptin concentration, body fat and age at menarche (AAM). The study was part of a larger cohort study that followed 349 multiethnic adolescent girls (ages 9-14yr at baseline) and an additional 180 girls at exam 3 in Hawaii (Female Adolescent Maturation Study). Anthropometry was obtained by measurement, Tanner stages by clinical examination, dietary intake by 3-day diet record and physical activity by a standardized questionnaire. DNA was obtained from mouthwash or blood and was genotyped using a fluorescent 5' endonuclease assay and the ABI FAST 7900HT Real-Time PCR System for allelic discrimination. Dual energy X ray Absorptiometry (DXA) was used to determine body fat mass. The study did not show association between *LEP/LEPR* variants and plasma leptin. However, the association between plasma leptin and body fat measures (measured using both DXA and anthropometry) was substantiated. A difference in *LEPR* rare homozygote genotype (AA) and the common homozygote genotype (GG) in DXA trunk-to-periphery fat ratio (TPFR) was found (girls with *LEPR* Q223R AA genotype had lower TPFR compared to GG). It was also found that protein intake (adjusted for BMI) was negatively associated with plasma leptin level. In a COX regression model, girls with AG genotype for *LEP* G-2548A reached menarche earlier than the AA genotype. The study also substantiated the evidence of heavier and taller girls reaching menarche early. Importantly, this study demonstrated these associations in understudied girls of Asian White ancestry. This present study provides new information on the *LEP/LEPR* genetic variants, body fat and age at menarche in an Asian White population.

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CHAPTER 1

INTRODUCTION

1.1 Introduction to the dissertation

Age at menarche (AAM) is an important biological milestone of pubertal development in females. The mean AAM of a population reflects numerous health aspects, including the timing of pubertal maturation, growth, nutritional status, and environmental conditions (1). Risk factors for breast cancer, such as estrogen, are beneficial for the prevention of osteoporosis. Women whose menarche occurs early have a longer duration of exposure to higher levels of estrogens; thus, the age at which a girl reaches menarche is an important indicator of her lifetime exposure to estrogen which, in turn, is associated with risk for breast cancer and obesity but protection from osteoporosis (2-4). This dissertation examines the factors influencing AAM, with a focus on plasma leptin and body fat, diet and genotypes.

1.2 Breast Cancer

Breast cancer is a disease in which abnormal cells from the breast grow out of control.

Breast cancer is the most common form of cancer, in women, and is the second most common cause of cancer death in White, Black, Asian, Pacific Islander, American Indian, and Alaska Native women (5). According to WHO, more than 1.2 million women are diagnosed with breast cancer annually worldwide. In 2005, approximately 190,000 women in the US were diagnosed with breast cancer and approximately 50,000 women died from breast cancer (5)

Early menarche and late menopause have been shown to increase the lifetime risk of breast cancer. It is hypothesized that breast cancer risk is increased because of greater cumulative exposure to estrogen. For each two-year delay in menarche, the risk of breast cancer has been estimated to be reduced by 10% (2). Data indicate that higher endogenous estrogen exposure (e.g. pregnancy, early menarche and late menopause, obesity) or exogenous estrogen exposure (e.g. oral contraceptive, hormone replacement therapies) is associated with an increased probability of breast cancer diagnosis (2). Since menarche at a young age is associated with earlier onset of regular menstrual cycles, this early exposure to the hormonal milieu associated with regular ovulatory menstrual cycles plays an important etiologic role in breast cancer (3, 6, 7). Also, some studies have reported that women with early menarche have higher estrogen levels for several years after menarche and probably throughout their reproductive lives (3, 8). Thus, early menarche is an important risk factor for developing breast cancer, which makes age at menarche an important biologic phenomenon.

1.3 Osteoporosis

Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and to an increased susceptibility to fractures. The probability of developing osteoporosis and sustaining a fracture are dependent on two major phenomena: peak bone mass achieved as a young adult and rate of bone loss over the succeeding years (9). About 85-90% of adult bone mass is acquired by age 18 years in girls and 20 years in boys. Osteoporosis is a major public health threat for an estimated 44 million Americans, or 55 percent of the people 50 years of age and older. In 2005, osteoporosis-related fractures were responsible for an estimated \$19 billion in costs.

Of the 10 million Americans estimated to have osteoporosis, eight million are women (10). Known risk factors are age, low sex hormones, and low estrogen levels in women. Estrogen, both endogenous (11) and exogenous (12-14), protects against osteoporosis. In a 29 year follow up of more than 60,000 women, Jacobsen et al. (4) found a protective effect of early menarche, a long reproductive period, and a high age at first birth on the risk of hip fracture mortality, all of which point towards a protective effect of estrogen on osteoporosis. The later the menarche, and the earlier the menopause, the higher the degree of osteoporosis (15). It has been reported that long exposure to estrogen reduces the hip fracture mortality through the well established positive relationship between estrogen and bone mass (4).

Thus, risk factors for breast cancer are beneficial for prevention of osteoporosis, raising a question of optimal adolescent growth and maturation rates to minimize risk for both conditions.

1.4 Obesity across the lifespan

A high prevalence of obesity (body mass index [BMI, kg/m²] ≥ 30) illustrates that obesity continues to be a major health concern for adults, children and adolescents in the United States. Results from the 2007-2008 National Health and Nutrition Examination Survey (NHANES), indicate that an estimated 17% of children and adolescents ages 2-19 years are obese. Among pre-school age children 2-5 years of age, obesity increased from 5 to 10.4% between 1976-1980 and 2007-2008 and from 6.5 to 19.6% among 6-11 year olds (16). This rate of obesity raises concern because of implications of obesity for the health of Americans. There are numerous adverse effects of obesity on general, and especially cardiovascular (CV) health (17).

Cessation of gonadal estrogen production at menopause is associated with an increase in the waist-to-hip ratio and size of the visceral adipose tissue depot, i.e., development of a more android body habitus (18-21). Also estrogen administration to postmenopausal women is associated with a lowering of the waist-to-hip ratio (22). Obesity is associated with a increased risk of breast cancer in postmenopausal women, and the risk increases with time since menopause (23), which can be attributed to low estrogen levels during menopause. Among several hormones, estrogens promote, maintain, and control the typical distribution of body fat and adipose tissue metabolism through still unknown

mechanisms. These steroids are known to regulate fat mass, adipose deposition and differentiation, and adipocyte metabolism (24). Moreover, estrogen deficiency during menopause in women results in increases in total body fat and visceral fat, which confirms linkage between obesity with obesity-related disorders (25).

1.5 Age at menarche (AAM)

Age at menarche is an important milestone of pubertal development in females.

Menarche is the end-point of a complex series of developmental events, defined by the dynamic interaction between genetic factors and environmental cues, ultimately leading to the attainment of reproductive capacity. The timing of AAM is an indicator of the start of regular exposure to endogenous estrogen and to other hormones. The proximate cause of menarche is an increase in secretion of gonadotropin releasing hormone (GnRH) in the hypothalamus, which is the primary regulator of Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH).

Age at menarche is known to be a sensitive indicator of the environment where a human population lives, as it can be influenced by factors as varied as diet, physical activity, (26-28), body composition (29), genetics and socio-economic inequalities in a society (30, 31). The mean AAM reflects numerous health aspects of a population, including the timing of pubertal maturation, growth and nutritional status, and environmental conditions (1). In the United States, the mean AAM decreased from over 14 years old prior to 1900 (32) to less than 12.5 years old currently (1). Published data on the incidence of breast cancer, among a large cohort of French women, provide further

evidence that the overall risk of breast cancer increases with younger age at menarche (relative risk 1.52; 95% confidence interval [CI], 1.03–2.22) (33). Secular trends in the average AAM may, in fact, affect the future epidemiology of breast cancer.

The data suggest that women whose menarche occurs early, not only have a longer duration of exposure to estrogens during years that are probably important in the initiation of breast cancer but, in addition, their exposures are at a higher level during those years and probably at later ages also (3).

Thus, the age at which girl reaches menarche is an important indicator of her lifetime exposure to estrogen, which is associated with risk for breast cancer, obesity and protection against osteoporosis. Age at menarche, age at menopause and overall years of menstruation are important indicators of health of pre and postmenopausal women.

1.5.1 Age at menarche (AAM) and body fat

A trend for earlier timing of menarche in several countries has been documented for the period from the mid-1800s to the mid-1900s and, presumably, is related to improved health and nutrition (32). It has been established that overweight girls tend to mature earlier than lean girls. Initially Frisch & Revelle (34) proposed a “critical weight” needed to be achieved in order to initiate and sustain menses in young girls and that this relationship could explain the secular trend toward earlier menarche during the previous century (did not adjust for height or body size). One problem with this theory is that, although the average weight at menarche in this study varied little with the age at

menarche, there was a broad distribution of weights (between 35 and 60 kg), at the time when individual girls reached menarche. Thus, weight is an important factor, but by no means the only determinant of age at menarche. Later Frisch (29) modified her theory to suggest that a critical fat mass (17% of body weight) was required for menarche and a higher fat mass (22% of body weight) was required for maintenance of reproductive capacity (girl reaching menarche). Body fatness may trigger neuroendocrine events that lead to the onset of menses (35).

As a child approaches puberty, the percentage of body fat increases and appears to signal (to the brain) the onset of puberty and reproductive maturation. Similarly, ceasing of menstruation occurs in females with extremely low body fat stores (patients with anorexia nervosa, ballet dancers, or long distance runners) because of hypothalamic reproductive dysfunction, resulting in abnormal gonadotrophin secretion: there is an age-inappropriate secretory pattern of (lower) LH and FSH, resembling that of prepubertal children. The secretion of LH and the responses to Luteinizing hormone releasing hormone (LHRH) (also known as Gonadotropin-releasing hormone (GnRH)) are reduced in direct correlation with the amount of weight loss.

Although the statistics on which Frisch's theory was made have been criticized (36, 37), there are insufficient studies to refute or support this theory. The lack of evidence is partly due to the small number of studies that longitudinally assess girls' body composition before, during and after menarche. In a recent longitudinal study of healthy White girls (38), using Dual Energy X ray Absorptiometry (DXA) to assess body

composition, the variability in weight (kg), percent body fat (%BF) and total body fat (TBF), was studied two years prior and two years after menarche. At menarche, there was a large variation in TBF, % BF and weight (CV=48.6%, 27.4%, 19.2%, respectively) compared to before menarche. The variation in TBF, %BF and weight decreased with an increase in biological age (years from menarche). Reductions in variation of weight, %BF and TBF were seen at menarche, compared to before menarche but not after menarche. Reductions in variability of body fat and weight were not apparent at menarche, which does not provide support for the hypothesis that a critical body mass/fat is required for menarche.

The relationship of body size (measured by height, BMI) and menarche is consistent, irrespective of the study design (Table 1.1). From longitudinal and cross sectional studies (26, 27, 39-41) girls who were taller and heavier reached menarche earlier. Although, the longitudinal study design, and for some studies the sample size, (Moisan 640, Maclure 194, Koprowski 679, Koo 187; Petridou 345) were strengths, there were limitations. Associations were likely underestimated because of random error in reporting menarche. Anthropometry was collected over a relatively short period of time, with respect to the occurrence of menarche. Socioeconomic status, ethnicity, and hereditary factors were not considered in the relationship of body fat and age at menarche. Physical activity was inconsistently handled. Finally, a cross sectional study design (41) might not be appropriate since exposures like height, weight and diet change over time.

1.5.2 Age at menarche (AAM) and body fat distribution

Recent studies (Table 1.1) have been conducted that show that AAM is related to central adiposity or type of body fat distribution more than the total body fat. In a retrospective historical cohort of 7349 Chinese women, age at menarche before 12.5 years was associated with the presence of central obesity (measured by waist circumference) in post menopausal women (42). The cross-sectional design, and recalled age of menarche are limitations to this study, however, the study demonstrated good reliability on repeat questioning of age of menarche. Random misclassification may have made their findings conservative. Their findings would be biased if either women with healthy metabolic profiles and early age of menarche, or women with unhealthy metabolic profiles and late age of menarche, were disproportionately excluded. There was no evidence for this type of bias.

Lassek and Gaulin (43) examined cross-sectional anthropometric data from the third National Health and Nutrition Examination Survey (NHANES III) and reported that some girls aged 10-14 years attained menarche despite low overall body fat, but they had proportionately more fat in gluteofemoral depots and less fat in the upper body compared with those who had not yet reached menarche. In this study, the body fat estimate was derived from thigh circumference, skinfold thickness, and fat volume, and calculated by multiplying fat area by femoral length; body fat was estimated from the sum of triceps and subscapular skinfolds using the method of Slaughter et al. (44). The same relationships were found in women aged 20-29 years. Because the data used in the study were cross-sectional, the relationship between body fat distribution and the odds of

menarche should be interpreted with caution. Also using triceps and subcapular skinfold thicknesses to estimate total body fat may be biased as it does not incorporate any measure of lower body fat. The study by Slaughter et al. (44) replicated findings from longitudinal studies, where total body fat and weight were not significantly related to the timing of menarche when combined in multivariate analyses with measures of skeletal growth (height, breadths). But, the study by Lassek (43) found that, under the same multivariate conditions, age at menarche was more strongly related to fat distribution than to skeletal growth, suggesting that body fat distribution may be a more important factor.

Table 1.1: Studies examining Age at menarche (AAM) in females, body size and shape (Outcome variable is self reported age at menarche for all the studies listed)

Authors, yr	Study Design (follow-up yr)	Ancestry	N	Age (yr)	Independent variables	Potential confounders adjusted	Results related to AAM
Frisch and Revelle, 1970 (45)	Data from 3 longitudinal studies	White	181	Adolescents	Weight (kg)	None	Body mass of 48kg and / or body fat of 22% were needed to trigger a change in metabolic rate that leads to menarche.
Moisan J et al., 1990 (26)	Longitudinal	White	640	9.5-12.5	Height (cm), Weight (kg) and BMI (kg/m ²)	Age (yr)	Taller and heavier girls reached menarche earlier.
Maclure et al., 1991 (27)	Longitudinal (4 yr)	White	194	9-10	Height (cm), Weight (kg) and BMI (kg/m ²)	None	Taller and heavier girls reached menarche early. Did not adjust for physical activity.
Petridou E et al., 1996 (41)	Cross sectional	White	345	9-16	Height (cm) and BMI (kg/m ²)	Physical activity (inactive, average, active, very active) and energy intake (kcal)	Taller and heavier girls reached menarche earlier.
Koprowski et al., 1999 (39)	Longitudinal (3 yr)	White, Hispanic, Asian/Pacific, African American	679	8-13	Height (cm) and BMI (kg/m ²)	Age (yr) and ancestry	Taller and heavier girls reached menarche earlier. Asian/Pacific Islander girls reached menarche earlier than others. Physical activity not measured.
Lassex et al., 2007 (43)	Cross sectional (NHANES III)	Multi ethnic	69	10-14	Hip and waist circumference (cm), skinfold thickness (cm)	None	The higher the hip and thigh circumference, the earlier the menarche. The higher the waist circumference, the later the menarche.
Heys M et al., 2007 (42)	Cross sectional	Asian	7349	50-94	Waist circumference (cm)	Age (yr), highest educational attainment and number of pregnancies.	Age of menarche before 12.5 years was associated with the presence of central obesity (waist circumference ≥ 88cm)
Sherar LB, 2007 (38)	Longitudinal (7 yr)	White	61	8-15	Weight (kg), DXA Total body fat (%) and body fat (kg) and	None	Average weight at menarche (49.1 kg) in this sample was not significantly greater than 48 kg (Frisch, 1987). Average %BF at menarche (26.7%) was significantly greater than 22% (Frisch, 1987).

DXA: Dual Energy X ray Absorptiometry

1.5.3 Age at menarche (AAM) and diet

As mentioned earlier, a trend for earlier timing (age) of menarche in several countries has been assumed to be related to improved health and nutrition (32). Various anthropometric, socio-economic, familial, nutritional and lifestyle predictors have been studied in relation to AAM (46-48). The most consistent finding has been an inverse association of weight and height with AAM (studies listed in Table 1.2). Available studies are less clear regarding the relationship with other factors, particularly dietary factors. Results from some of the studies (on Asian and White population since 1990) examining the relationship between AAM and diet are listed in Table 1.2.

One study reported that high dietary energy intake delayed AAM significantly in white girls adjusting for physical activity (41) and another study also reported the same association but did not measure physical activity (39). Three studies did not show any association between AAM and dietary energy intake (27, 40, 49) and these three studies did not adjust for physical activity.

The studies by Moisan et al., (26) and Maclure et al., (27) were longitudinal in design and food intake data were collected before menses. Moisan et al. collected three-day diet records, Maclure et al. collected two semi quantitative food frequency questionnaires (FFQ) four years apart and Koo et al., (40) collected semi-quantitative FFQ at baseline. Another longitudinal study by Koprowski et al., (39), used a semi quantitative FFQ collected at baseline, which was at age 10-11 yr old, to predict AAM. The validity of the FFQ was not reported. An inverse association between dietary energy intake and AAM

was found only in Hispanics. This association in Hispanics could be due to under-reporting of total energy intake among girls with highest BMI. Under-reporting of dietary intake by overweight or obese subjects has been documented previously (50-53). Also, socio-economic and hereditary factors that may influence AAM were not considered in the analysis. Other issues are that the time lag between initial assessment of dietary intake and menarche ranged from <1 wk to 3.2yr; it is also critical to measure early childhood diet and diet at least six months prior to menarche, because of expected changes in girl's diets with maturation (39). Study by Petridou et al., (41) reported that high energy intake and moderate physical activity associated with reaching menarche late based on a cross-sectional analysis, which might not be appropriate since exposures, like diet, change over time and introduce information bias (eg. diet and AAM are collected at a single point in time in the study).

Limitations of dietary records are that they tend to underestimate current food consumption (54), while FFQ overestimates food consumption (55) and is specific to a population. Non-differential misclassification may occur, which can attenuate observed associations (i.e., bias the risk estimates towards the null). Since an adolescent girl's diet changes from year to year, studies should consider doing repeated dietary assessments. Studies did not address the possible effect of diet that occurs earlier in childhood or in utero. Socio-economic status was not adjusted for in the models. Associations are likely to be biased towards the null because any association would have been underestimated because of random error in reporting AAM and diet. Additionally, it is possible that girls remember reaching regular cycles as their AAM, rather than remembering the first notice

of menstrual bleeding. Possibly regular cycling is more associated with body habitus than the first menstrual bleeding; but the latter is generally assumed to be more memorable.

Studies also have shown association of AAM with other dietary factors. An association found where AAM was associated with Vitamin A could be due to chance because the study (27) tested many hypotheses. On the other hand, Vitamin A may be a marker for fruit and vegetable intake (56), which may be protective for early AAM. Also a weak positive association found with total dietary fat may be confounded by physical activity, which was not adjusted for in the statistical models.

An association between higher dietary fiber intake (adjusted for energy intake) with girls reaching menarche later, was shown by Koo et al., (40). Increased dietary fiber intake may reduce the availability of circulating estrogen and thereby influence pubertal development and menarche. The use of a prospective design and annual follow-up of the cohort in Koo's study reduced the possibility of recall bias. The completeness of the follow-up and adjustment for a wide range of potentially relevant confounding variables also strengthen their results. In addition, their study is the first one to focus on components of dietary fiber (soluble and insoluble). A major limitation of this study was that the length of the follow-up period was relatively short. Of the 589 subjects included in the analyses, 402 of them had not reached menarche at the end of the follow-up. Reducing the number of censored observations by a longer follow-up period could increase study power and minimize the possibility of follow-up bias arising from insufficient duration of follow-up (57).

In conclusion, the findings are inconsistent with the hypothesis that differences in pre-menarcheal dietary intake or utilization can influence menarche. Differences in study design and methodology make comparisons of studies difficult. The results of association between higher dietary energy intake and AAM are conflicting, and also unable to support a hypothesis of an independent role of dietary macronutrients. The lack of control for physical activity and other factors may confound the relationship. Thus, it is not clear how variation in dietary patterns influences AAM. Ethnicity, socio-economic status, physical activity, hormonal changes, and genotype variations need to be considered in future studies.

Table 1.2 Studies examining Age at menarche (AAM) and diet (Outcome variable is self reported age at menarche for all the studies listed)

Author, year	Study Design (follow-up yr)	Ancestry	N	Age (yr)	Independent variables	Potential confounders adjusted	Results related to AAM
Petridou et al., 1996 (41)	Cross sectional	White	345	9-16	Energy intake and macronutrients adjusted for energy.	Height, weight, BMI, Daily inactive period, Playing pattern.	Girls with high energy intake and moderate physical activity reached menarche late.
Moisan et al., 1990 (26)	Longitudinal but the data analyzed as a Nested Case control	White	640	9.5-12.5	Energy intake and other nutrients adjusted for energy.	Age, and weight.	Girls with high mono-unsaturated fat intake reached menarche early. Girls with high Vit A intake reached menarche late. Energy intake was not associated with menarche.
Maclure et al., 1991 (27)	Longitudinal (4 yr)	White	194	9-10	Energy intake and other nutrients adjusted for energy.	Age, height and BMI.	Girls with high Vit A, B-12, C, thiamine and omega 3 fatty acid intake reached menarche early. Energy intake was not associated with menarche.
Koo et al., 2001 (40)	Longitudinal (3 yr)	White	187	6-14	Energy intake and other nutrients adjusted for energy.	Age, weight, maternal age at menarche	Energy intake not associated with age at menarche. Energy adjusted fiber intake associated with reaching age at menarche late.
Koprowski et al., 1999 (39)	Longitudinal (3 yr)	White, Hispanic, Asian/Pacific, African American	679	8-13	Energy intake and macronutrients adjusted for energy.	Age, ethnicity, height, BMI.	White girls with high intake of total energy reached menarche late. Hispanic girls with high intake of energy reached menarche early. Carbohydrates, protein and fat intake were not associated with age at menarche. Asian/Pacific Islanders reached menarche earlier than others.

1.6 Leptin hormone

Leptin, the hormone encoded by the obesity (*ob*) gene or leptin gene (*LEP*), is a 146 amino acid protein with structure similar to that of cytokines (58) and a mass of ~16kD. Leptin is principally secreted by adipose tissue and its concentration in the blood is highly correlated with the amount of adipose tissue in the body. Smaller amounts of leptin are also secreted by cells in the epithelium of the stomach and in the placenta. The main physiological pool of leptin is in the blood, where it circulates in two forms: free and bound to soluble leptin receptors (59, 60). Accordingly, leptin clearance from the blood is described by the two-pool model. The free form is rapidly removed from serum with a half-life of 3-4 min, while the bound form is retained in blood for a much longer period (half-life - 71 min) (61). It has been calculated that ~80% of total body leptin clearance can be attributed to the kidney (62, 63). The balance between free and bound leptin is a potential regulator of leptin bioavailability (60, 64). Leptin expression and release were shown to depend on adipocyte size in humans (65, 66).

Leptin plays an important role in the regulation of energy expenditure, body fat homeostasis, and in the reproductive processes (67-69). Leptin acts by binding to specific leptin receptors in the brain and activating the Janus protein-tyrosine kinase and Signal Transducers and Activators of Transcription system (JAK-STAT system), which results in altered expression of many hypothalamic neuropeptides (67). Altered expression of neuropeptide Y (NPY), and possibly other neuropeptides, by leptin results in changes of energy homeostasis and activation of several neuroendocrines axes, including the hypothalamic-pituitary-gonadal axis (HPG axis).

Leptin has recently been shown to be of critical importance for normal function of the female reproductive system in rodents (70). Since body weight and adiposity appear to play a critical role in the timing of puberty in humans and rodents, and leptin levels rise with increasing adiposity, Ahima et al. studied the effects of once daily injections of recombinant leptin on the onset of puberty in female mice weaned on day 21 and fed ad libitum. There was a linear increase in body weight during the study period, which was not altered by the dose of leptin used. Mice injected with leptin had an earlier onset of three classic pubertal parameters (i.e., vaginal opening, estrus, and cycling) compared with saline-injected controls. Leptin is the first peripheral molecule demonstrated to accelerate the maturation of the reproductive axis in normal rodents. Based on the above findings, Ahima et al. proposed that leptin is the signal that informs the brain that energy stores are sufficient to support the high energy demands of reproduction, and may be a major determinant of the timing of puberty.

1.6.1 Central role of leptin in hypothalamic and pituitary regulation of gonadotropin secretion

In the hypothalamus, leptin accelerates gonadotropin-releasing hormone (GnRH) pulsatility, but not pulse amplitude in arcuate hypothalamic neurons in a dose-dependent manner (71). It is believed that leptin may facilitate GnRH secretion predominantly via indirect mechanisms (Fig 1.1), acting through interneurons secreting neuropeptides, such as cocaine and amphetamine regulated transcript peptide (CART) (72, 73), galanin-like peptide (74), and/or melanocortin-concentrating hormone (MCH) in the hypothalamic zona incerta (75). In addition, leptin may increase release of nitric oxide (NO) from

adrenergic interneurons, which then induces GnRH release from GnRH neurons by activating both guanylate cyclase and cyclooxygenase 1 (76).

Other downstream effectors of leptin are important in the control of feeding, but their influence on puberty is less well defined. Leptin administration has been shown to decrease the expression of neuropeptide Y (NPY) in the arcuate nucleus, and consequently to remove the inhibitory action of NPY on GnRh release (77).

In addition to the stimulatory effect on the HPG axis at the hypothalamic level, leptin has direct effects on the anterior pituitary as well (78) (Fig 1.1). In the anterior pituitary, leptin may directly stimulate the release of luteinizing hormone (LH) and, to a lesser extent, follicle-stimulating hormone (FSH) via nitric oxide synthase activation in the gonadotropes (79).

1.6.2 Role of leptin in tissues of the body periphery

It is possible that the major site of action of leptin may differ depending on the concentration of leptin in the blood. The direct action of leptin on the ovary may be of importance under certain conditions with elevated concentrations of leptin in the blood, such as with obesity. It was recently shown that the cerebrospinal fluid/plasma leptin ratio is lower in obese compared to lean subjects, and it was suggested that this was due to a reduced efficiency of the transport of leptin from plasma to cerebrospinal fluid (80, 81). This creates a situation where peripheral tissues may be exposed to very high leptin concentrations while the central nervous system is exposed to only moderately increased

levels of leptin. Conversely, leptin action at the hypothalamic level may be of relatively greater importance during conditions with low concentrations of leptin in the blood, as in women with low BMI and in *ob/ob* mice (82, 83).

Leptin's role at the periphery: gonadal organs

Leptin has been found to exert equally important effects on gonadal organs (Fig 1.1). The effects of leptin on gonads are implied by the expression of leptin receptors on the surface of ovarian follicular cells, including granulosa, theca and interstitial cells (84) as well as Leydig cells (85).

It is not known whether the effects of leptin on the reproductive system are exerted at the ovarian or pituitary/hypothalamic level or at both levels. The presence of leptin in follicular fluid and the absence of *ob* gene expression in the ovary indicate that leptin acts in an endocrine fashion on the ovary. Interestingly, leptin inhibits the production of estradiol, but not that of progesterone, in cultured rat granulosa cells (86), suggesting that it may promote a steroid microenvironment in the follicle similar to that present in polycystic ovary syndrome (PCOS) (87). Based on the tissue distribution of leptin receptors (84, 88-90), it is possible that leptin acts on the reproductive system both at the hypothalamic-pituitary -level (GnRH-FSH/LH) (82, 87) and directly on the ovary (84, 86).

Caprio et al. (1999) (85) demonstrated that a functional leptin receptor is expressed in rat Leydig cells and a mouse tumoral Leydig cell line, and have shown that leptin can exert

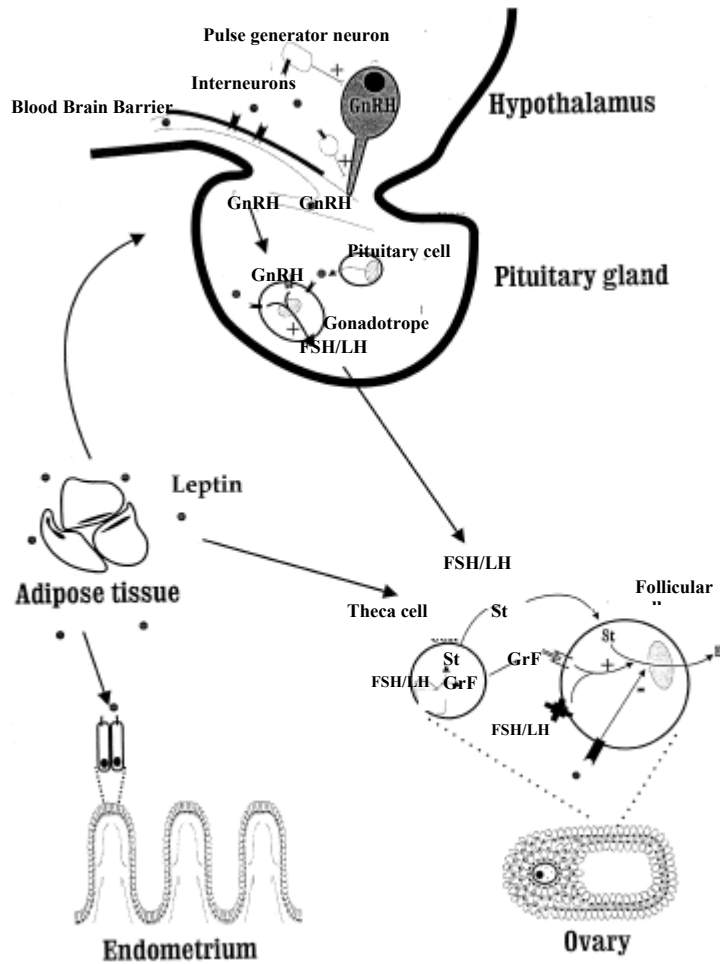
biological effects (leptin-induced inhibition of human chorionic gonadotropin (hCG) stimulated testosterone production) on rodent Leydig cells.

Leptin's role at the periphery: endometrium

Recent studies have shown that leptin receptors are expressed in the glandular and luminal tissues of endometrium throughout the menstrual cycle (91). Low leptin receptors seen during the early proliferative phase are followed by a gradual increase and peak in the early secretory phase of the menstrual cycle, which implies that leptin receptors may be regulated by ovarian steroids.

The relationship between leptin hormone and age at menarche (AAM) is discussed further in section 1.6.6.

Figure 1.1 Schematic diagram illustrating the interaction of leptin with the hypothalamic-pituitary-gonadal axis and endometrium.



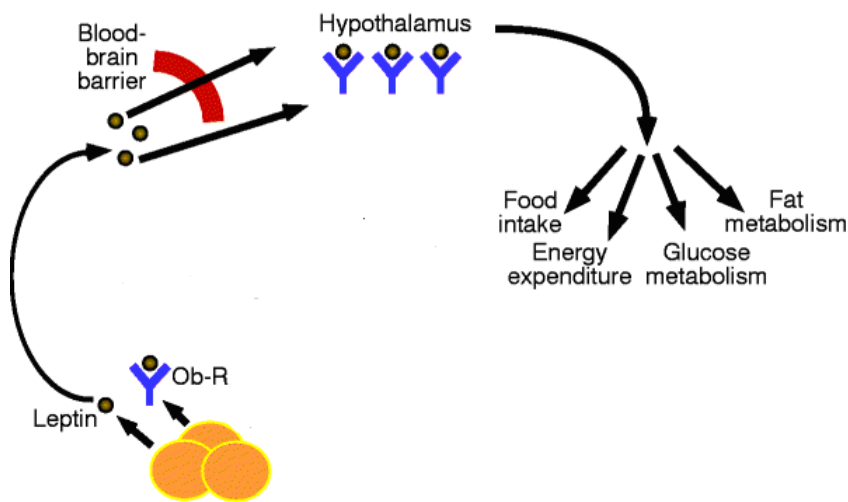
GnRH: Gonadotropin-releasing hormone; FSH: Follicular stimulating hormone; LH: Luteinizing hormone; St: Steroid precursors; GrF: Growth Factors; E: Estrogens

(Adapted from Moschos, Chan, & Mantzoros, 2002. Fig 1 (92))

1.6.3 Leptin in the regulation of energy balance

Leptin, secreted from adipocyte (Fig 1.2), signals the size of the fat stores to the brain and is also the main hormone of the 'tonic homeostatic regulatory system' of energy balance. Leptin is the afferent signal in a negative feedback loop that maintains constancy of adipose tissue mass. Increased leptin (from weight gain) results in negative energy balance (energy expenditure > food intake), whereas decreased levels of leptin (as in starvation) lead to positive energy balance (food intake > energy expenditure). Leptin acts through hypothalamus to decrease food intake and modulate glucose and fat metabolism.

Figure 1.2 Adipocyte leptin and the regulation of adipose tissue mass.



Ob-R: Obesity receptor (leptin receptor)

(Adapted from Jeffrey & Jeffrey, 1998, Fig 1(93))

1.6.4 Leptin hormone and sexual dimorphism

Even after correcting for body weight and fat mass, women have higher serum leptin levels than men (51, 94, 95). This sexual dimorphism in serum leptin concentrations has been associated with, or is causally related to, a number of factors. First, the pulse amplitude, but not the pulse frequency, of leptin secretion from adipose tissue is twofold to threefold higher in females than in males (96). Second, fat mass is increased in females, and there is differential fat distribution with a higher subcutaneous/visceral fat ratio in women than in men. Leptin mRNA expression is known to be higher in subcutaneous than the visceral fat depots (97, 98). Third, women have higher total serum leptin levels but lower leptin-binding protein levels than men, indicating higher free leptin levels (99). Finally, female adipose tissue may be more sensitive to hormones (i.e., insulin and glucocorticoids) or other substances that stimulate leptin production. It is known that sex steroids such as estrogens increase leptin levels (100), whereas androgens decrease leptin levels (101).

It has been reported that the sexual dimorphism of serum leptin levels may be present at birth (102-104) or even in utero during late gestation, but relevant data have not been consistent (105, 106). This has been attributed to the relatively elevated levels of sex steroids in neonates. Serum leptin levels are negatively correlated with testosterone levels (107), with male neonates having lower leptin levels in cord blood than females. Sexual dimorphism in circulating leptin concentrations is not observed during childhood or early puberty, but is evident in later puberty, suggesting a relationship between leptin and gonadal steroids, body fat mass, body mass index (BMI) and other anthropometric

parameters (108-115). Whether studied longitudinally from prepuberty to late puberty or cross-sectionally at different ages, serum leptin levels in boys appear to peak just before puberty or in early Tanner stages, followed by a decrease to baseline levels as testosterone levels rise (109-111, 115, 116). Girls, on the other hand, display a steady rise in leptin levels throughout puberty (109-111, 115).

The mean serum leptin concentration in girls increases from Tanner I to V with no statistically significant difference between them ($P = 0.068$, Kruskal-Wallis), as suggested by the weak positive correlation between leptin and pubertal Tanner stage ($r = 0.28$, $P < 0.094$) (117). In contrast, in boys, circulating leptin levels decrease from Tanner I to V ($r = -0.45$, $P < 0.0001$); although the mean values for each Tanner stage did not show significant differences ($P = 0.143$, Kruskal-Wallis test) (117).

Although leptin levels maintain a significant correlation with fat mass at all pubertal stages, this divergence of leptin levels between genders that occurs throughout puberty leads to significantly higher leptin levels, normalized to fat mass, in females during late puberty (115); this persists into adulthood.

Also, leptin-binding activity (leptin binding to soluble leptin receptor) has been found to be low at birth, high during prepubertal stages, and then to fall through puberty, raising the possibility that the fall in leptin-binding activity may lead to increased free leptin levels and, therefore, increased leptin activity (118).

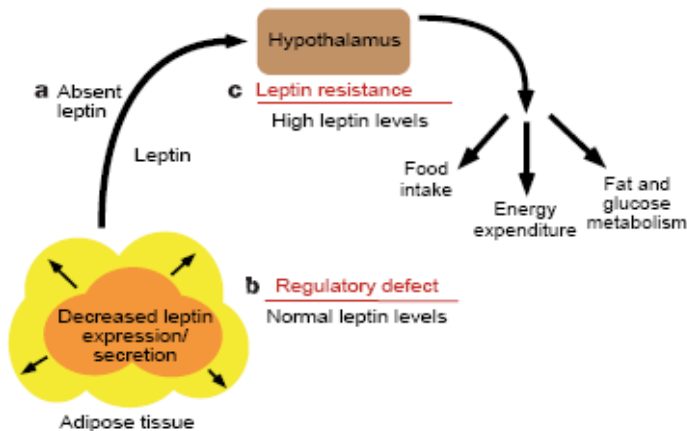
To what extent the prepubertal rise of serum leptin levels results in activation of the HPG axis and in initiation of sex steroid secretion is currently unknown because evidence is available only from observational studies. However, the prepubertal peak of serum leptin levels precedes the rise in free testosterone, GH, and IGF-I in boys who are followed longitudinally through puberty (119). The suppressive effect of testosterone on leptin production (120) may account for the decrease in serum leptin levels in boys after the initial prepubertal rise (116), whereas the rise of estrogen in girls may explain their higher serum leptin levels during the late stages of puberty (111).

1.6.5 Leptin hormone and body fat

Pathogenesis of obesity

There are three general ways in which alterations of the leptin regulatory loop could lead to obesity. a) Failure to produce leptin, as occurs in *ob/ob* mice, would result in obesity. b) Inappropriately low leptin secretion for a given fat mass so that the fat mass would expand until 'normal' leptin levels are reached, resulting in obesity (Fig 1.3). c) Finally, obesity could result from relative or absolute insensitivity to leptin at its site of action. Such resistance would be associated with increased circulating leptin, analogous to the increased insulin levels seen with insulin-resistant diabetes. In general, high plasma leptin levels are evident in obese rodents and humans. In a subset of cases, obesity is associated with normal levels of leptin. Differences in leptin production and leptin sensitivity could be the result of genetic, environmental and psychological factors and their interaction.

Figure 1.3 Pathogenesis of obesity



(Adapted from Jeffrey & Jeffrey, 1998, Fig 4 (93))

A number of studies have addressed the association of leptin with body fat measures but there are differences among these studies in the conclusions reached (Tables 1.3 & 1.4).

In such situations, systematic review of studies can be useful for assessing the likelihood and magnitude of association between leptin and body fat measures. A PUBMED search was performed from 1997 up through 2009 following the STROBE (121) checklist of items that should be addressed while doing a systematic review. The longitudinal and cross sectional studies in the systematic review are listed in Table 1.3 and 1.4

respectively, with an age range of 6 to 74 years, and included both males and females.

Longitudinal and cross-sectional studies with leptin measured from blood (as ng/ml) were included. No criteria were used for measuring the outcome variable (body fat) since there were too few studies using the same method. Fleish et al. (122) measured both change in BMI and DXA body fat as outcome variables, Johnson et al. (123) measured change in DXA body fat, Hodge et al. (124) measured change in BMI and Ahmed et al. (112) measured percent body fat using skinfold measures, Peltz et al. (125) measured percent body fat, Hu et al. (126) measured BMI and Demarath et al. (113) measured body fat by

the underwater weighing method. Studies were not considered for meta-analysis since they did not provide any estimates and standard errors (127, 128).

Longitudinal studies

Most of the studies were conducted on whites and African-Americans, except for the study by Hodge et al. which was on Asians and found no association between leptin and body fat. The study duration ranged from 2.5 to 8 years.

Three studies (122-124, 127) reported a significant positive association between log leptin and change in body fat measures, but the study by Ahmed et al. (112) reported a negative association of leptin and percent body fat as measured by skinfold thickness (Table 1.3).

Cross sectional studies

All cross sectional studies reported a positive association between leptin and body fat measures, irrespective of different methods being used to measure body fat variables. All except two studies were on subjects of white ethnicity (Table 1.4).

Limitations of the systematic review are as follows:

1. Leptin hormone is a recently discovered hormone found in the 1990's and there have been few longitudinal studies conducted on leptin and body fat, with follow-up. In the review there were three studies with 8 years of follow-up, two with 5 years of follow up and one with 2.5 years of follow up.

2. Measurement of the exposure variable was not consistent among these studies. The most recent studies have measured body fat using DXA; older studies have used BMI and skinfold thickness.
3. Even though publication bias cannot be ruled out completely, studies with low sample sizes and studies with negative or no association were published.
4. Studies were mostly conducted on whites and African Americans, although there were 2 studies on Asians and one on Mexicans.
5. The age groups studied varied substantially; hence it is difficult to generalize the findings to say that the association between body fat and leptin is consistent among adolescents.

Conclusion:

Obesity and early age at menarche are increasing health problems, not only in the industrialized western countries but, also in the developing countries. Recent progress in understanding leptin hormone and body fat measures may provide potential new targets to combat obesity. The literature review suggests that more longitudinal studies are needed with a longer follow-up, and among diverse ethnic populations. A consistent way of measuring and reporting the exposure variables is needed. Measuring body fat using new and standard technology, like DXA, is preferred to BMI or skinfold thickness.

Table 1.3 Longitudinal Studies included in the systematic review of leptin and body fat

Author (yr)	Follow-up (yr)	Ancestry	Age (yr)	N	Gender	Independent variable: Leptin level (ng/ml)	Dependent variable: Body fat	Estimates for body fat	SE	P
Hodge et al. 1997 (124)	5	Asian	25 to 74	1314	Male	Leptin (3 categories)	BMI (kg/m ²) change	0.003	0.052	0.96
				1574	Female	Leptin (3 categories)	BMI (kg/m ²) change	-0.039	0.058	0.5
Ahmed et al. 1999 (112)	8	Wht	8 to 16	20	Male	Leptin	Body fat % change (Skinfolds)	-1.04	4.11	0.81
				20	Female	Leptin	Body fat % change (Skinfolds)	-12.28	3.46	0.005
Johnson et al. 2001 (123)	5	Wht, AA	7.97 to 8.25	85	Male/Female	Leptin	DXA Fat mass (kg) change	0.154	0.037	<0.0001
Savoie et al. 2002 (127)	2.5	Wht, AA	7 to 18	33	Male	Leptin	BMI Z (kg/m ²) change	0.004	-	0.37
				35	Female	Leptin	BMI Z (kg/m ²) change	0.014	-	0.006
Fleish et al. 2007 (122)	8	Wht, AA, Oth	6 to 12	149	Male/Female	Log Leptin	Log BMI change	0.0667	0.0271	0.0001
						Log Leptin	Log DXA Fat mass (kg) change	0.1423	0.041	0.0007

Wht: White; Oth: Other; AA: African American

Table 1.4 Cross sectional studies included in the systematic review of leptin and body fat

Author (yr)	Ancestry	Age (yr)	N	Gender	Independent variable: Body fat	Dependent variable: Leptin level (ng/ml)	Estimates for outcome variable	SE	p
Hu et al. 2001 (126)	Asians	20 to 74	148	Male	BMI (kg/m ²)	Leptin	0.15	0.04	<0.01
			146	Female	BMI (kg/m ²)	Leptin	0.54	0.11	<0.01
Demarath et al. 1999 (113)	Wht	prepubertal	28	Male	Body fat (kg) (underwater weighting)	Leptin	1.1	0.19	<0.0001
		postpubertal	33	Male	Body fat (kg) (underwater weighting)	Leptin	1.19	0.05	<0.0001
		prepubertal	30	Female	Body fat(kg) (underwater weighting)	Leptin	1.24	0.22	<0.0001
		postpubertal	27	Female	Body fat (kg) (underwater weighting)	Leptin	1.38	0.18	<0.0001
Tanaka et al. 2005 (128)	Austro, Non-Austro	32 to 35	37 pairs	Both	% Body fat using SF	Log Leptin	0.58	-	0
Peltz et al. 2006 (125)	Mexican-Americans	18 to 30	110	Male	% Body fat using BI	Log Leptin	0.1	0.012	<0.001
			242	Female	% Body fat using BI	Log Leptin	0.03	0.009	<0.001

Wht: White; Oth: Other; AA: African American; SF: Skin fold thickness; BI: Bioelectrical impedance

1.6.6 Leptin hormone and its relationship with body fat and puberty

Animal studies have indicated that leptin plays an important role in initiating puberty (70, 82). Fasting mice develop both low leptin levels and hypogonadotrophic hypogonadism, which is reversed by leptin injection. The female *ob/ob* mouse is both overweight and sterile. Thinning of *ob/ob* mice by restricting their diet does not restore fertility, but injection of leptin into these mice allows them to ovulate, become pregnant, and have offspring (129). These observations suggest that leptin is the signal that informs the brain that energy stores are sufficient to support the high energy demands of reproduction, and may be a major determinant of the timing of puberty (70).

There is also evidence for a major role for leptin in puberty in humans (109, 130). As in mice, fasting in humans also results in low leptin levels and delayed puberty. Several studies, both cross-sectional and longitudinal, have shown a marked rise in serum leptin concentrations in young girls starting as early as age 7 years and continuing as they progress through puberty, at least until age 15 years (110, 112). These changes in leptin levels are paralleled by increasing body fat during female puberty. In at least one cross-sectional study, the rise in serum leptin was well established two years before clear increases in serum luteinizing hormone (LH) and estradiol levels were observed (111). This is consistent with the hypothesis that higher leptin level is one of the factors that is critical in allowing puberty to progress, rather than a result of the hormonal increases of puberty.

If the relationship between body fat and earlier menarche in humans is mediated by leptin, then one would predict that leptin levels would be related to age at menarche. This was examined in a study of 343 healthy, white girls from central Ohio who were recruited at Tanner stage 2 of puberty between the ages of 8.3 and 13.1 years (131). Menstrual history, height and weight, body composition by DXA, and leptin by radio immuno assay (RIA) were measured every six to 12 months during a 4-year period. As expected, leptin was highly correlated with body fat mass ($r=0.81$). Higher leptin levels, up to a level of 12 ng/mL, were associated with a decline in the AAM by approximately 1 month per 1-ng/mL increase in leptin. In addition, the group of girls who remained premenarcheal for the entire four years of the study had significantly lower leptin levels than the groups of girls who reached menarche during the study.

Lassek & Gaulin (43) reported that in young women with completed growth, age at menarche was related to body fat distribution. In this study, age at menarche was negatively related to waist circumference, stature, and biiliac breadth; and blood leptin levels were more strongly related to gluteofemoral fat than upper- body fat, suggesting that leptin may convey information about body fat distribution during puberty. Because the data used in this study are cross-sectional, the relationship between body fat distribution and the odds of menarche should be interpreted with caution. Subcutaneous gluteal fat contains more leptin mRNA than does abdominal fat (visceral fat) (132). In a multivariate analysis, adjusting for total body fat, hip circumference was a significant positive predictor of blood leptin levels, while waist circumference was not (43, 133). Hip circumference explained 36% of the variance in blood leptin levels, while total body

fat explained an additional 2%, and waist circumference was not related (133). In another study of a group of female anorectic patients and low-weight women, including restrained eaters, a leptin threshold of 1.85 ng/l was defined, below which menstrual cycles were disrupted (134). As discussed in section 1.6, mice injected with leptin had an earlier onset of three classic pubertal parameters (i.e., vaginal opening, estrus, and menstrual cycling) compared with saline-injected controls (70) suggesting that leptin is the signal that informs the brain that energy stores are sufficient to support the high energy demands of reproduction, and may be a major determinant of the timing of puberty. In humans, Farooqi (135) showed that administration of recombinant human leptin resulted in the onset of puberty at an appropriate developmental age in human congenital leptin deficiency.

In a clinical trial by Farooqi (135), recombinant human leptin therapy in four children (Child A 8yr, Child B 2yr, Child C & D 3yr) with congenital leptin deficiency was undertaken. After 12 months of leptin therapy, Child A had pulsatile secretion of FSH and LH at night consistent with the early stages of puberty. Subsequently Child A went on to progress through the stages of pubertal development, with clinical development of secondary sexual characteristics like growth spurt, behavioral changes associated with pubertal development, enlargement of the ovaries on ultrasound with observation of follicles, and an increase in uterine size. When further followed, Child A later had her first menstrual period and regular menstrual cycles. For Child B, basal LH and FSH concentrations remained in the prepubertal range after 12 months of treatment. Similarly, there has been no evidence of premature pubertal development in Children C and D (age

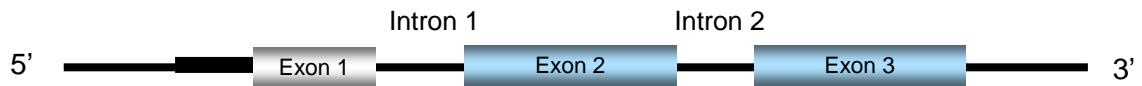
4 and 6 years). These studies in human congenital leptin deficiency suggest that leptin is permissive for the onset of puberty in humans, but only at an appropriate developmental stage.

Summary

Animal experiments and human observational studies suggest that the rise in leptin levels may be the earliest known signal of the initiation of puberty and may contribute to the activation of the HPG axis, resulting in increased sex steroid production and subsequent activation of the GH/IGF-I axis. Given the importance of sex steroids, GH/IGF-I, and other hormonal factors in initiating the complex signals for the onset of puberty and growth, leptin appears to be a necessary but not sufficient factor for the initiation of puberty.

1.7 Leptin gene (*LEP*)

Figure 1.4 *LEP* gene



In the 1970s, studies on lean and obese mice strongly suggested that circulatory factors can influence bodyweight, but these remained unidentified until the *LEP* gene (also known as *ob* gene) was isolated by positional cloning in 1994. The *LEP* gene encodes the leptin hormone (58). *LEP* gene has been localized in humans on the 7q31.3 chromosome and consists of 3 exons separated by 2 introns (136). Leptin mRNA levels and leptin secretion rates are three- to fivefold higher in subcutaneous than omental adipose tissue(137), which may be related to differences in adipocyte size as omental adipocytes are smaller than subcutaneous adipocytes (98, 138). Leptin mRNA levels and rates of leptin secretion in both subcutaneous and omental adipose tissues are positively correlated with body mass index, and with adipocyte volume (98, 139).

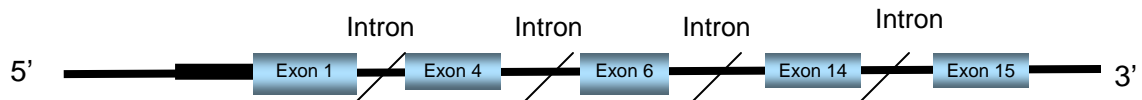
Early studies suggested that the adipocyte was the only source of leptin, but recent work indicates that the leptin gene is also expressed in the placenta (140-142), stomach (143), skeletal muscle (144), and mammary gland (145). It was also shown that leptin gene is expressed in rat hypothalamus, cortex, cerebellum and pituitary gland (146, 147).

In rodents and humans, adipocyte leptin gene expression is regulated by various factors. In adiposites, glucocorticoids (141) increase leptin mRNA, whereas dibutyryl cAMP (dbcAMP) and adrenergic agonists (148) down-regulates leptin gene expression. Insulin

and steroid hormones, such as estradiol, also increase gene expression (69, 149, 150). However, little is known about regulation of the leptin gene in the brain.

1.8 Leptin receptor gene (*LEPR*)

Figure 1.5 *LEPR* gene



The leptin hormone acts by combining to the leptin receptors, which maps in humans to the 1p31 chromosome. Leptin receptors are expressed in the hypothalamus and in most peripheral organs and tissues, such as brain, lung, skeletal muscle, and kidney (137).

They are members of the class I cytokine receptors. Leptin exerts its action after binding to leptin receptors in the brain, activating the Janus protein-tyrosine kinase and Signal Transducers and Activators of Transcription system (JAK-STAT), which results in altered expression of many hypothalamic neuropeptides (67).

At least five isoforms of leptin receptor (*Ob-R*) exist, primarily because of alternate splicing. A protein isoform is any of several different forms of the same protein arising from the same gene by alternative splicing. *LEPR* isoforms are divided into three types: short form, long form and soluble form. The short and long isoforms contain identical extracellular and transmembrane domains and differ in the length of the intracellular amino acid sequence (88, 151-155). Experiments have shown that only the long isoform of the leptin receptor has signaling capacity (156). Karlsson et al. (84) and occurs

predominantly in hypothalamic nuclei (157). The short form, expressed ubiquitously in human tissue, is less clear. The soluble leptin receptor (sOB-R) is an extracellular domain, which is mostly secreted into the blood stream by the liver (153), and which binds with circulating leptin and regulates the concentration of free leptin (154). This soluble receptor isoform could modulate steady-state leptin levels by complexing free leptin in the circulation, preventing the hormone from degradation and clearance (158). Therefore, high sOB-R levels in the blood, as determined in lean humans (159), may act as a potential reservoir of bioactive leptin.

High leptin levels could down-regulate leptin receptors, since expression (mRNA) of the long and short isoforms of the leptin receptor are markedly reduced in the hypothalamus and liver of obese rats, which have enhanced plasma leptin concentration (160). *LEPR* expression appears to be reduced by testosterone in Leydig cells (161), while estradiol administration increases the *LEPR* expression in ovariectomized rats.

Soluble leptin receptor (sOB-R) was measured using ligand-immunofunctional assay in a study by Kratzsch et al. (162), during childhood, puberty and adolescence in a cohort of 581 healthy children and adolescents. During childhood and adolescence, a serum sOB-R level showed a characteristic decrease, independently of gender, but was significantly inversely related to age, stage of puberty, and IGF-I level. High levels of sOB-R in emaciation mirror a compensatory up-regulation of the sOB-R that may lead to a suppression of leptin action (162).

1.9 Single Nucleotide Polymorphisms (SNPs)

Human single nucleotide polymorphisms (SNPs) represent the most frequent type of human population DNA variation. One of the main goals of SNP research is to understand the genetics of the human phenotype variation and especially the genetic basis of complex human diseases. It is estimated that around 90% of human genetic variations are differences in single bases of DNA, known as single nucleotide polymorphisms (SNPs) (163). Single nucleotide polymorphisms are DNA sequence variations that occur when a single nucleotide (A,T,C,or G) in the genome sequence is altered. For a variation (polymorphism) to be considered a SNP, it must occur in at least 1% of the population. Among them, non-synonymous single nucleotide polymorphisms (nsSNPs), also known as single amino acid polymorphism (SAPs), that cause amino acid changes in proteins have the potential to affect both protein structure and protein function (164). A synonymous substitution (also called a *silent* substitution) is the substitution of one base for another in an exon of a gene coding for a protein, such that the amino acid sequence produced is not modified.

SNPs can occur in coding (gene) and noncoding regions of the genome. Rare mutations in the genes encoding leptin (165) and the leptin receptor (166) result in obesity syndromes and hypogonadotropic hypogonadism. Reports of humans with mutations in the *LEPR* (166) or the *LEP* gene (165, 167) who fail to undergo puberty provide important evidence for the central role of leptin in facilitating human pubertal development. In humans, several polymorphisms have been identified in the *LEP* and *LEPR* genes.

The literature and searched public databases (Haplotype Map, also known as HapMap) were surveyed for SNPs that have been identified in the *LEP* and *LEPR* genes. The International HapMap Project is a multi-country effort to identify and catalog genetic similarities and differences in human beings. Public and private organizations in six countries are participating in the International HapMap Project. (Japan, the United Kingdom, Canada, China, Nigeria, and the United States). The goal of the International HapMap Project is to compare the genetic sequences of different individuals to identify chromosomal regions where genetic variants are shared. In the initial phase of the Project, genetic data were being gathered from individuals of African, Asian, and European ancestry.

SNPs were selected for the present study based on:

1. Functional SNPs (SNPs altering gene function or expression) known to affect plasma leptin levels – a focus of this dissertation
2. Allele frequencies of the SNPs reported to be present in Asians and Whites – the ancestral groups in the Female Adolescent Maturation (FAM) study population of this dissertation

SNPs identified for inclusion in this study are *LEP* G-2548A, *LEP* A19G, and *LEPR* Q223R (Table 1.5).

Table 1.5 Summary of SNPs that met study criteria

Gene	Nucleotide base change	SNP #	Location
<i>LEP</i> G-2548A	Guanine to Adenine	rs7799039	Promoter region
<i>LEP</i> A19G	Alanine to Glycine change	rs2167270	Untranslated region of Exon 1
<i>LEPR</i> Q223R	Glutamine to Arginine change	rs1137101	Exon 6

1.10 Genetic variants in leptin gene (*LEP*), plasma leptin and body fat

Leptin plays an important role in neuro-endocrine function, body weight and energy homeostasis (168). One of the common polymorphisms, the G-2548A leptin promoter variant, has been shown to be associated with either variation in serum leptin levels or with the degree of obesity in obese and overweight subjects (148, 169-172). However, the reported data are conflicting. Mammés et al (169), found that the less frequent -2548A allele is associated with higher leptin levels at baseline ($p=0.04$), irrespective of BMI, and that the G-2548A polymorphism predicts weight loss in response to a low calorie diet. In a subsequent study (170), the same researchers reported that male, but not female, subjects homozygous for the -2548A allele had higher leptin concentrations, adjusted for fat mass ($p=0.05$). Hoffstedt et al. (171) found that nonobese women with the A/A genotype had higher serum leptin levels, adjusted for BMI ($P=0.03$), and significantly higher adipose tissue leptin mRNA levels ($P=0.01$) than did carriers of the -2548G allele. In another study, Le Stunff et al. (172) also reported evidence of association between this genetic variant and circulating leptin levels in two independent cohorts of obese White girls. In contrast with the previous two studies (170, 171), girls with the A/A genotype

(referred to as -/- genotype) exhibited lower mean leptin levels compared to those with G/A and G/G genotypes (20-25% lower, $P < 0.02$).

The discordant observations in these studies could be attributed to age related and/or gender specific effects of the polymorphism (171, 172). In addition to the potential confounding effect of age and gender, it has also been suggested that fat mass and gender may play a role as effect modifiers (172). Finally, it is also possible that soluble leptin receptor (sOB-R) (the main leptin binding protein in plasma and determinant of free leptin), which was not measured in the previous studies, may also confound or modify the association between leptin and the polymorphism of interest (168).

Yiannakouris et al. (173) studied the associations between the *LEP* G-2548A polymorphism of the leptin gene promoter, not only with total leptin but also with sOB-R and free leptin. This study reported no association between the G-2548A polymorphism and BMI, fat mass or plasma total leptin levels but AA genotype females had higher soluble leptin receptor levels compared to carriers of the G allele. The effect modification by gender shown in this study raises the possibility of an interaction between this polymorphism and an Estrogen Response Element (a short sequence of DNA within the promoter of a gene that is able to bind a specific estrogen receptor complex and therefore regulate transcription) of the leptin gene promoter. The study was not powered for subgroup analysis (for males and females) but the fact that the associations studied in females are of statistical significance provides reassurance regarding the validity.

The above studies cannot exclude the possibility that the polymorphism is in linkage disequilibrium with other regulatory sequences influencing transcription of the leptin gene. Linkage disequilibrium occurs when genotypes at the two loci are not independent of each other or the frequency of each allele within the haplotypes is not the same as the frequency of that allele in the population as a whole. The level of linkage disequilibrium is influenced by a number of factors including genetic linkage if loci on the same chromosome are physically close to one another so the cross over between them are rare, genetic drift or allelic drift is the change in the relative frequency with which a gene variant (allele) occurs in a population due to random sampling), non-random mating, and population structure.

The other *LEP* polymorphism that results due to A to G nucleotide substitution at 19 of the untranslated Exon 1 (leading to amino acid change from Alanin [A] to Glycine [G]) of the *LEP* gene the reported to be associated with lower leptin levels in two White populations (174). Subjects homozygous for the G allele had significantly lower leptin levels compared with AG or AA genotypes among massively obese individuals and in a cohort of randomly chosen white women. However, this study failed to show any association between *LEP* A19G variant and BMI (Table 1.6).

1.11 Genetic variants in leptin receptor gene (*LEPR*), plasma leptin and body fat

A to G substitution changing amino acid glutamine to arginine at codon 223 in Exon 6 is common to all *LEPR* isoforms (175). Subjects carrying the *LEPR* G223R were shown to be associated with enhanced gene expression, increased circulating leptin levels, obesity and prediction of a small percentage of body weight and composition variability, in a Mediterranean population (176). A study by Quinton et al. (177) indicated that the polymorphic variations in the leptin receptor gene may be associated with variation in ligand binding and, as such, inter-individual differences in leptin levels and fat mass may reflect altered functional parameters (expression levels or ligand binding kinetics) of the leptin receptor in white postmenopausal women. Studies with *LEPR* are summarized in Table 1.6.

The association between *LEPR* and serum leptin levels is conflicting (176, 178-181). Potential limitations of these studies include their moderate size. They did not explore associations between body composition variables and polymorphisms separately in subgroups divided by gender and BMI because of the small size of the subgroups. However, they adjusted for gender and BMI (considered as both a dichotomous and a continuous variable) in the statistical analysis. All the previous studies mentioned used BMI, not the DXA measured total body fat, as a measure of overweight/obesity. Finally, they performed detailed statistical analysis using several different genotype models as well as relevant linear regression analysis to assess predictors of percent variability of obesity.

Discrepancies in results may be also attributed to differences in population, with respect to not only genetic background, but also in culture, traditions, climate, type of diet, lifestyle and prevalence of exposure to common environmental risk factors for obesity and related disorders.

1.12 *LEP/LEPR* and Asian population

There are very few studies focused on the association between *LEP* and *LEPR* gene variants, serum leptin and body fat among Asian populations. A study by Wang et al. (182) reported *LEP* -2548 G/G homozygote plays a genetically recessive role in the development of extreme obesity in Taiwanese aborigines but there was no relationship between *LEPR* Q223R variant and obesity. The study did not show any relationship between *LEP* and *LEPR* gene variants and serum leptin levels. The weakness in this study was a sample size limitation of extremely obese patients (n=24), which caused difficulty in analyzing the epistatic effect (when the effects of *LEP* gene is modified by *LEPR* gene) because of its rare frequency. A study by Woo et al. (183) reported the mean leptin concentration was significantly higher in the cases (breast cancer) with genotype G/G than those with genotype A/G of *LEPR* Q223R variant. This case control study had a small sample of 45 pre and post menopausal females.

Table 1.6 Studies examining Genetic variations at *LEP/LEPR* genes, serum leptin (dependent variable) and body fat

Authors (yr)	Study Design	N	Age (yr)	Ethnicity/Sex	Independent variable (polymorphisms)	Potential confounders adjusted	Results
Hagner et al. 1998 (174)	Cross sectional	515	32-56	White males/females	A19G	Sex, BMI	Low serum leptin levels among obese who have G allele. No association between genotypes and BMI.
Le Stunff et al. 2000 (172)	Cross sectional	237	9.5-14.5	White girls	G-2548A	Body mass (bioelectric impedance) and stage of puberty	Low serum leptin levels in girls with A allele. Serum leptin and body fat relationship is significantly dependent on genotype.
Mammes et al. 2000 (170)	Cross sectional	424	36-46	White males/females	G-2548A	Sex, BMI	Overweight, especially men showed a significant trend to carry more G alleles. In men, the G alleles lowered leptin levels.
Yiannakouris et al. 2001 (176)	Cross sectional	118	14-26	White males/females	Q223R	Sex, age	Presence of R allele in the homozygous form is a significant predictor of both BMI and %fat mass. RR genotype also had lower serum leptin levels compared to QQ or QR.
Wauters et al. 2001 (179)	Cross sectional	280	28-56	White females	Q223R	Age	No association with polymorphism and body weight, BMI or fat mass. Postmenopausal women, with Gln223Gln homozygote have more total abdominal fat than Arg223 allele carriers.
Quinton et al. 2001 (177)	Cross sectional	220	62-72	White females	Q223R		Subjects carrying A allele had significantly higher BMI and fat mass than those not carrying the A allele.
Hoffstedt et al. 2002 (171)	Cross sectional	39	25-62	White females	G-2548A	BMI	Serum leptin and leptin secretion were higher in females with A allele.
Yiannakouris et al. 2003 (173)	Cross sectional	118	14-26	White males/females	G-2548A	Age, BMI	No association between this polymorphism and BMI, fat mass or plasma total leptin levels. AA genotype females had higher soluble leptin receptor compared to carriers of the G allele.

1.13 *LEP/LEPR* and Age at Menarche

Although there are number of studies showing an association of leptin levels and AAM, there is only one study (184) showing an association between genetic variations at *LEP* genes and AAM. Based on the role of leptin in initiating or permitting the onset of puberty, it would be anticipated that there would be an association between alleles of the *LEP/LEPR* genes and age at menarche. Since it has also been proposed that the trend for an earlier age of onset of menarche is associated with the trend for increased obesity (185), the fact that leptin is involved in both suggests that the *LEP/LEPR* genes could play a role.

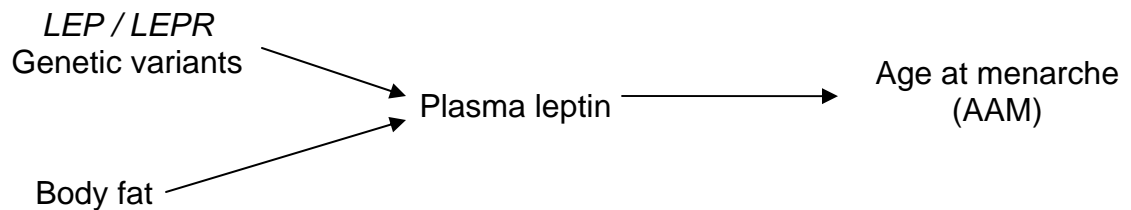
A study by Commings et al. (184), mostly on obese adult females, reported that the variants at the *LEP* gene may have a significant effect on age at menarche. The subjects for this study were obtained from the Loma Linda University (Loma Linda, CA) Center for Health Promotion and were participants in a study of the effectiveness of an anorexic agent in obese women. The age, sex, race, weight, height, waist-hip ratio, age of menarche, and maternal age were determined for each subject. Of the 144 obese subjects, 39 were able to bring a friend who were non- or less obese, for a total of 183 subjects. The BMI for the obese subjects ranged from 24 to 76 with a mean of 38.8 (SD 7.2). The BMI for the “non- or less-obese” subjects ranged from 19 to 35 with a mean of 23.5 (SD 2.8). The mean age at menarche was 12.3y for those with the *LEP*1875<208/<208 genotype (D7S1875 is a dinucleotide repeat), 12.4y for heterozygotes, and 12.1y for those with the *LEP*1875 ≥208/≥208 genotype among white females but was not significant (F ratio = 0.72, P = 0.48). There was a significant ($P < 0.006$) interaction of

*LEP*1875 and maternal age but neither independent variable was significant, by itself, in predicting AAM. This was due to an “association crossover effect” in which the *LEP*1875 by age of menarche effects were in opposite directions for those with a maternal age of <30 years compared to those with a maternal age of >30 years. Possible recall bias for AAM should be noted, although studies have reported a good recall for AAM in adult females (39, 186).

1.14 Goal and Study Aims

Goal: The goal of this dissertation is to examine the inter-relationship of the genetic variations at the *LEP* and *LEPR* genes, plasma leptin, AAM, and body fat in Asian and white adolescent girls in Hawaii (Fig 1.6).

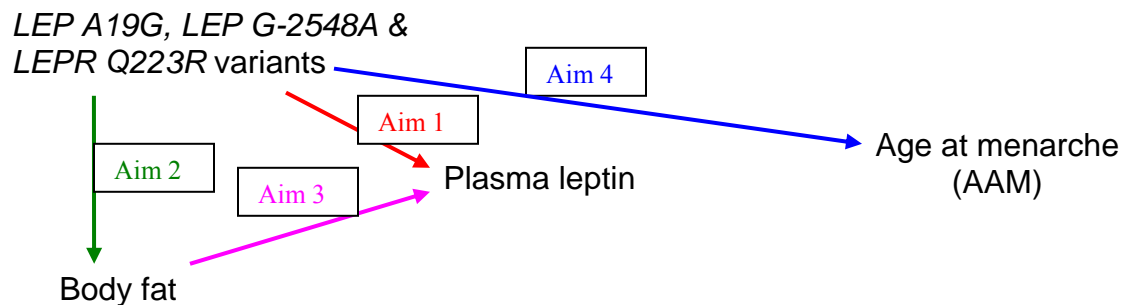
Figure 1.6 Conceptual Framework



Significance: Limited studies have shown association between *LEP/LEPR* genetic variants and AAM, and those were mostly in Whites. Asian and White (and Asian White mix) girls in Hawaii provide a unique group of girls to further explore the relationship of genetic variants, body fat and AAM. Also, this study uses DXA, which is considered a gold standard for body fat assessment, instead of BMI.

This study will help increase our current knowledge of the complex association of how carrying a certain polymorphism can affect the girl's age at reaching menarche, which affects the risk of breast cancer, osteoporosis, and obesity. In the future, gene association studies may provide for tailoring of medical care to an individual's genetic background.

Figure 1.7 Analytical framework



Study aims:

Aim 1: To determine whether selected SNPs in *LEP* and *LEPR* genes are associated with plasma leptin levels in adolescent girls in Hawaii.

Aim 2: To determine whether selected SNPs in *LEP* and *LEPR* genes are associated with total body fat and type of body fat distribution in adolescent girls in Hawaii.

Aim 3: To determine whether total body fat and body fat distribution are associated with plasma leptin levels in adolescent girls in Hawaii.

Aim 4: To determine whether selected SNPs in *LEP* and *LEPR* genes are associated with AAM in adolescent girls in Hawaii.

CHAPTER 2

METHODS

2.1 Introduction

This study is part of the “Female Adolescent Maturation” Studies (FAM Principal Investigator, Dr. Rachel Novotny for Exams 1 and 2; Dr. Loic LeMarchand and Dr. Rachel Novotny were co-PI’s for Exam 3), supported by DOD Grant # BC032028, USDA Grant # 99-00700, NIH/NCRR/RCMI P20 RR119091 award, and a Cancer Research Centre of Hawaii (CRCH) Developmental Funds award. The FAM study was designed to investigate dietary, physical activity, and more recently, hormonal and genetic factors that may influence adolescent growth, development and maturation. These factors have implications for future breast cancer and osteoporosis risk. The goal of this dissertation is to examine the inter-relationships of the genetic variations at the *LEP* and *LEPR* polymorphisms, plasma leptin, age at menarche (AAM), and body fat in these adolescent girls in Hawaii.

2.2 Design

The study consisted of two cross sectional studies (exam 1 n=348; exam 3 n=283) with nested cohorts of n=160 (who participated in exams 1 & 2); n=103 (who participated in exams 1 & 3) and n=73 (who came for all 3 exams). The FAM Study received approval of the Institutional Review Board (IRB) of Kaiser Permanente Hawaii (where the exam 1 assessments/exams were conducted), the University of Hawaii (UH) and the UH Clinical Research Center (CRC) at Kapiolani Medical Center (where the exams 2 and 3 were conducted). Recruitment letters, assent and consent forms found in Appendix A. The

study consists of exam 1 and follow-up exams (exams 2 and 3). At exam 3, (n=283) new participants were recruited. Exam 1 (baseline) data were collected in 2000-2001; exam 2 data were collected in 2002-2003; and exam 3 data were collected in 2004-2005.

2.3 Subjects

For the longitudinal (nested cohort) component of this study, only subjects that participated in exam 1 were eligible for exam 2 and 3; at exam 3, exam 1 girls and also we recruited an additional cross-sectional sample of 180 girls were eligible, using the same selection criteria as the FAM cohort girls. The inclusion criteria for exam 1 were as follows: Kaiser Permanente Oahu member, female adolescents of White or Asian ancestry, and aged 9 to 14 years old. Other eligibility requirements included non-smoking; not using or have used oral corticosteroids, oral contraceptives or other steroid hormones, and no pre-existing medical conditions such as thyroid, chronic asthma, and epilepsy requiring medication. At exam 3, the new girls were aged 13 to 18 years, which is similar to the FAM cohort girls at exam 3. At exam 3 the oral contraceptive exclusion criteria was relaxed and only required an exclusion criterion that the girl remained nulliparous.

Potential subjects were mailed a recruitment letter and asked to mail back a reply slip, indicating their willingness to participate (either “yes”, “no” or “more information desired”). The Study Coordinator called subjects who had mailed back an affirmative response slip and subjects that failed to mail any response. Subjects who mailed a negative response were not further contacted. During the recruitment call, the study

coordinator screened for study eligibility and scheduled an appointment for the visit. Recruitment letters, recruitment calls and appointments for exams 1 and 2 were timed two years (± 2 months) apart and exams 2 and 3 timed 3 years (± 6 months) apart.

From an initial sample of 3,717 age and sex eligible Kaiser Permanente members, 1,106 responded to the mailing. Of the 1,106, 812 met the ethnic ancestry criteria. Of the 812, 348 participated at exam 1 (Fig 2.1). Participation rate at exam 1 was 9.4%. From the 348 exam 1 participants, 160 girls (46%) attended exam 2. If any contact at all had been made with the participant, but no appointment had been scheduled, the subject was classified as “not interested”. Subjects that made an appointment but did not attend the visit (and no reschedule was possible) were classified as “no show”. Subjects were classified as “unavailable” when there was no response to the recruitment letter, and no contact with the participant had been achieved, or when subjects left the state and were willing but unable to attend the exam. Sixty seven subjects (19%) were unavailable. Six subjects (2%) were not able to attend exam 2, but agreed to mail back the questionnaires and mouthwash specimens although only two of these subjects actually sent back the questionnaires and mouthwash specimen. At exam 3, 285 new girls (8%) (Fig 2.1) were age, sex and ancestry eligible from the initial sample of 3,375 Kaiser Permanente members available at that time. Of the 285 new eligible girls only 180 girls (63%) participated at exam 3. From 348 FAM cohort girls recruited at exam 1, 103 (26%) participated at exam 3. Eighteen subjects had incomplete information at exam 3; either they dropped out of the study or could not be contacted.

Figure 2.1 Recruitment from Kaiser Permanente membership

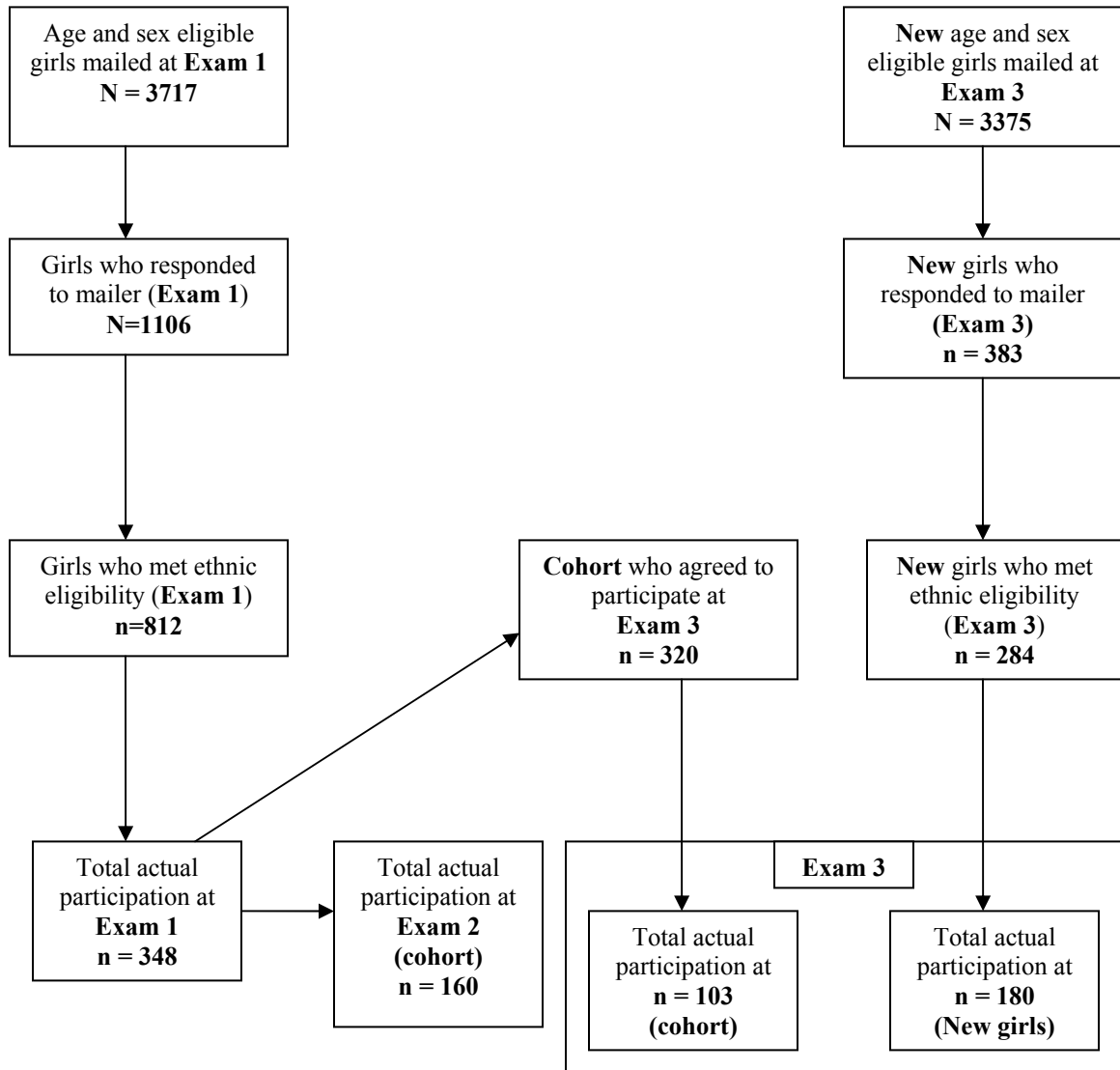
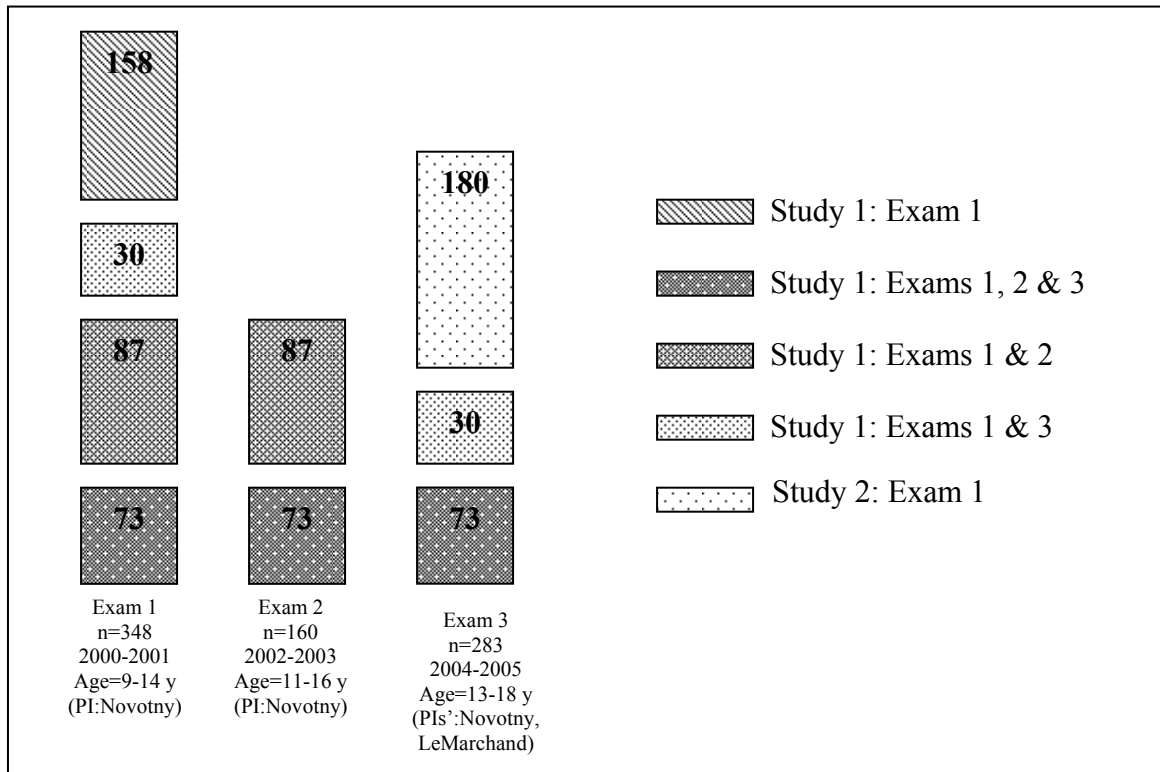


Figure 2.2 gives a description of the number of subjects at each exam. There were 73 girls who came for all three exams. Eighty seven came for exam 1 and 2. Thirty girls came for exam 1 and exam 3. There were additional 180 girls who were recruited at exam 3.

Figure 2.2 Total numbers at each exam



2.4 Measures

Signed assent and consent forms were obtained from the study participant and their parent/guardian. The variables used in the analysis were obtained from the questionnaires, the clinical examination at UH CRC and the UH CRCH blood collection for hormones and the genotyping. The questionnaires used in all the three exams are found in Appendix B.

2.4.1 Questionnaires

All subjects were required to complete a series of five questionnaires prior to their visits to Kapiolani Clinical Research Center at each exam. The week before the visit, subjects received the packet of questionnaires in the mail. The questionnaires were:

1. Background Questionnaire
2. Health Questionnaire
3. Menstrual Questionnaire
4. Physical Activity Questionnaire
5. Three day Diet Records

Table 2.1 Summary of FAM measures by exam

FAM Measures	Exam 1	Exam 2	Exam 3
Date	2000-2001	2002-2003	2005-2007
N	349	160	283
Age range (yr)	9-14	11-16	13-18
Family Background Questionnaire ^a	X	X	X
Health Questionnaire ^a	X	X	X
Menstrual Questionnaire ^a	X	X	X
Physical Activity Questionnaire ^a	X	X	X
3-Day Diet record ^a	X	X	X
Anthropometry ^b	X	X	X
Tanner staging ^c	X	X	X
DXA body fat measures ^d		X	X
Genotypes ^e		X	X
Hormones ^f			X

^aFilled by the subject with the help of the parent/guardian

^bExam 1 by Yihe Daida; Exam 2 and 3 by CRC research staff

^cExam 1 by Kaiser nurse practitioner; Exam 2 and 3 CRC research staff

^dExam 2 and 3 by DXA technician

^eCancer Research Center of Hawaii

^fIARC, Unit of Hormones and Cancer, Lyon, France

Background Questionnaire

The background questionnaire was completed by a parent or a guardian. Additional background questionnaire data were incorporated at exams 2 and 3 to include personal information on parents (e.g. their current height and weight and their education level), family information (breast cancer and diabetes occurrence in the family), and child's weight and length at birth.

Health Questionnaire

The subject was required to complete a health questionnaire concerning factors affecting breast cancer and bone metabolism (e.g. contraceptive pill intake, acne and asthma medication) and whether any bones had been broken. The health questionnaire also

gathered information on girl's menstrual history, such as age at which she reached menarche (age at menarche), regularity of the cycles, and number of pads/tampons used. At exam 1 this was a separate questionnaire; later, at exams 2 and 3, menstrual questions were integrated with the health questionnaire. At exam 3, subjects were given calendars to mark their menstrual cycles.

Age at menarche

The girls (with the help of the guardian as needed) were asked if they had started menstruation and, if so, in what month and year it occurred. Age at menarche (AAM) was calculated from the self reported date of menarche (month and year) and the date of birth. In cases where the subject did not report month and year of menarche, the reported age at menarche was used in analysis (exam 1 = 35, exam 2 = 46, exam 3 = 51). If AAM was missing at exam 3, for the FAM cohort girls, AAM from the previous exams were considered. At exam 3 there were 20 girls who had not reached menarche, they were contacted after the study period and ten of them had reached menarche. In a study by Koo & Rohan (186), 66% and 45% of girls recalled their age at menarche accurately with a mean interval of 323 and 649 days respectively, demonstrating that the accuracy of recall decreases as the number of days to recall increases.

Birth weight

Birth weight was obtained both by linkage birth certificates (from the Hawaii State Vital Statistics Office, for 76% of FAM participants, n=397) and by recall (n=110) of the parent. The Spearman correlation coefficient for birth data from recall and birth certificates was 0.9 for birth weight, indicating that this information was correctly

remembered by the mothers. Default values for birth weight and gestational age were set to birth certificate values; and, if missing, the recalled values were use (n=110).

Physical activity

Past Year Physical activity questionnaire was used, which was validated for children (187). It queries activities that were performed at least ten times during the past year. For each activity, subjects were required to record how many months (and which months), how many times a week, and for how many minutes each day they spent doing that activity. The values from this questionnaire were transformed into metabolic equivalents (METs) (188) based on the average MET hours per week doing that activity during the past year.

Formula:

Month x (4.3 wk/month) x (days/wk) x (minutes/day) / (60 minutes/hour) (52 weeks/year) = average hrs / wk / activity for the past year

The average hours per week was then multiplied by a specified Metabolic Equivalent Unit (MET) value, to calculate the adjusted hours per week for that activity.

Hrs/wk x MET= _____ MET-hrs/wk for that activity

For exam 1, MET values were retrieved from the work by Lee et al. (189). The METs for the physical activities reported by subjects at exams 2 and 3 that were not reported in exam 1 were retrieved from The Compendium of Physical Activities Tracking Guide (http://prevention.sph.sc.edu/tools/docs/documents_compendium.pdf).

When the activity reported was not listed in the compendium, a mean of the METs corresponding to the most similar activity was assigned. When the activity reported had more than one value for different intensities or variations of a sport, the mean of the set of MET values was taken. Finally, MET values were summed for all activities to describe the usual physical activity based on the year prior to the visit.

Food records

A three-day food record (two weekday and one weekend) previously developed for exam 1 (190) was also completed by subjects at exams 2 and 3. Subjects (with the help of parents) were asked to record their food intakes on the Thursday, Friday and Saturday of the week prior to the visit. In addition, a dietary supplement questionnaire was provided for each food record day, to assess the subject nutrient intake from items not reflected in the food record. Detailed examples of entries were included on the food record and supplement intake forms, along with a measuring cup and spoon and a ruled edge of the paper, to help the subject estimate the portion sizes. The mean of three day intake is used in the analysis.

2.4.2 Anthropometry

Anthropometric measures were taken at Kaiser Permanente Honolulu Clinic for exam 1 (by Yihe Daida) and at the UH Clinical Research Center located at Kapiolani Medical Center for exams 2 and 3 (CRC staff). Weight was measured with a digital scale (Seca) in pounds and kilograms. Height and sitting height were measured using a digital stadiometer (Measurement Concepts, North Bend WA). Skinfold measurements were

taken at the subscapular, biceps, triceps, iliac and calf sites using a Lange Skinfold Caliper from Beta Technology Incorporated (Cambridge, Maryland).

Two measures were taken. If they were more than two decimals apart (eg. Skinfold: difference of 2mm; Height: difference of 2cm), then a third measurement was taken. An average of the two closest measures was used in the analysis. Circumferences (arm, abdomen, buttocks) were measured with an inextensible measuring tape (Rollfix, Hoechst. Mass, Germany). Biiliac and Biacromial breadth were measured with a Lafayette Caliper. A standardized measurer (Dr Novotny) trained the Project Coordinator Yihe Daida who took the measurements at exam 1 and the CRC DXA study Coordinator Jane Yakuma who took the measurements at exam 2 and 3 and was standardized with Yihe Daida according to Lohman et al. (191).

2.4.3 Tanner Pubertal Staging

Pubertal staging was performed according to Tanner (192). At exam 1 the study coordinator was standardized with the nurse practitioner (Mary Kawaski) who performed the Tanner staging at Kaiser Permanente. The Study coordinator and the nurse assessed the 3 subjects by clinical examination and the data were recorded onto a datasheet. The agreement between measurements was 100% for both Tanner breast and pubic hair stages. At exams 2 and 3 Tanner pubertal staging was performed by the standardized research staff at UH Clinical Research Center (UH CRC). The study coordinators were also further trained by adolescent physician (Dr. Robert Bidwell, MD) of Kapiolani Medical Center.

2.4.4 Dual Energy X ray Absorptiometry (DXA)

Dual Energy X ray Absorptiometry was assessed at exams 2 & 3 only using Lunar Prodigy by GE, at UH CRC by a trained DXA technician (Jane Yakuma). A urine test was done prior to the scan to screen for pregnancy and the scan was not conducted in the case of a positive pregnancy result (n=1 at exam 2).

2.4.5 Genotype

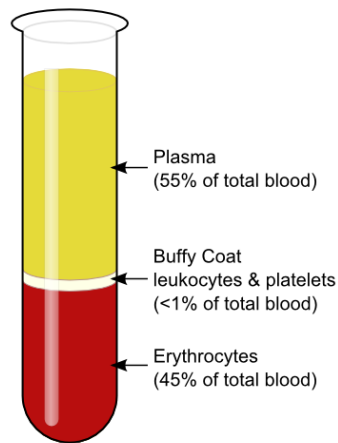
Single nucleotide polymorphisms (SNPs) included in this study were selected based on: functional SNPs (SNPs altering gene function or expression) known to affect plasma leptin levels – a focus of this dissertation and allele frequencies of the SNPs reported to be present in Asians and Whites – the ancestral groups in the Female Adolescent Maturation (FAM) study population of this dissertation. SNPs identified for inclusion in this study are *LEP* G-2548A, *LEP* A19G, and *LEPR* Q223R.

Genotyping was done at the Cancer Research Center of Hawaii. At exam 2 buccal cells were collected (by CRC research staff) with a non-invasive mouthwash and spit method (193) and frozen at -80°C until extraction. The frozen mouth wash was thawed and spun to separate the cells and the supernatant was dumped. At exam 3, fasting blood samples were collected by CRCH research staff and for the subjects who refused the blood sample, buccal cells were collected with mouth wash. Blood sample was centrifuged to separate the buffy coat, which is the fraction of an anticoagulated blood sample that contains most of the white blood cells and platelets (Fig 2.2). The CRC visit will be timed

in the morning (with blood draw between 7:30 and 9:00 am) and as to correspond to the 20-24th day (luteal phase) of the menstrual cycles.

A study by Thomas (2007) compared blood and buccal cells collected by the mouthwash and spit method and concluded that mouthwash samples are a good alternative to blood samples, for epidemiologic studies.

Figure 2.3 Blood components after centrifugation



DNA was extracted at exams 2 and 3 using QIAamp DNA blood Midi kit with no modifications done to the protocol. The DNA concentration of the product was measured by spectrophotometry, normalized to 50ng/ul.

Genotyping was performed using a fluorescent 5' endonuclease assay and the ABI FAST 7900HT Real-Time PCR System for allelic discrimination (TaqMan; Applied Biosystems, Foster City, CA). Genes, db SNP RS# and Applied Biosystems assay ID for the SNPs genotyped for this study are listed in Table 2.2. Amplification reactions were

carried out in ABI 9700 thermal cyclers and allelic discrimination was determined on the ABI FAST 7900HT Real-Time PCR System. Amplification reaction for Nanogen primers and probes consist of PCR master mix from Sigma (cat. M4693) and JumpStart Taq. Nucleotide-specific PCR primers and fluorogenic probes were designed using Primer Express (Applied Biosystems) and MGB Eclipse Probe Systems (Nanogen).

Table 2.2 Applied Biosystem assay IDs

<u>Gene Symbol</u>	<u>dbSNP*</u>	<u>Assay ID (ABI)</u>	<u>Gene Name</u>
<i>LEPR</i> Q223R	rs1137101	C__8722581_10	Leptin Receptor
<i>LEPA</i> 19G	rs2167270	C__15966471_20	Leptin
<i>LEP</i> G-2548A	rs7799039	C__1328079_10	Leptin

*Single Nucleotide Polymorphism Database developed and hosted by the National Center for Biotechnology Information (NCBI)

2.4.6 Laboratory

Fasting blood was collected and hormonal assay was performed at the International Agency for Research on Cancer (IARC), Unit of Hormones and Cancer, Lyon, France. The plasma samples were shipped to IARC in dry ice. Total plasma leptin was only analyzed at exam 3. Total leptin concentration was measured by radioimmunoassay (Linco, St Charles, Missouri, USA). The assay had a sensitivity=0.5 ng/ml, specificity=100%. Duplicate measures for 19 blind samples were used to calculate the coefficient of variation (CV). The CV was 3%. Coefficient of variation is a measurement of precision (relative or estimated SE) expressed as percent. The lower the CV, the better the test.

2.4.7 Ancestry (Ethnicity)

The Ancestral background of the parents was determined at exam 1. Capturing ethnicity in a highly mixed population such as the population in Hawaii is complicated. In order to obtain the most accuracy, each parent was asked to detail their ancestral background and percentage for each. Girl's ancestry was calculated based on the parent's reported race/ethnicity (e.g. *Father*: 50% Japanese and 50% White; *Mother*: 25% Japanese and 75% White then *Girl*: 37.5% Japanese and 62.5% White) (Novotny & Daida, 2009).

For the purpose of this genetically focused study, we grouped ancestry based on geographical approximation as follows (Fig 2.3):

South East Asian: Thai, Laotian, Vietnamese, and Cambodian.

Malay: Filipino, Malaysian, and Indonesian.

North East Asian: Japanese (Okinawans), Chinese, and Korean.

White (European): Reported as Whites, German, French, Scandinavian, Russian, Irish, Portuguese, Spanish, and Italian.

Other: Hawaiian, Pacific islanders, Tongan, Samoan, African American, American Indian, Hispanics, Puertorican, Indian, Armenian, Mongolian, and Mexican. (Numbers of individuals in each of these ethnic groups were too small to create geographic ancestral groups and, hence, were grouped together as “other”)

Figure 2.4 Geographic grouping of Ancestries



White, NEA, SEA, and Malay

Table 2.3 Sample size available from each exam

	<u>Exam 1</u>	<u>Exam 2</u>	<u>Exam 3</u>	
Total Number Recruited	349	160	FAM cohort 103	New cohort 180
Family Background Questionnaire	338	157	103	166
Health Questionnaire	334	157	98	172
Physical Activity Questionnaire	340	157	95	165
Diet Records	322	154	98	169
Anthropometry	348	160	103	180
Tanner Breast stage	346	160	89	147
Tanner Pubic stage	346	160	89	135
DXA		159	101	180
Genotypes		81	103	179
Hormone			80	127

2.5 Data entry and cleaning

At exam 1, data were entered using an excel spread sheet (Yihe Daida). At exam 2 data were double entered using an access database and were randomly checked (Samantha Stavar). At exam 3 data were double entered using a Fox Pro database. Data entering and data cleaning were done by the data manager (Vinutha Vijayadeva). Two entries were compared and any data that did not match were corrected using the original hard copy of the data. The first level of data cleaning was done to check for duplicates. The second level of cleaning was done to check for missing entries. A third level of cleaning was done to check for outliers, by calculating the mean, maximum and minimum values of every continuous variable and frequency tables for categorical data.

2.6 Calculated variables

The formulas used to obtain calculated variables we calculated are detailed in the following Table 2.4.

Table 2.4 Calculated variables

Calculated variable	Formula	Units
Age	Date of the visit – Date of birth	Decimal years
Age at menarche	Date of menarche – Date of birth	Decimal years
Time since menarche	Date of the visit – date of menarche	Decimal years
Weight	Weight in pounds * 0.45359	Kilograms
Height	Height in cm /100	Meters
BMI	Weight (Kg)/(Height in m) ²	Kg/m ²
DXA trunk-to-periphery fat ratio	DXA trunk fat (g) / [DXA arms (g) +DXA legs (g)]	Ratio

2.7 Statistical Analysis

Data analysis was limited to subjects who had genotype data (n=364). The data analysis was performed using Statistical Analysis Software (SAS, Version 9).

All the dependent variables were tested for normality based on coefficients of skewness and kurtosis and the distribution of these variables is discussed in section 2.8. Normal distributions produce a coefficient of skewness of 0 and kurtosis of 3 (small variations can occur by chance alone). Negative values for skewness indicate data that are skewed left and positive values for the skewness indicate data that are skewed right. Positive kurtosis indicates a "peaked" distribution and negative kurtosis indicates a "flat" distribution. If the dependent variables were highly skewed to the right then exponential regression models were used, which are similar to using the logarithm of the variable.

Dummy variables were coded for having a particular genotype for each single nucleotide polymorphism (SNP) (e.g. dummy variables for *LEP* A19G: AG genotype = 1, AA genotype = 0 & GG genotype = 0).

Three alternate ways of doing genotype statistical analysis are: (1) *A one way ANOVA across the three genotypes with 2 degrees of freedom*. This would lower the statistical power, compared to a simple comparison of means for homozygote genotypes. (2) *Regressing the dependent variable on an allele count of 0, 1, or 2 for one of the two alleles*. This is a compromise between models, although some might object to what they would see as an arbitrary assumption of the additive model. (3) *Placing the heterozygotes*

arbitrarily with one homozygote group. This approach involves a guess about inheritance or performing two tests of significance. Then p value needs to be adjusted for additional comparisons if two significance tests are done and would not assure the correct model of inheritance since the mean for heterozygotes might fall between the two homozygotes and so not be dominant or recessive, or might have a greater or lesser mean than either homozygote genotype.

For the present study, a fourth (virtually) model-free analysis was used to compare only the two homozygote genotype (AA vs GG) means, not making any possibly false assumptions about where the heterozygote means fall. Including the heterozygote and separating them out with their own dummy variable gives some information about the effect of covariates, like age, in the regression model. So, they are included in the analysis even though they are not being tested as a main effect.

2.8 Study Design for Specific Aims

Aim 1: To determine whether selected SNPs in Leptin (LEP) and Leptin receptor (LEPR) genes are associated with plasma leptin levels in adolescent girls in Hawaii

This is a cross sectional analysis with the 207 girls who provided blood that is needed for hormone analysis (all from exam 3).

Dependent variable: Total plasma leptin (ng/ml). The distribution for the plasma leptin residuals from the regression model with age, ancestry and genotypes had a coefficient of skewness = 2.44 and a coefficient of kurtosis = 7.88. The plasma leptin residuals distribution was grossly non-normal but a log transformation made it approximately bell shaped (Fig 2.4 & 2.5) and for this reason exponential regression models, with GEE estimation, were used. GEE provides standard errors (SE) and confidence intervals (CI), which are robust to clustering and heteroscedasticity (unequal error variance about the regression line) (194).

Independent variables: *LEP* and *LEPR* genotypes were tested in separate models.

Covariates: Ancestral groups (North East Asian [NEA], Malay, White & Other mix) were included in models as potential confounders. Adjusting for age improves the precision of the model (i.e. variables included to remove any variability in the estimate of the exposure on the outcome contributed by other known predictors, hence gaining precision of the effect estimate by narrowing the CI, as a result of controlling).

Figure 2.5 Distribution of plasma leptin residuals before log transformation (coefficient of skewness=2.44, coefficient of kurtosis=7.88)

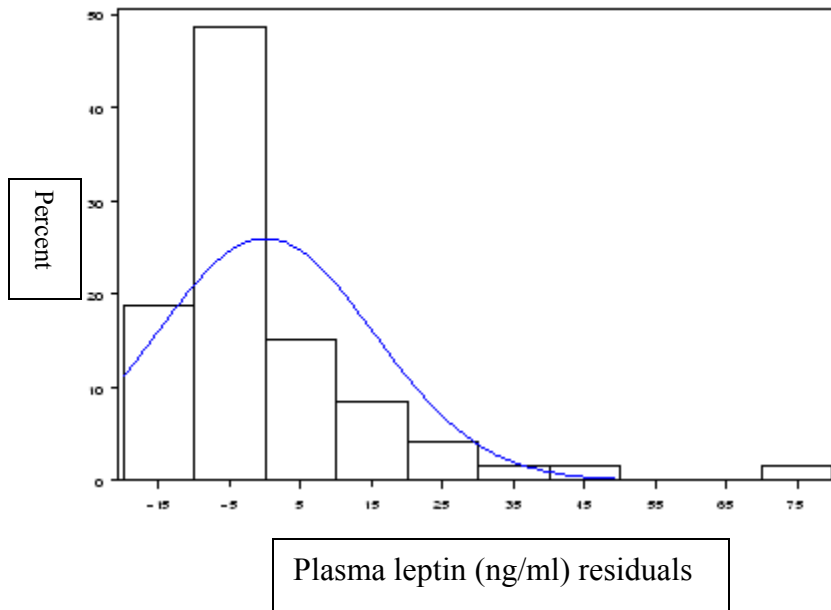
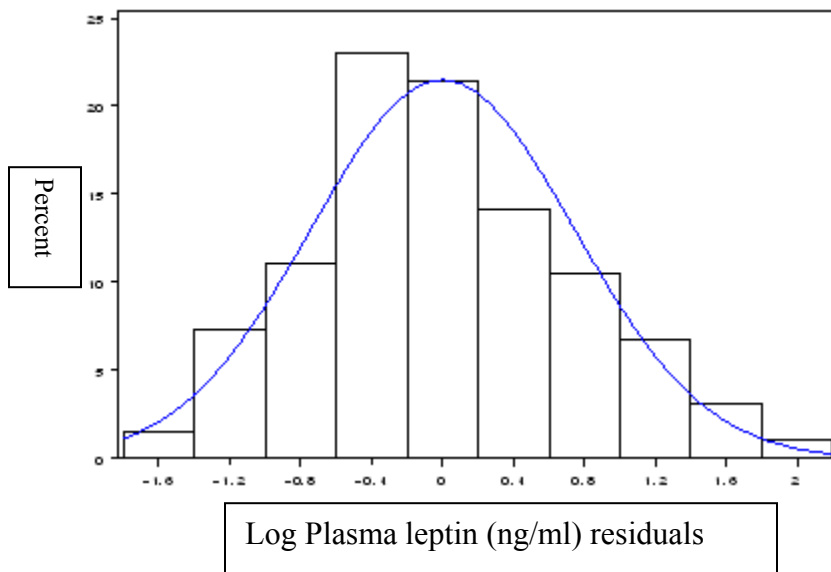


Figure 2.6 Distribution of plasma leptin residuals after log transformation (coefficient of skewness=0.32, coefficient of kurtosis=-0.18)



Statistical power was computed to detect the mean plasma leptin levels in standard deviation units of 1.0, 0.5, 0.25 and 0.2, between the two homozygote genotypes for varying allele frequencies and an alpha of 0.5. Table 2.5 demonstrates statistical power of 0.80 and above to detect 0.5 ng/ml of plasma leptin between two homozygote genotypes.

Table 2.5 Statistical Power for standardized differences of plasma leptin by allele frequencies from the FAM (n= 207, alpha=0.05)

<u>Variants</u>	<u>Allele frequencies</u>	<u>Standardized difference</u>			
		<u>1.0</u>	<u>0.5</u>	<u>0.25</u>	<u>0.20</u>
<i>LEP A19G</i>	(A) 0.31	1.00	0.84	0.31	0.22
<i>LEP G-2548A</i>	(A) 0.57	1.00	1.00	0.82	0.63
<i>LEPR Q223R</i>	(A) 0.31	1.00	0.84	0.31	0.22

Aim 2: To determine whether SNPs in LEP and LEPR genes are associated with total body fat and type of body fat distribution in adolescent girls in Hawaii

Pooled data from exams 1, 2, and 3 were analyzed in a cross sectional design. Three hundred and sixty six girls had genotype data. Repeated DXA body fat variables and skinfold thickness were used.

Dependent variable: DXA total body fat (g), DXA trunk fat (g), DXA trunk-to-periphery fat ratio (from exams 2 & 3), waist and hip circumferences (cm), waist to hip ratio, and skinfolds (mm) (from exams 1, 2 & 3). The distributions of body fat variables were checked (Fig 2.6 & 2.7) and exponential models were used when the dependent variables were skewed to the right.

Figure 2.7 Distribution of DXA total body fat (coefficient of skewness=1.48; coefficient of kurtosis=2.72)

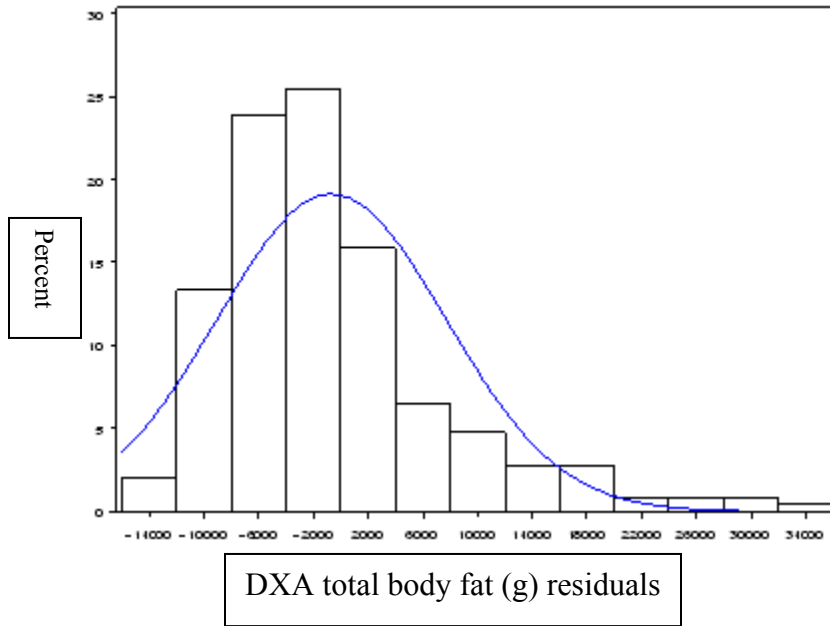
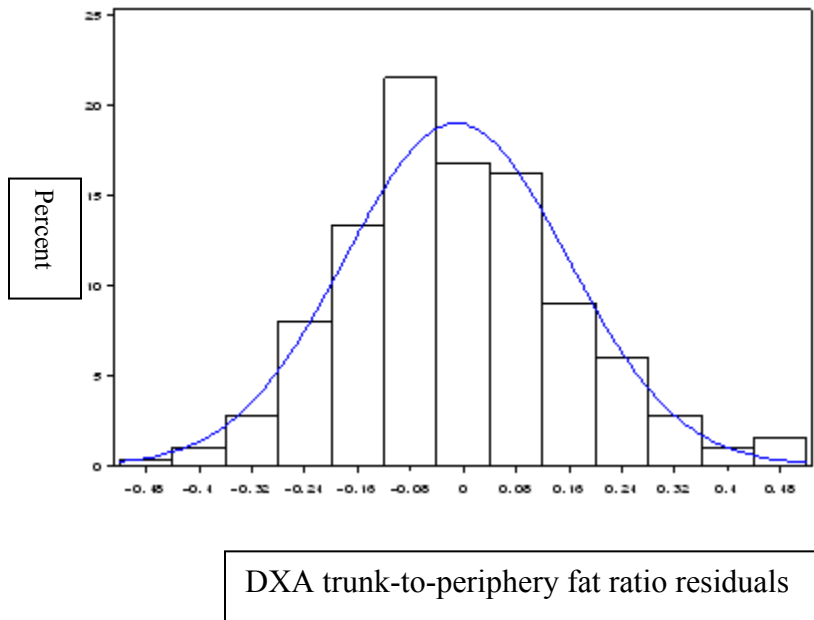


Figure 2.8 Distribution of DXA trunk-to-periphery fat ratio (TPFR) (coefficient of skewness=0.34; coefficient of kurtosis=0.17)



Other distributions of dependent variables that were examined were DXA trunk fat, peripheral fat (arm+leg), hip circumference, abdominal circumference and waist-to-hip ratio (WHR) (coefficient of skewness= 1.59, 1.43, 0.47, 1.25, 1.2; coefficient of kurtosis=3.57, 2.63, 0.94, 2.34, 3.49 respectively).

Based on the coefficient of skewness and coefficient of kurtosis, exponential models were used for DXA total body fat, trunk fat, waist circumference and waist-to-hip ratio. For other approximately normally distributed dependent variables, multiple linear regression models were used.

Independent variables: *LEP* and *LEPR* genotypes in separate models.

Covariates: Ancestral groups (North East Asian [NEA], Malay, White & Other mix) were included in models as potential confounders. Adjusting for age improves the precision of the model.

Statistical power was computed to detect the mean DXA body fat / skinfold thickness in standard deviation units of 1.0, 0.5, 0.25 and 0.2, between the two homozygote genotypes for varying allele frequencies and an alpha of 0.5. Table 2.6 demonstrates statistical power of 0.80 and above to detect 0.5 gm of DXA total body fat or 0.5 mm of skinfold thickness between two homozygote genotypes.

Table 2.6 Statistical Power for standardized differences of various body fat variables by allele frequencies (n=207; alpha=0.05)

Variants	Allele frequencies	Standardized difference			
		1.0	0.5	0.25	0.20
<i>LEP A19G</i>	(A) 0.31	1.00	0.84	0.31	0.22
<i>LEP G-2548A</i>	(A) 0.57	1.00	1.00	0.82	0.63
<i>LEPR Q223R</i>	(A) 0.31	1.00	0.84	0.31	0.22

Aim 3: To determine whether total body fat or type of body fat distribution is associated with plasma leptin levels in adolescent girls in Hawaii

A cross sectional design was used with the 207 girls that provided blood samples (needed for the hormone analysis).

Dependent variable: Total plasma leptin (ng/ml) was the dependent variable. Exponential models, with GEE adjustments were used.

Independent variables: DXA total body fat (g), trunk fat (g), trunk-to-periphery fat ratio, and sum of skinfolds (mm) were tested in separate models.

Potential confounders: Age and ancestral groups (NEA, Malay, White & Other mix) were included in the model as potential confounders.

Aim 4: To determine whether selected SNPs in LEP and LEPR genes are associated with AAM in adolescent girls in Hawaii

The above aim will be addressed in two parts.

4a: A longitudinal analysis with 305 girls from exams 1, 2 and 3 with a COX regression model.

Dependent variable: event of reaching menarche

Censoring variable: girls who achieved menarche = 1 and girls who did not reach menarche = 0 (girls who have not reached menarche will be censored).

Girls from exam 1 who were lost for follow-up, were set to be censored at the last exam they were seen (n=102).

Independent variables: *LEP* and *LEPR* genotypes in separate models adjusting for ancestral groups (NEA, Malay, White & Other mix) as potential confounders.

4b: In the second part of the analysis COX regression using selected girls who were premenarcheal at exam 1.

Dependent variable: Time to reach menarche since exam 1

Censoring variable: girls who achieved menarche = 1 and girls who did not reach menarche = 0 (girls who have not reached menarche will be censored).

Independent variables: body fat or type of body fat distribution in separate models adjusting for potential confounders age and ancestral groups (NEA, Malay, White & Other mix). Age is a potential confounder because; the proportion of girls who have attained menarche at a given year might differ across populations because of different age

distributions (due to migration of different populations into Hawaii, there may be confounding between age at a given year and ancestry).

Girls who were lost to follow-up were censored at the last exam they were seen. For example: Premenarcheal girls at exam 1, who did not come to either exam 2 or 3, were censored at exam 1. Girls with missing menarche status were excluded.

Table 2.7 shows minimal detectable Hazards ratio (HR) to reach menarche (number of events = 325) for various allele frequencies with a power of 0.80 and an alpha of 0.5. Hazards ratios of 1.57 and 1.73 can be obtained with allele frequencies of 0.57 and 0.31 respectively.

Table 2.7 Minimal detectable Hazards Ratio (HR) to reach menarche by variant allele frequencies from FAM (number of events = 325)

<u>Variant</u>	<u>Allele frequencies</u>	<u>HR</u>
<i>LEPR</i> Q223R	0.31	1.73
<i>LEP</i> A19G	0.31	1.73
<i>LEP</i> G-2548A	0.57	1.57

CHAPTER 3

RESULTS

3.1 Description of study population

Participants for this study were recruited from eligible members of Kaiser Permanente Health Maintenance Organization (KPH), Oahu, Hawaii. In Hawaii, 95% of the population is insured (195) with 25% at KPH (Total Oahu's population 900,000 and 227,000 insured by KPH <http://www.family-health-insurance.org/kaiserpermanentehawaii.html>).

The Kaiser Permanente membership is demographically representative of the State (Table 3.1).

For the purposes of comparison and reporting, both Hawaii state data and Kaiser ethnic ancestry data (from which FAM sample was drawn), were classified according to the state's algorithm (195) (which addresses the issue of mixed ancestry), and further collapsed by the Office of Management and Budget (OMB) algorithm (196). The FAM sample was collected differently from the state census (197) which asks for the ethnicity which the person identifies most with, while in the FAM study, the data were collected asking the percentages of each ethnicity for each of the parents.

The Kaiser membership includes a slightly greater proportion of females, White and mixed races and a lower proportion of Asians, compared to the state population. The data reflect the fact that females tend to use health care services more frequently (showing more females in Kaiser data than in state demographics), though data are broadly

comparable. Overall comparison of Kaiser Permanente with State data shows a high level of comparability (Table 3.1).

Table 3.1 Race/Ethnic comparison between KPH and Hawaii State Data based on the Office of Management and Budget (OMB) Race/Ethnic categories

	KPH – EMR* Sample (%) (2008)	Hawaii State Data (%) (2000)
Sex		
Females	59.9	49.8
Males	40.1	50.2
Ethnic Category		
Hispanic or Latino	5.1	7
Not Hispanic or Latino	94.9	93
Racial Category		
American Indian/Alaska Native	0.3	0.3
Asian	34.8	41.6
Native Hawaiian/Pacific Islander	9.1	9.4
Black or African American	1	1.8
White	30.3	24.3
Mixed Race	24.5	21.4

* EMR: Electronic Medical Record

Table 3.2 summarizes the mean proportion of each ancestral group in the FAM sample (total sum of the proportions does not add up to 1 due to rounding errors) which is grouped based on the ethnic proportion of the subject. By this classification, Whites comprise the largest proportion of the FAM population, followed by North East Asians (NEA).

Table 3.2 Ancestral proportions found in the FAM sample

<u>Ancestry (Range)</u>	<u>N</u>	<u>Mean</u>	
		<u>Proportion</u>	<u>SD</u>
North East Asian (0-1)	338	0.35	0.37
South East Asian (0-1)	338	0.01	0.09
Malay (0-1)	338	0.10	0.22
White (0-1)	338	0.42	0.35
Other mix (0-1)	338	0.11	0.17

We also compared the FAM sample’s parental educational attainment with Oahu’s census table (197). Since the census data are not presented by sex, combined father and mother’s educational attainment within the FAM sample is presented for the comparison. FAM data showed a higher educational attainment at both categories “Some college (no degree) and or associate degree” and “bachelor’s degree” (Table 3.3). About quarter of the FAM study population’s parents completed some college. Mothers had a higher percent of graduating from college than fathers within the FAM sample.

Table 3.3 Parent's Educational Attainment, FAM sample compared with census data for Oahu (197)

Education level	FAM			Census Oahu *
	Father (%)	Mother (%)	Total (%)	(%)
Some college (no degree) and or Associates degree (<i>Completed post high school training, excluding college / Completed some college/community college</i>)	30	32	62	29
Bachelor's degree (<i>Graduated from a four-year college or university</i>)	18	27	45	19

* (<http://hawaii.gov/dbedt/info/economic/databook/2008-individual/03/>)

Hawaii Health Survey reported 95.4% insured, 3.4% uninsured and 2.2% unknown (195). Based on the above comparisons, and that Kaiser Permanente is the second largest insurance provider for the state, we conclude that the FAM study group can be broadly generalized to the population of Oahu.

Table 3.4 describes mean birth weight and mean age at menarche (AAM) for our Asian White population which is 3.23 kg and 12.30 yr respectively.

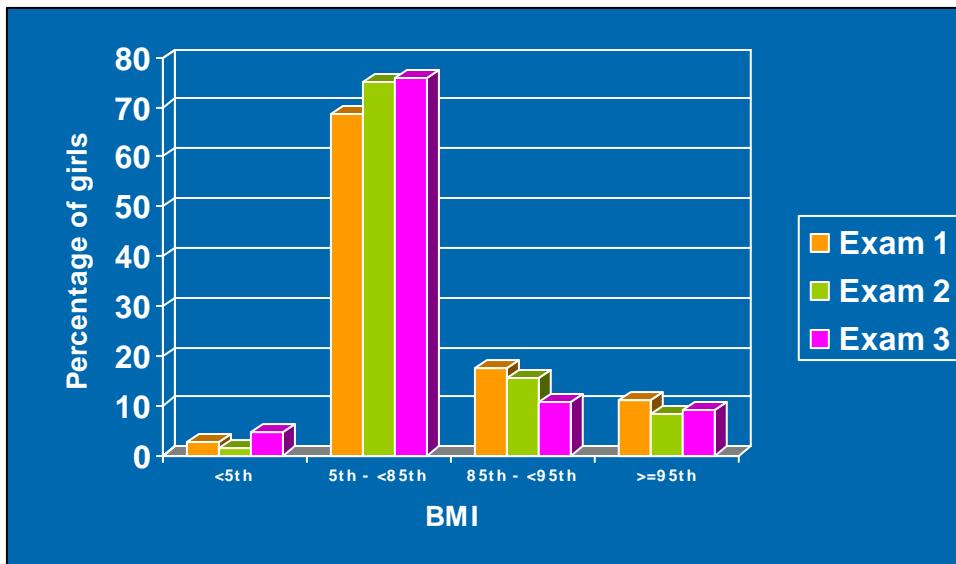
Table 3.4 Birth weight and AAM of FAM girls

Variable	N	Mean	SD
Birth weight, kg	346	3.23	0.50
Age at menarche, yr	318	12.30	1.21

BMI percentile categories

BMI percentile categories (198) at each exam are shown in Fig 4.1. Most of the study population was in the healthy weight group at all three exams. The mean BMI percentile, at exam 1, exam 2 and exam 3 are 61.2 ± 29.0 (n=183); 58.1 ± 28.2 (n=155); 57.6 ± 28.4 (n=270) showing some skewing to the right.

Figure 3.1 Body Mass Index Percentile Categories by Exam



Compared to NHANES data (2007-2008) which reported ~17% of children and adolescents aged 2-19 are obese, our data also reported ~ 10% obese and ~ 20% overweight (16). The percent obese in this sample is lower compared to the NHANES, This may be due to under representation of Asians in NHANES data.

Tanner pubertal stages

Two girls had missing data for Tanner pubic hair and Tanner breast stage at exam 2; fifty nine were missing Tanner pubic hair and 47 were missing Tanner breast at exam 3. Fig 4.2 and 4.3 show the proportion of participant's breast and the pubic hair Tanner stages at each exam. Girls at exam 1 and 2 were in early Tanner stages but at exam 3 most of them had reached Tanner 4 or 5 for both breast and pubic hair.

Figure 3.2 Proportion reaching Tanner Breast stage (%) by exam

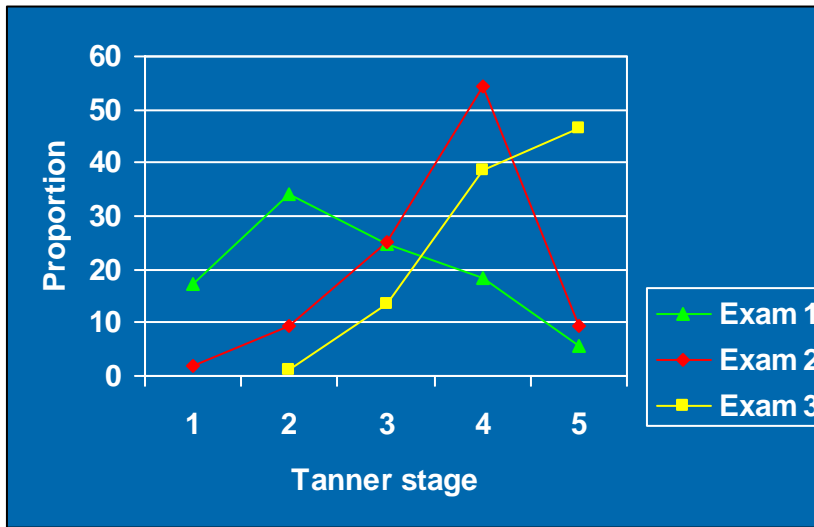
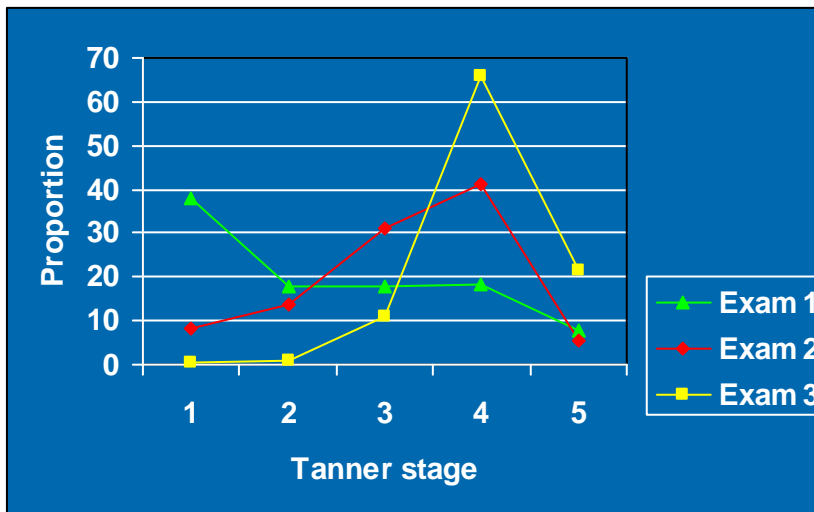


Figure 3.3 Proportion reaching Tanner Pubic Hair stage (%) by exam



3.2 Leptin (LEP) and Leptin receptor (LEPR) genotype and allele frequencies

The genotype distribution and allele frequencies of *LEP* and *LEPR* in our population are described in Table 3.5. Forty seven percent of girls were common homozygotes (GG) and 41% were heterozygotes for *LEPR* Q223R. Forty five percent of girls were common homozygote (GG) and 46% were heterozygote for *LEP* A19G. Thirty one percent of girls were common homozygote (AA) and 50% were heterozygote for *LEPR* G-2548A.

Table 3.5 *LEP* and *LEPR* genotype distribution and allele frequencies

Polymorphisms	Genotype distribution		Allele	
		n (%)		Frequency
<i>LEPR: Q223R</i> (<i>rs1137101</i>) <i>n=363</i>	AA	39 (11)	A	0.31
	GA	151 (41)	G	0.69
	GG	173 (47)		
<i>LEP: A19G</i> (<i>rs2167270</i>) <i>n=364</i>	AA	29 (8)	A	0.31
	GA	170 (46)	G	0.69
	GG	165 (45)		
<i>LEP: G-2548A</i> (<i>rs7799039</i>) <i>n=364</i>	AA	115 (31)	A	0.57
	GA	184 (50)	G	0.43
	GG	65 (18)		

Hardy-Weinberg Equilibrium

The Hardy-Weinberg Equilibrium (HWE) states that both allele and genotype frequencies in a population remain constant over time, that is, they are in equilibrium. Hardy-Weinberg Equilibrium was computed only for 100% North East Asian (NEA) and 100% White girls. Hardy-Weinberg Equilibrium test for the other girls was not computed because of the very small number of girls, in the Malay (n=5) and the South East Asian (SEA) (n=6) groups. The Pearsons chi-square tests for the deviation from HWE are in Table 3.6a. From the table below, all the three variants are in HWE for both ancestral groups except for *LEP* A19G in White ancestry, which is not in HWE. The chi-square for HWE for *LEP* A19G variant was significant (ChiSq = 10.51, p=0.0012), meaning that the *LEP* A19G variant in 100% White girls in this population was not in HWE. It was confirmed with the genotype lab that there was no problem calling this SNP and that the clusters were very clear.

Table 3.6a Allele frequencies by ancestry and chi-square test for HWE

Ancestral group	<i>LEPR O223R</i>			<i>LEP A19G</i>			<i>LEP G-2548A</i>		
	A allele	ChiSq	p	A allele	ChiSq	p	A allele	ChiSq	p
White (n=45)	0.57	0.299	0.584	0.37	10.509	0.0012	0.39	0.015	0.903
NEA (n=56)	0.11	0.806	0.369	0.28	1.303	0.254	0.64	0.249	0.618

LEP and LEPR allele frequencies by ancestry

The allele frequencies and standard errors by proportion of ancestry (Table 3.6b) were also computed using Proc GENMOD with NOINT option. The allele frequency is calculated for the ancestral proportion of 1. None of the 6 South East Asian (SEA) girls had A alleles and thus they were not included in the analysis. Girls with White ancestry had the highest frequency of A alleles and North East Asian (NEA) has lowest frequency of A alleles for *LEPR* Q223R. For *LEP* A19G polymorphism, Whites had the highest and NEA has the lowest frequency of A alleles. For *LEP* G2548A, NEA had the highest and the Whites had the lowest frequency of A alleles.

Table 3.6b Allele frequencies by ancestry

<u>Ancestral group</u>	<u>LEPR Q223R</u> n=330		<u>LEP A19G</u> n=331		<u>LEP G-2548A</u> n=331	
	<u>A allele</u>	<u>SE</u>	<u>A allele</u>	<u>SE</u>	<u>A allele</u>	<u>SE</u>
White	0.589	0.035	0.392	0.031	0.422	0.037
NEA	0.107	0.025	0.232	0.036	0.702	0.038
Malay	0.134	0.057	0.316	0.063	0.622	0.064
Other mix	0.136	0.090	0.361	0.086	0.584	0.095

Comparison of LEP and LEPR allele frequencies among studies

For the purpose of comparison with previous studies, only girls of 100% White ancestry or 100% NEA were included in Tables 3.7, 3.8 and 3.9 from our multiethnic population. The International HapMap Project is a multi-country effort to identify and catalog genetic similarities and differences in human beings. In the initial phase of the Project, genetic data were being gathered from African, Asian, and European ancestry. Most published studies were conducted on Whites.

Compared to the HapMap data (199) (Haplotype Map), our White subjects have high A allele frequency for *LEPR* Q223R. For other previous studies, A allele frequency ranged between 0.60 to 0.65 in non-obese subjects and between 0.52 to 0.70 in obese subjects of White ancestry (Table 3.7). Allele A frequency in our NEA group (which includes Chinese, Japanese and Korean) is similar to the Chinese and Japanese from the HapMap data (Table 3.7).

Table 3.7 *LEPR* Q223R frequency comparisons

Author	Ancestry	Age, yr	n	Sex	Allele	
					A	G
FAM study, 2010	NEA	8-18	56	Female	0.11	0.89
	White	8-18	45	Female	0.57	0.43
HapMap data (199)	Chinese	-	90	Male/Female	0.11	0.89
	Japanese	-	88	Male/Female	0.14	0.86
	White	-	118	Male/Female	0.45	0.55
Wauters et al. (179)	White	28-56	280	Female (overweight, obese)	0.52	0.48
Quinton et al. (177)	White	62-72	220	Female (non-obese)	0.60	0.40
Yiannakouris et al. (176)	White	14-26	89	Male/female (obese)	0.70	0.30
			29	Male/female (non-obese)	0.62	0.38
Mendoza et al. (181)	Mexico	12-17	55	Male/female (obese)	0.70	0.30
			48	Male/female (non-obese)	0.63	0.38
Portoles O et al. (200)	White (Spain)	18-70	303	Male/female (obese)	0.69	0.31
			606	Male/female (non-obese)	0.65	0.35
Duarte et al. (180)	White (Rio)	19-61	200	Male/female (obese)	0.57	0.44
			150	Male/female (non-obese)	0.61	0.39

Compared to the HapMap data, our White subjects have low A allele frequency for *LEP* G-2548A. For other previous studies, A allele ranged between 0.46 to 0.70 in non-obese subjects and between 0.36 to 0.73 in obese subjects of White ancestry (Table 3.8). Allele A frequency in our NEA (which includes Chinese, Japanese and Korean) is lower compared to the Chinese and Japanese from the HapMap data (Table 3.8).

Table 3.8 *LEP* G -2548A frequency comparisons

Author	Ancestry	Age, yr	n	Sex	Allele	
					A	G
FAM study, 2010	NEA	8-18	56	Female	0.64	0.35
	White	8-18	45	Female	0.39	0.61
HapMap data (199)	Chinese	-	90	Male/Female	0.74	0.26
	Japanese	-	90	Male/Female	0.78	0.22
	White	-	116	Male/Female	0.51	0.49
Le Stunff et al. (172)	White	9.5-14.5	237	Female	0.61	0.39
Mammes et al. (170)	White	36-46	218	Male/female (obese)	0.36	0.64
			628	Male/female (non-obese)	0.46	0.54
Yiannakouris et al. (173)	White	14-26	118	Male/female	0.53	0.47
Portoles O et al. (200)	White (Spain)	18-70	303	Male/female (obese)	0.50	0.50
			606	Male/female (non-obese)	0.51	0.49
Duarte et al. (180)	White (Rio)	19-61	200	Male/female (obese)	0.73	0.27
			150	Male/female (non-obese)	0.70	0.30

Compared to the HapMap data, our White subjects have slightly higher A allele frequency for *LEP* A19G. Allele A frequency in our NEA group (which includes Chinese, Japanese and Korean) is also higher compared to the Chinese and Japanese from the HapMap data (Table 3.9).

Table 3.9 *LEP* A19G frequency comparisons

Author	Ancestry	Age, yr	n	Sex	Allele	
					A	G
FAM study, 2010	NEA	8-18	95	Female	0.28	0.72
	White	8-18	68	Female	0.37	0.63
HapMap data (199)	Chinese	-	88	Male/Female	0.22	0.78
	Japanese	-	90	Male/Female	0.20	0.80
	White	-	118	Male/Female	0.30	0.70
Hager J et al. (174)	White	32-56	395	Male/Female	0.67	0.33

3.3 Specific aims

Aim 1: To determine whether selected SNPs in LEP and LEPR genes are associated with plasma leptin levels in adolescent girls in Hawaii

A cross sectional study with 207 girls with hormone data at exam 3 was used.

Since the dependent variable, plasma leptin, was grossly skewed to the right, exponential models with GEE adjustment for robust standard errors were used. Independent variables, *LEP* and *LEPR* genotypes in separate models, were adjusted for the potential confounders of age and ancestry. Descriptive statistics and genotype distribution for aim 1 are provided in Tables 3.10 & 3.11.

Table 3.10 Descriptive statistics for Aim 1

Variable	N	Mean	SD
Age, yr	198	15.55	1.43
North East Asian proportion (0-1)	193	0.33	0.36
Malay proportion (0-1)	193	0.12	0.24
White proportion (0-1)	193	0.44	0.33
Other mix proportion (0-1)	193	0.1	0.16
DXA total body fat, kg	202	18.38	8.4
Leptin, ng/ml	203	17.66	16.57

Table 3.11 Genotype distribution for Aim 1

Polymorphisms	Genotype frequency	
		n (%)
<i>LEPR: Q223R</i>	AA	21 (10)
	GA	80 (39)
	GG	100 (49)
<i>LEP: A19G</i>	AA	20 (10)
	GA	99 (49)
	GG	83 (41)
<i>LEP: G-2548A</i>	AA	59 (29)
	GA	106 (52)
	GG	38 (19)

Plasma leptin level by ancestry

The plasma leptin level by proportion of the ancestral group was obtained using SAS GENMOD with NOINT option. The means of plasma leptin are for the ancestral proportion of 1. The Whites had the highest plasma leptin level (22.73 ng/ml) and the Malays had the lowest (12.45 ng/ml) (Table 3.12). Once adjusted for DXA total body fat, Whites had the highest and the other mix group had the lowest but the difference between the ancestral groups was reduced (Table 3.12).

Table 3.12 Plasma leptin (ng/ml) by ancestry (n=193)

Ancestry	Unadjusted (model 1)			Adjusted for DXA total body fat (model 2)		
	Mean plasma leptin	95% CI		Mean plasma leptin	95% CI	
White	22.73	17.15	30.12	5.44	3.99	7.41
NEA	12.86	10.20	16.21	4.48	3.56	5.64
Malay	12.45	8.23	18.83	4.02	2.91	5.55
Other mix	13.28	6.12	28.79	2.87	1.89	4.36

Exponentiated (antilog) estimates (Exp β), confidence interval (CI) and their p values for plasma leptin by genotypes, after adjusting for the age and ancestry, are provided in Tables 3.13a, 3.14a & 3.15b. The mean plasma leptin level, after adjusting for mean age, ancestry and mean DXA total body fat, is provided in Tables 3.13b, 3.14 b& 3.15b.

There was no significant difference in plasma leptin level among the *LEPR* Q223R genotypes. NEA and Malays had lower leptin levels compared to Whites (approximately 45% lower) (Model 1; Tables 3.13a). Adjusted for DXA total body fat, the ancestral differences were decreased, but DXA total body fat was a significant predictor of plasma leptin (Model 2; Tables 3.13a). BMI instead of DXA total body fat yielded the same results (results not shown). The interaction between DXA total body fat and rarer homozygote (AA) genotype was significant ($p=0.04$) (results not shown). After stratifying, girls with AA genotype had approximately 52% less plasma leptin, only if the girls had less than the median (16.7 kg) DXA total body fat ($n=95$; $p=0.01$) (results not shown).

Adjusting for mean age and ancestry, the girls with the common homozygote genotype (GG) for *LEPR* Q223R has the highest and the girls with heterozygote genotype (GA) has the lowest mean plasma leptin level. Once the mean DXA total body fat was adjusted, GG genotype still had the highest mean plasma leptin level and the rarer homozygote genotype (AA) had the least, although they did not differ significantly (Table 3.13b).

Table 3.13a *LEPR* Q223R variants and plasma leptin (exponential models with GEE adjustments)

Variable	Leptin, ng/ml (n=191)				Leptin, ng/ml (n=190)			
	Model 1			p	Model 2			p
	Exp β	95% CI			Exp β	95% CI		
Intercept	23.234	5.715	94.453		6.740	2.177	20.870	
Age, yr	0.999	0.914	1.093	0.989	0.990	0.912	1.075	0.818
NEA	0.570	0.361	0.899	0.016	0.784	0.533	1.155	0.218
Malay	0.554	0.311	0.986	0.045	0.707	0.459	1.089	0.115
Other mix	0.605	0.236	1.550	0.295	0.514	0.285	0.927	0.027
DXA total body fat, kg					1.065	1.056	1.075	<.0001
GA	0.949	0.708	1.274	0.729	0.925	0.740	1.155	0.489
AA	0.968	0.588	1.595	0.899	0.833	0.555	1.250	0.378

White ancestry and common homozygote (GG) genotype are reference

Table 3.13b Mean plasma leptin level by *LEPR* Q223R genotypes

Genotype	Adjusted for mean age and White ancestry			Adjusted for mean age, White ancestry and mean DXA total body fat		
	Mean	95% CI		Mean	95% CI	
	AA	22.28	13.54	36.68	15.31	9.79
GA	21.85	16.50	28.93	16.98	13.83	20.85
GG	23.02	15.63	33.89	18.37	13.52	24.94

Mean age (y) = 15.51

Mean DXA total body fat (kg) = 18.19

There was no significant difference in plasma leptin level between the *LEP A19G* homozygote genotypes. NEA and Malays had mean lower leptin levels compared to Whites (approximately 45% lower) (Model 1; Tables 3.14a). Adjusted for DXA total body fat, the ancestral difference was diminished but DXA total body fat was a significant predictor of plasma leptin (Model 2; Tables 3.14a). BMI instead of DXA total body fat yielded the same results (results not shown). The interaction between DXA total body fat and genotypes was not significant (results not shown).

Adjusting for mean age and ancestry, the girls with the rarer homozygote genotype (AA) for *LEP A19G* had the highest and the girls with the common homozygote genotype (GG) had the lowest mean plasma leptin level. Once mean DXA total body fat was adjusted, AA genotype still had the highest and the heterozygote genotype (GA) had the lowest mean plasma leptin level but they were not significantly different from the mean for the GG homozygote (Table 3.14b)

Table 3.14a *LEP A19G* variants and plasma leptin (exponential models with GEE adjustments)

Variable	Leptin, ng/ml (n=192)				Leptin, ng/ml (n=191)			
	Model 1				Model 2			
	Exp β	95% CI		p	Exp β	95% CI		p
Intercept	22.545	6.379	79.671		5.867	1.942	17.729	
Age, yr	0.999	0.924	1.080	0.981	0.998	0.924	1.078	0.967
NEA	0.573	0.374	0.878	0.011	0.800	0.546	1.171	0.250
Malay	0.559	0.318	0.982	0.043	0.751	0.489	1.153	0.190
Other mix	0.558	0.222	1.407	0.217	0.518	0.299	0.896	0.019
DXA total body fat, kg					1.066	1.056	1.076	<.0001
GA	1.021	0.789	1.322	0.873	0.866	0.705	1.064	0.170
AA	1.133	0.687	1.867	0.625	1.110	0.742	1.659	0.612

White ancestry and common homozygote (GG) genotype are reference

Table 3.14b Mean plasma leptin level by *LEP A19G* genotypes

Genotype	Adjusted for mean age and White ancestry			Adjusted for mean age, White ancestry and mean DXA total body fat		
	Mean	95% CI		Mean	95% CI	
AA	25.16	15.18	41.73	20.32	13.26	31.13
GA	22.68	16.85	30.55	15.86	12.59	19.97
GG	22.21	15.60	31.63	18.31	13.77	24.35

Mean age (y) = 15.51

Mean DXA total body fat (kg) = 18.1

There was no significant difference in plasma leptin level among the *LEP* G-2548A homozygote genotypes. NEA and Malays had lower mean leptin levels compared to Whites (approximately 40% lower) (Model 1; Tables 3.15a). Adjusted for DXA total body fat, the difference among the ancestral groups diminished and DXA total body fat was a significant predictor of plasma leptin levels (Model 2; Tables 3.15a), although, heterozygote genotype (GA) for *LEP* G-2548A variant had a 20% lower mean plasma leptin level compared to the common homozygote genotype (AA) ($p=0.048$) (Table 3.15a). BMI instead of DXA total body fat, yielded the same results (results not shown). The interaction between DXA total body fat and genotypes was not significant (results not shown).

Adjusting for mean age and ancestry, the girls with the rarer homozygote (GG) for *LEP* G-2548A had the highest and the girls with the heterozygote genotype (GA) had the lowest plasma leptin levels and this was the same even after adjusting for mean DXA total body fat, but neither differed significantly from the mean of the AA homozygote (Table 3.15b).

Table 3.15a *LEP G-2548A* variants and plasma leptin level (exponential models with GEE adjustments)

Variable	Leptin, ng/ml (n=193) Model 1				Leptin, ng/ml (n=192) Model 2			
	<u>Exp β</u>	<u>95% CI</u>		<u>p</u>	<u>Exp β</u>	<u>95% CI</u>		<u>p</u>
Intercept	24.071	6.959	83.346		7.411	2.532	21.693	
Age, yr	0.992	0.920	1.068	0.825	0.986	0.918	1.060	0.712
NEA	0.602	0.396	0.914	0.017	0.816	0.571	1.167	0.266
Malay	0.596	0.343	1.036	0.067	0.759	0.501	1.151	0.194
Other mix	0.543	0.215	1.367	0.195	0.506	0.293	0.872	0.014
DXA total body fat, kg					1.065	1.056	1.075	<.0001
GA	0.958	0.724	0.976	0.762	0.802	0.644	0.998	0.048
GG	1.385	0.955	1.047	0.086	1.052	0.773	1.432	0.744

White ancestry and common homozygote (AA) genotype are reference

Table 3.15b Mean plasma leptin level by *LEP G-2548A* genotypes

Genotype	Adjusted for mean age and White ancestry			Adjusted for mean age, White ancestry and mean DXA total body fat		
	<u>Mean</u>	<u>95% CI</u>		<u>Mean</u>	<u>95% CI</u>	
AA	21.14	14.24	31.38	18.98	13.92	25.88
GA	20.25	15.59	26.30	15.22	12.58	18.40
GG	29.28	19.65	43.64	19.98	14.31	27.89

Mean age (y) = 15.51

Mean DXA total body fat (kg) = 18.19

Summary of Aim 1 findings

1. Whites had a higher plasma leptin level than NEA or Malays, but this was not significant after adjusting for DXA total body fat.
2. No association was found between plasma leptin level and *LEPR* variants
3. Girls with *heterozygote genotype LEP G-2548A* (AG) had 20% lower plasma leptin levels compared to the AA (p=0.048).

Aim 2: To determine whether SNPs in LEP and LEPR genes are associated with total body fat and type of body fat distribution in adolescent girls in Hawaii

To explore the above aim, concatenated data from exam 1, exam 2 and exam 3 were used in a cross sectional analysis, which included repeated measurements for some girls.

Exponential models with GEE adjustment (GEE gives robust standard error for repeated measures) were used when dependent variables were grossly skewed to the right.

Distributions of dependent variables were checked after taking the residuals, and adjusting for age, ancestry and genotype. Except for trunk-to-periphery fat ratio (TPFR), hip circumference and BMI percentile, all the other dependent variables were tested in exponential models. Independent variables, *LEP* and *LEPR* genotypes were tested in separate models adjusting for the potential confounders of age and ancestry. Table 3.16 gives the descriptive statistics for aim 2.

Table 3.16 Descriptive statistics for Aim 2

Variable	N	Mean	SD
Age [*] , y	350	14.09	1.59
Height [*] , cm	363	157.09	8.24
Weight [*] , kg	363	52.99	12.58
Waist circumference [*] , cm	363	67.55	8.74
Hip circumference [*] , cm	363	91.15	9.61
DXA total body fat ^{**} , kg	360	17.22	8.31
DXA trunk fat ^{**} , kg	360	8.35	4.49
DXA peripheral fat (arm+leg) ^{**} , kg	360	8.27	3.87
DXA trunk-to-periphery fat ratio ^{**}	360	0.993	0.166

*Mean of 3 exams

**Mean of 2 last exams (when DXA was available)

In most of the models, girls with North East Asian ancestry had lower total body fat, trunk fat and peripheral fat (measured as DXA and anthropometry) except for trunk-to-periphery fat ratio (Tables 3.17- 3.22).

The rarer homozygous genotype (AA) for the *LEPR* Q223R variant was negatively associated only with DXA TPF_R ($p=0.028$) (Table 3.17). This variant was not associated with waist, hip circumference, and waist-to-hip ratio (WHR) or BMI percentile (Table 3.18). The variants *LEP* A19G and G-2548A were not associated with waist, hip circumference, WHR or DXA body fat variables, except for the rarer homozygous genotype(AA) for G-2548A variant, which approximated a negative association with TPF_R only ($p=0.066$) (Tables 3.19, 3.20, 3.21 & 3.22). The sum of skinfolds (subscapular, biceps, triceps, iliac, and calf) was a tested for difference among genotypes across all three SNPs and there was no significant difference.

Table 3.17 *LEPR* Q223R variant predicting DXA body fat variables (n=398) (Exponential models except for DXA trunk-to-periphery fat ratio, with GEE adjustment)

Variable	DXA total body fat, g			DXA trunk fat, g			DXA peripheral fat, g			DXA trunk-to-periphery fat ratio						
	Exp β	95% CI	p	Exp β	95% CI	p	Exp β	95% CI	p	β	95% CI	p				
Intercept	6633.58	4550.08	9670.16	2490.40	1745.33	3553.54	2708.91	1998.20	3672.77	0.757	0.620	0.894				
Age, yr	1.071	1.045	1.098	<0.01	1.091	1.068	1.114	<0.01	1.084	1.064	1.103	<0.01	0.015	0.007	0.023	0.00
NEA	0.786	0.660	0.937	0.01	0.775	0.641	0.936	0.01	0.756	0.644	0.888	<0.01	0.060	0.003	0.118	0.04
Malay	0.917	0.694	1.211	0.54	0.927	0.681	1.262	0.63	0.875	0.669	1.145	0.33	0.081	-0.011	0.173	0.09
Other mix	1.182	0.832	1.679	0.35	1.207	0.813	1.792	0.35	1.174	0.847	1.629	0.34	0.032	-0.086	0.150	0.60
GA	1.011	0.897	1.141	0.85	0.980	0.861	1.117	0.76	1.010	0.903	1.129	0.86	-0.026	-0.065	0.014	0.20
AA	0.909	0.754	1.096	0.32	0.898	0.729	1.105	0.31	1.002	0.827	1.214	0.98	-0.073	-0.137	-0.008	0.03

White ancestry and the common homozygous (GG) genotype are reference categories

Table 3.18 *LEPR* Q223R variant predicting anthropometrically measured fat variables (n=576) (Exponential models with GEE adjustment)

Variable	Waist circumference, cm				Hip circumference, cm			Waist-to-hip Ratio				BMI percentile*				
	Exp β	95% CI		p	β	95% CI		p	Exp β	95% CI		p	β	95% CI		p
Intercept	52.64	49.67	134.21		49.87	45.57	54.16		0.929	0.90	0.96		60.63	47.38	73.88	
Age, yr	1.02	1.02	12.45	<0.01	3.05	2.81	3.29	<0.01	0.984	0.98	0.99	<0.01	0.001	-0.64	0.64	1.00
NEA	0.94	0.90	-2.99	0.00	-4.98	-8.03	-1.93	0.00	0.996	0.98	1.01	0.65	-7.60	-17.77	2.57	0.14
Malay	0.97	0.90	-0.90	0.37	-3.87	-8.76	1.02	0.12	1.007	0.98	1.04	0.65	0.51	-15.56	16.57	0.95
Other mix	1.05	0.96	1.00	0.32	3.83	-2.97	10.63	0.27	1.005	0.97	1.04	0.81	9.41	-12.51	31.33	0.40
GA	0.97	0.97	-0.19	0.85	-0.07	-2.14	2.00	0.95	0.999	0.99	1.01	0.82	-1.66	-8.56	5.24	0.64
AA	0.97	0.93	-1.15	0.25	-0.69	-4.16	2.77	0.69	0.983	0.97	1.00	0.06	-2.29	-13.15	8.56	0.68

White ancestry and the common homozygous (GG) genotype are reference categories

*CDC growth charts (http://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html)

Table 3.19 *LEP A19G* variant predicting DXA body fat variables (n=400) (Exponential models except for DXA trunk-to-periphery fat ratio, with GEE adjustment)

Variable	DXA total body fat, g				DXA trunk fat, g				DXA peripheral fat, g				DXA trunk-to-periphery fat ratio			
	Exp β	95% CI		p	Exp β	95% CI		p	Exp β	95% CI		p	β	95% CI		p
Intercept	6602.48	4580.21	9518.57		2458.48	1768.52	3417.28		2732.85	2043.26	3654.81		0.730	0.602	0.859	<0.01
Age, yr	1.071	1.046	1.096	<0.01	1.090	1.069	1.112	<0.01	1.084	1.065	1.104	<0.01	0.015	0.007	0.024	0.00
NEA	0.800	0.687	0.932	0.00	0.794	0.671	0.939	0.01	0.748	0.647	0.863	<0.01	0.079	0.027	0.132	0.00
Malay	0.944	0.731	1.219	0.66	0.960	0.723	1.274	0.78	0.874	0.681	1.122	0.29	0.098	0.010	0.187	0.03
Other mix	1.182	0.832	1.679	0.35	1.232	0.828	1.832	0.30	1.153	0.832	1.597	0.39	0.053	-0.063	0.168	0.37
GA	1.000	0.894	1.117	0.99	0.985	0.870	1.115	0.81	0.993	0.891	1.107	0.90	-0.023	-0.059	0.014	0.22
AA	0.950	0.788	1.144	0.59	0.937	0.763	1.150	0.53	0.946	0.787	1.136	0.55	-0.013	-0.093	0.066	0.74

White ancestry and the common homozygous (GG) genotype are reference categories

Table 3.20 *LEP A19G* variant predicting anthropometrically measured fat variables (n=578) (Exponential models with GEE adjustment)

Variable	Waist circumference, cm			Hip circumference, cm			Waist-to-hip Ratio			BMI percentile*						
	Exp β	95% CI		p	Exp β	95% CI		p	Exp β	95% CI		p	β	95% CI		p
Intercept	52.26	49.48	55.20		49.80	45.83	53.78		0.924	0.898	0.950		60.58	48.89	72.26	
Age, yr	1.020	1.017	1.023	<0.01	3.074	2.839	3.309	<0.01	0.984	0.983	0.986	<0.01	0.035	-0.592	0.662	0.91
NEA	0.945	0.911	0.980	0.00	-4.988	-7.672	-2.303	0.00	1.000	0.983	1.018	0.96	-7.434	-16.672	1.804	0.11
Malay Other mix	0.975	0.912	1.044	0.47	-3.823	-8.393	0.746	0.10	1.013	0.984	1.044	0.37	0.771	-14.330	15.873	0.92
GA	1.051	0.956	1.155	0.31	3.733	-3.072	10.539	0.28	1.007	0.970	1.046	0.70	10.094	-11.745	31.932	0.37
AA	0.993	0.965	1.021	0.61	-0.425	-2.417	1.567	0.68	0.998	0.986	1.010	0.74	-2.496	-9.134	4.142	0.46
AA	0.994	0.945	1.045	0.80	-1.430	-4.499	1.639	0.36	1.009	0.984	1.034	0.50	-4.388	-15.350	6.573	0.43

White ancestry and the common homozygous (GG) genotype are reference categories

*CDC growth charts (http://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html)

Table 3.21 *LEP G-2548A* variant predicting DXA body fat variables (n=400) (Exponential models except for DXA trunk-to-periphery fat ratio, with GEE adjustment)

Variable	DXA total body fat, g			DXA trunk fat, g			DXA peripheral fat, g			DXA trunk-to-periphery fat ratio						
	Exp β	95% CI		p	Exp β	95% CI		p	Exp β	95% CI		p	β	95% CI		P
Intercept	6557.08	4512.02	9530.00		2399.94	1729.00	3331.24		2648.11	1980.10	3541.48		0.736	0.607	0.866	
Age, yr	1.071	1.046	1.097	<0.01	1.091	1.070	1.113	<0.01	1.084	1.065	1.104	<0.01	0.016	0.008	0.024	0.00
NEA	0.798	0.685	0.930	0.00	0.793	0.670	0.939	0.01	0.756	0.655	0.872	0.00	0.072	0.018	0.126	0.01
Malay	0.952	0.738	1.229	0.71	0.962	0.727	1.274	0.79	0.877	0.686	1.123	0.30	0.100	0.007	0.192	0.03
Other mix	1.174	0.826	1.670	0.37	1.219	0.818	1.814	0.33	1.155	0.834	1.599	0.38	0.042	-0.073	0.156	0.48
GA	1.006	0.897	1.127	0.92	1.017	0.896	1.154	0.80	1.042	0.935	1.161	0.46	-0.029	-0.070	0.012	0.17
GG	0.983	0.847	1.141	0.82	0.962	0.815	1.136	0.65	1.008	0.874	1.163	0.91	-0.053	-0.109	0.004	0.07

White ancestry and the common homozygous (AA) genotype are reference categories

Table 3.22 *LEP G-2548A* variant predicting anthropometrically measured fat variables (n=579) (Exponential models with GEE adjustment)

Variable	Waist circumference, cm				Hip circumference, cm				Waist-to-hip Ratio				BMI percentile*			
	Exp β	95% CI		p	β	95% CI		p	Exp β	95% CI		p	β	95% CI		p
Intercept	52.185	49.368	55.158		49.528	45.436	53.619		0.925	0.899	0.952		59.779	47.227	72.330	
Age, yr	1.020	1.017	1.023	<0.01	3.064	2.829	3.299	<0.01	0.984	0.983	0.986	<0.01	0.006	-0.623	0.634	0.99
NEA	0.946	0.912	0.981	0.00	-4.984	-7.695	-2.274	0.00	1.002	0.985	1.019	0.84	-7.078	-16.555	2.399	0.14
Malay	0.980	0.916	1.049	0.56	-3.697	-8.244	0.850	0.11	1.017	0.987	1.047	0.28	2.723	-12.366	17.812	0.72
Other mix	1.049	0.955	1.153	0.32	3.559	-3.212	10.330	0.30	1.008	0.971	1.046	0.67	9.337	-12.253	30.926	0.40
GA	1.000	0.970	1.030	0.98	0.552	-1.457	2.560	0.59	0.993	0.980	1.006	0.30	-0.210	-7.272	6.853	0.95
GG	0.997	0.959	1.036	0.88	-0.777	-3.384	1.831	0.56	1.005	0.988	1.022	0.55	-1.731	-10.870	7.408	0.71

White ancestry and the common homozygous (AA) genotype are reference categories

*CDC growth charts (http://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html)

Since *LEPR* Q223R was associated with TPF_R, the model was further explored, adjusting for Tanner stages. The rarer homozygote (AA) genotype had lower TPF_R than the common homozygote (GG) genotype even after adjusting for Tanner breast stage (but not Tanner pubic hair stage) in separate models (Table 3.23). But both breast and pubic hair stage were positively associated with TPF_R. North East Asian ancestry was not significant. When both the Tanner breast and pubic hair stage were adjusted simultaneously, neither the Tanner stages nor the rarer homozygote genotype (AA) predicted TPF_R (data not shown).

Table 3.23 TPF_R predicted by *LEPR* Q223R genotypes adjusting for Tanner stages

Variable	DXA trunk-to-periphery fat ratio n=343			DXA trunk-to-periphery fat ratio n=354				
	β	95% CI	p	β	95% CI	p		
Intercept	0.774	0.631	0.918	0.745	0.604	0.886		
Age, yr	0.006	-0.006	0.017	0.95	0.009	-0.001	0.020	0.07
NEA	0.080	0.018	0.142	2.53	0.060	-0.001	0.121	0.06
Malay	0.082	-0.013	0.178	1.69	0.072	-0.027	0.171	0.15
Other mix	0.030	-0.105	0.165	0.43	0.021	-0.110	0.151	0.76
Tanner breast, stages 1-5					0.024	0.005	0.043	0.016
Tanner pubic hair, stages 1-5	0.028	0.003	0.054	0.02				
GA	-0.016	-0.059	0.027	0.46	-0.023	-0.065	0.020	0.30
AA	-0.066	-0.136	0.005	0.07	-0.076	-0.147	-0.005	0.03

White ancestry and common homozygote (GG) genotype are reference categories

The model with Tanner breast and *LEPR* Q223R predicting TPF_R in the above table (Table 3.23) was further adjusted for physical activity and energy intake. The rarer homozygote (AA) still had lower TPF_R than the common homozygote (AA) even after adjusting for Tanner breast, physical activity and energy intake (Table 3.24). North East Asian ancestry was not significant after these adjustments.

Table 3.24 TPF_R predicted by *LEPR* Q223R genotypes, adjusting for Tanner breast, physical activity and energy intake

Variable	DXA trunk-to-periphery fat ratio n=336			p
	β	95% CI		
Intercept	0.779	0.627	0.931	
Age, yr	0.008	-0.003	0.019	0.13
NEA	0.056	-0.006	0.117	0.08
Malay	0.046	-0.051	0.142	0.35
Other mix	0.023	-0.112	0.156	0.74
Physical activity, METs	0.004	-0.320	0.327	0.98
Energy intake, kcal	-0.016	-0.047	0.016	0.33
Tanner breast, stages 1-5	0.029	0.010	0.047	0.003
GA	-0.033	-0.076	0.010	0.13
AA	-0.084	-0.154	-0.014	0.02

White ancestry and common homozygote are reference categories

Based on the above model (Table 3.24), mean TPF_R by genotypes are provided below in Table 3.24a. Girls with the common homozygous genotype (GG) had the highest and the rarer homozygous genotype (AA) had the lowest TPF_R, and p for trend = 0.02.

Table 3.24a Mean TPF_R by *LEPR* Q223R genotypes, adjusting for mean age, mean physical activity, mean energy intake, mean Tanner breast and ancestry

<u>Genotype</u>	<u>Mean</u>	<u>95% CI</u>	
AA	0.87	0.82	0.93
GA	0.92	0.88	0.97
GG	0.96	0.90	1.02

Mean age (yr) = 13.36
 Mean physical activity (METs) = 60.16
 Mean energy intake (kcal) = 1793.79
 Mean Tanner breast (1-5) = 3.36

Summary of Aim 2 results

1. The rarer homozygote (AA) for *LEPR* Q223R had lower DXA TPF_R adjusting for Tanner breast, physical activity and energy intake.
2. Variants at *LEP* A19G and G-2548A were not associated with waist, hip circumference, WHR or DXA body fat variables.

Aim 3: To determine whether total body fat or body fat distribution is associated with plasma leptin levels in adolescent girls in Hawaii

A cross sectional study design with 207 girls with hormone data was used. Since plasma leptin was grossly non-normal, exponential models with GEE adjustments were used.

Independent variables, DXA total body fat, DXA trunk fat, DXA peripheral, DXA trunk-to-periphery fat ratio, and sum of skinfolds were used in separate models. Also in separate models, dietary factors influencing plasma leptin were explored. Models were adjusted for age and ancestry as potential confounders. Descriptive statistics for aim 3 are shown in Table 3.25. The genotype distribution was presented earlier in Table 3.11.

Table 3.25 Descriptive statistics for aim 3

<u>Variable</u>	<u>N</u>	<u>Mean</u>	<u>SD</u>
Age, yr	198	15.55	1.430
North East Asian proportion	193	0.33	0.360
Malay proportion	193	0.12	0.240
White proportion	193	0.44	0.330
Other mix proportion	193	0.10	0.160
DXA total body fat, kg	202	18.38	8.400
DXA trunk fat, kg	202	8.98	4.540
DXA peripheral fat, kg	202	8.74	3.850
DXA trunk-to-periphery fat ratio	202	1.01	0.170
Waist circumference, cm	203	69.39	8.38
Hip circumference, cm	203	95.73	8.39
Waist-to-hip ratio	203	0.72	0.040
Sum of skinfold thickness, mm	200	92.07	34.440
Skinfold trunk-to-periphery fat ratio	200	0.75	0.160
Plasma leptin, ng/ml	203	17.66	16.570

All the body fat variables measured by both DXA and anthropometry were significantly associated with plasma leptin level (Tables 3.26 & 3.27).

For every kilogram (kg) increase in DXA total body fat, mean plasma leptin level increased by 6.5% (Table 3.26, Model 1). For a kg increase in DXA trunk fat, leptin increased by 12% and a kg increase in DXA peripheral fat, leptin increased by 15% (Table 3.26, Models 2 & 3). For every unit increase in DXA trunk-to-periphery fat ratio, plasma leptin level increased by about 210% (Table 3.26, Model 4).

Table 3.26 Association between DXA body fat variables and plasma leptin level

Model	Variable	Plasma leptin level (ng/ml) (n=192)			
		exp β	95% CI		p
1	Intercept	6.243	1.947	20.015	
	Age, yr	0.991	0.913	1.076	0.83
	NEA	0.824	0.582	1.168	0.28
	Malay	0.739	0.483	1.132	0.16
	Other mix	0.525	0.299	0.922	0.02
	DXA total body fat, kg	1.065	1.055	1.075	<0.0001
2	Intercept	6.903	2.037	23.397	
	Age, yr	0.995	0.914	1.083	0.90
	NEA	0.803	0.565	1.141	0.22
	Malay	0.738	0.477	1.141	0.17
	Other mix	0.517	0.296	0.906	0.02
	DXA trunk fat, kg	1.120	1.101	1.139	<0.0001
3	Intercept	6.195	2.006	19.131	
	Age, yr	0.987	0.912	1.069	0.75
	NEA	0.845	0.595	1.198	0.34
	Malay	0.734	0.483	1.116	0.15
	Other mix	0.532	0.299	0.946	0.03
	DXA peripheral fat, kg	1.149	1.125	1.175	<0.0001
4	Intercept	7.750	1.547	38.830	
	Age, yr	0.995	0.908	1.089	0.91
	NEA	0.571	0.379	0.860	0.01
	Malay	0.567	0.339	0.950	0.03
	Other mix	0.518	0.241	1.114	0.09
	*DXA trunk-to-periphery fat ratio	3.106	1.660	5.811	0.0004
5	Intercept	1.544	0.442	1.555	0.50

Age, yr	0.996	0.917	2.501	0.92
NEA	0.709	0.507	1.661	0.04
Malay	0.638	0.416	1.516	0.04
Other mix	0.443	0.252	1.287	0.00
BMI, kg/m ²	1.123	1.104	3.017	<0.0001

*Trunk-to-periphery fat ratio = (DXA trunk fat)/(DXA peripheral fat)

Waist and hip circumferences and standardized waist-to-hip ratio (WHR) were positively associated with leptin level. For a 1 cm increase in waist and hip circumferences, leptin level increased by about 6% (Table 3.27). For every 1 mm increase in sum of skinfold thickness, plasma leptin level increased by 2% and for every 0.1 unit increase in trunk-to-periphery fat skinfold ratio, plasma leptin level increased by 160% (Table 3.27, Models 4 & 5). Peripheral (biceps + triceps + calf skinfolds) and trunk (subscapular + iliac skinfolds) skinfold measures were also positively associated with leptin level (results not shown).

North East Asian and Malay ancestries were significant positive predictors of plasma leptin level only in the models adjusting for DXA TPF and trunk-to-periphery fat skinfold ratio (Table 3.26, Model 4 & Table 3.26, Model 5). Once anthropometrically measured variables were adjusted for DXA total body fat, the association seen in Table 3.27 disappeared (Table 3.28). All the models were also analyzed adjusting for total height of the girl and results did not differ (results not shown).

In a separate model, physical activity was not a predictor of plasma leptin, adjusting for age, ancestry, energy intake and body weight (kg) at exam 3.

Table 3.27 Association between anthropometrically measured body fat variables and plasma leptin level

Model	Variable	Plasma leptin level (ng/ml) (n=190)			
		exp β	95% CI		p
1	Intercept	0.374	0.106	1.323	
	Age, yr	0.993	0.915	1.078	0.87
	NEA	0.804	0.575	1.124	0.20
	Malay	0.745	0.483	1.148	0.18
	Other mix	0.485	0.281	0.836	0.01
	Waist circumference, cm	1.059	1.050	1.068	<0.0001
2	Intercept	0.086	0.026	0.280	
	Age, yr	0.968	0.886	1.058	0.47
	NEA	0.815	0.557	1.193	0.29
	Malay	0.805	0.508	1.276	0.36
	Other mix	0.474	0.257	0.877	0.02
	Hip circumference, cm	1.063	1.051	1.075	<0.0001
3	Intercept	0.166	0.010	2.843	
	Age, yr	1.014	0.926	1.110	0.77
	NEA	0.650	0.444	0.952	0.03
	Malay	0.569	0.348	0.932	0.03
	Other mix	0.531	0.265	1.063	0.07
	Waist to hip ratio [†] (WHR) (standardized)	1.307	1.154	1.479	<0.0001
4 n=190	Intercept	4.527	1.266	16.192	
	Age, yr	0.998	0.916	1.087	0.96
	NEA	0.785	0.574	1.073	0.13
	Malay	0.679	0.445	1.035	0.07
	Other mix	0.542	0.315	0.933	0.03
	Sum of skinfold thicknesses*, mm	1.015	1.013	1.017	<0.0001
5 n=190	Intercept	11.807	2.493	55.913	
	Age, yr	0.997	0.910	1.091	0.94
	NEA	0.565	0.375	0.853	0.01
	Malay	0.507	0.281	0.914	0.02
	Other mix	0.520	0.209	1.291	0.16
	Trunk-to-periphery fat skinfold ratio ^{**}	2.594	1.351	4.980	0.004

[†]Standardized WHR = WHR/SD

*Sum of skinfold thicknesses = subscapular+iliac+triceps+biceps+calf

**Trunk-to-periphery skinfold fat ratio = (subscapular+iliac)/(triceps+biceps+calf)

Table 3.28 Association between anthropometrically measured body fat variables and plasma leptin level after adjusting for DXA total body fat (n=192)

Model	Variable	Plasma leptin level (ng/ml)			
		exp β	95% CI		p
1	Intercept	12.870	1.509	109.804	
	Age, yr	0.992	0.913	1.077	0.84
	NEA	0.819	0.571	1.176	0.28
	Malay	0.732	0.475	1.128	0.16
	Other mix	0.530	0.293	0.959	0.04
	DXA total body fat, g	1.000	1.000	1.000	<0.0001
	Waist circumference, cm	0.986	0.958	1.015	0.35
2	Intercept	5.073	0.613	41.997	
	Age, yr	0.990	0.903	1.085	0.83
	NEA	0.825	0.575	1.185	0.30
	Malay	0.743	0.465	1.187	0.21
	Other mix	0.522	0.300	0.908	0.02
	DXA total body fat, g	1.000	1.000	1.000	<0.0001
	Hip circumference, cm	1.003	0.972	1.035	0.86
3	Intercept	28.551	1.106	736.935	
	Age, yr	0.990	0.899	1.089	0.83
	NEA	0.799	0.543	1.176	0.25
	Malay	0.746	0.456	1.220	0.24
	Other mix	0.512	0.285	0.922	0.03
	DXA total body fat, g	1.000	1.000	1.000	<0.0001
	Waist to hip ratio	0.921	0.798	1.063	0.26
4 n=189	Intercept	5.041	1.403	18.120	
	Age, yr	0.997	0.915	1.085	0.94
	NEA	0.817	0.576	1.159	0.26
	Malay	0.732	0.459	1.169	0.19
	Other mix	0.543	0.308	0.957	0.03
	DXA total body fat, g	1.000	1.000	1.000	0.01
	Sum of skinfolds, mm	1.005	0.997	1.013	0.19
5 n=189	Intercept	6.185	1.702	22.479	
	Age, yr	0.998	0.919	1.083	0.95
	NEA	0.832	0.572	1.209	0.33
	Malay	0.774	0.475	1.261	0.30
	Other mix	0.552	0.304	1.001	0.05
	DXA total body fat, g	1.000	1.000	1.000	<0.0001
	Trunk-to-periphery fat skinfold ratio	0.834	0.465	1.495	0.54

*Sum of skinfold thicknesses = subscapular+iliac+triceps+biceps+calf

**Trunk-to-periphery fat skinfold ratio = (subscapular+iliac)/(triceps+biceps+calf)

Dietary predictors of plasma leptin level

Dietary predictors of plasma leptin (energy intake and macronutrients and dietary fiber adjusted for energy) were tested. A cross sectional study design with 207 girls who had hormone data at exam 3 was used. Since plasma leptin was grossly non-normal, exponential models with GEE adjustment for standard errors were used, adjusting for potential confounders of age and ancestry. Dietary descriptive statistics are provided in Table 3.29a.

Table 3.29a Descriptive statistics for dietary variables (n=198)

<u>Variable</u>	<u>Mean</u>	<u>SD</u>
Energy intake, kcal	1,769	527
Protein, g	67.33	22.06
Fat, g	68.2	25.51
Carbohydrate, g	225.42	76
Fiber, g	11.88	5.17

The Acceptable Macronutrient Distribution Range (AMDR) (201) was calculated for all the macronutrients (Table 3.29b). Girls in this study, on an average were getting 15% of their calories from protein, 34% from fat and 51% from carbohydrates.

Table 3.29b Acceptable Macronutrient Distribution Range (AMDR) (n=198)

<u>Variable</u>	<u>Mean</u>	<u>SD</u>
Protein	15.46	3.39
Fat	34.47	6.52
Carbohydrate	50.95	8.26

Adjusting for age only, there was no difference in dietary variables among the 100% ancestral groups (Whites, NEA, Malay & Other) (result not shown). None of the dietary variables (energy intake, fat, carbohydrate or fiber), except for protein, were associated with plasma leptin level after adjusting for age, ancestral group, and BMI (Table 3.30a). A gram increase in protein intake was associated with approximately a 1% decrease in plasma leptin. Adjusting for DXA total body fat (instead of BMI), protein was not a significant predictor of plasma leptin level (Table 3.30b). Adjusting for physical activity and LEP/LEPR variants in separate models, protein was not significant (results not shown). In a model in which all dietary factors (energy, protein, carbohydrates, fat and fiber intake) were included simultaneously (adjusting for age, ancestry and BMI), none of them were significant (results not shown).

Table 3.30a Dietary factors affecting plasma leptin level adjusting for BMI (exponential models with GEE adjustment; n=191)

<u>Model</u>	<u>Independent variables</u>	<u>exp β</u>	<u>95% CI</u>		<u>p</u>
1	Energy intake, kcal/1000	0.917	0.771	1.090	0.32
2	Protein, g	0.992	0.985	1.000	0.04
3	Fat, g	0.999	0.993	1.006	0.86
4	Carbohydrates, g	1.001	0.999	1.004	0.32
5	Fiber, g	1.008	0.985	1.031	0.49

All models were adjusted for age, ancestry, BMI and energy intake except for model 1 was adjusted for age, ancestry, and BMI only.

Table 3.30b Dietary factors affecting plasma leptin level adjusting for DXA total body fat (exponential models with GEE adjustment; n=191)

<u>Model</u>	<u>Independent variables</u>	<u>exp β</u>	<u>95% CI</u>		<u>p</u>
1	Energy intake, kcal/1000	0.975	0.823	1.153	0.76
	Protein, g	0.994	0.987	1.001	0.08
3	Fat, g	0.999	0.993	1.005	0.79
4	Carbohydrates, g	1.001	0.999	1.003	0.29
5	Fiber, g	1.012	0.989	1.034	0.30

All models were adjusted for age, ancestry, DXA total body fat and energy intake except for model 1 was adjusted for age, ancestry, and DXA total body fat only.

Summary of Aim 3 results

1. Body fat variables measured by DXA (total body fat, trunk fat, peripheral fat and trunk-to-periphery fat ratio [TPFR]) and anthropometry (waist, hip circumferences, waist-to-hip ratio [WHR], skinfolds) were significant positive predictors of plasma leptin.

2. Protein intake was negatively associated with plasma leptin, adjusted for BMI, but not for DXA total body fat.

Aim 4: To determine whether selected SNPs in LEP and LEPR genes are associated with AAM in adolescent girls in Hawaii

4a: For the first part, menarche was used as an event when the independent variables like genotypes and birth weight were used in the model. A longitudinal study design was used for 366 girls from exams 2 and 3 with time from birth to an event of reaching menarche. A COX regression model was used with achieved menarche as a censoring variable. Independent variables *LEP* and *LEPR* genotypes were examined in separate models adjusting for age and ancestry as potential confounders. Girls who were lost to follow up were also censored at the last exam they were seen (n=102). For example: Premenarcheal girls at exam 1 who did not come to either exam 2 or 3, were censored at exam 1. The descriptive statistics provided in Table 3.31 show that there was not much difference between the girls who were lost to follow-up (at exam 1 and exam 3) and the rest of the girls, except that lost girls tended to be younger.

Table 3.31 Comparison between girls who were lost to follow-up* with the rest of the girls

<u>Variable</u>	<u>Not lost to follow-up</u>			<u>Lost to follow-up</u>		
	<u>N</u>	<u>Mean</u>	<u>SD</u>	<u>N</u>	<u>Mean</u>	<u>SD</u>
Age, yr	383	14.72	1.95	102	10.95	1.16
White	383	0.42	0.35	99	0.48	0.35
NEA	383	0.37	0.38	99	0.27	0.33
Malay	383	0.11	0.23	99	0.12	0.24
Other	383	0.11	0.17	99	0.12	0.19
Birth weight, lb	364	7.18	1.13	90	7.18	1.23

*Lost to follow-up at exam 1 and exam 3

At exam 1, 50% had reached menarche by 11.73 yr (25% by 11.04 yr and 75% by 12.5 yr)

At exam 2, 50% of had reached menarche by 12.07 yr (25% by 11.50 yr and 75% by

12.55 yr). At exam 3, 50% of had reached menarche by 12.5 yr (25% by 11.50 yr and

75% by 13.49 yr).

Table 3.32 demonstrates that neither birth weight nor genotype affected the age of the girl's event of reaching menarche, after adjusting for birth weight, except for girls who were heterozygous for *LEP* A-2458G; they had a hazards ratio of 1.4 to reach menarche earlier ($p = 0.02$) (Table 3.32 Model 4).

Table 3.32 Genotypes predicting time to reach menarche in a COX regression model
(n=305; events=286; censored=19)

Independent variables	β	Error	p	Hazards Ratio	95% CI	
Model 1 (n=358; events=338; censored=20)						
NEA	0.338	0.181	0.061	1.403	0.984	1.999
Malay	0.319	0.251	0.205	1.375	0.84	2.25
Other ancestry	0.383	0.372	0.304	1.466	0.707	3.042
Birth weight, kg	-0.095	0.124	0.447	0.91	0.713	1.161
Model 2						
NEA	0.274	0.219	0.212	1.315	0.856	2.021
Malay	0.212	0.289	0.463	1.237	0.701	2.18
Other ancestry	0.304	0.412	0.460	1.356	0.604	3.041
Birth weight, kg	-0.161	0.139	0.248	0.852	0.648	1.119
<i>LEPR</i> Q223R AG	-0.139	0.136	0.306	0.870	0.666	1.136
<i>LEPR</i> Q223R AA	-0.050	0.219	0.818	0.951	0.619	1.46
Model 3						
NEA	0.355	0.200	0.076	1.427	0.963	2.113
Malay	0.258	0.278	0.353	1.294	0.751	2.23
Other ancestry	0.344	0.398	0.387	1.411	0.647	3.076
Birth weight, kg	-0.174	0.139	0.209	0.840	0.64	1.103
<i>LEP</i> A19G AG	0.210	0.128	0.102	1.233	0.959	1.586
<i>LEP</i> A19G AA	-0.209	0.222	0.348	0.812	0.525	1.255
Model 4						
NEA	0.315	0.204	0.122	1.371	0.919	2.045
Malay	0.186	0.284	0.514	1.204	0.689	2.103
Other ancestry	0.439	0.393	0.263	1.551	0.719	3.348
Birth weight, kg	-0.175	0.140	0.209	0.839	0.638	1.103
<i>LEP</i> A-2458G AG	0.312	0.138	0.024	1.366	1.043	1.79
<i>LEP</i> A-2458G GG	0.007	0.181	0.971	1.007	0.706	1.435

For the second part of the aim, the time to reach menarche since exam 1 was computed and used as a dependent variable when independent variables of body fat or type of body fat distribution were used in the models. Premenarcheal girls at exam 1 were selected for this analysis. A COX regression model was used, with time to reach menarche since exam 1 as the dependent variable, and achievement of menarche as a censoring variable.

Factors influencing time to reach menarche since exam 1 among premenarcheal girls were examined (Tables 3.33 - 3.67). As expected, girls who were heavier (weight, kg) and taller (height, cm) reached menarche earlier after adjusting for the potential confounders of age and ancestral group (Table 3.33, Models 1 & 2). Physical activity, energy intake and macronutrients did not affect time to reach menarche (Table 3.34).

In separate models, body size variables were tested and body mass index (BMI) was the only variable that had a significant hazards ratio of 1.04 (Table 3.36, Model 2). None of the ancestral groups had an effect on time to reach menarche. Body fat measured by skinfolds had no effect on time to reach menarche (Table 3.36) (DXA was not available for this analysis that included events from exam 1).

There were only 22 girls who were premenarcheal at exam 2 and hence no analysis was done with these girls.

Table 3.33 Weight, height and BMI variables at exam 1 predicting premenarcheal girl's time to reach menarche in a COX regression model (n=122 and number of events=102)

Independent variables	β	Error	p	Hazards Ratio	95% CI	
Model 1						
Age, yr	0.388	0.089	<.0001	1.47	1.238	1.754
NEA	0.381	0.317	0.23	1.46	0.786	2.722
Malay	-1.039	0.651	0.11	0.35	0.099	1.267
Other ancestry	0.306	0.955	0.75	1.36	0.209	8.819
Weight, kg	0.025	0.010	0.01	1.03	1.005	1.047
Model 2						
Age, yr	0.284	0.112	0.01	1.33	1.066	1.655
NEA	0.488	0.325	0.13	1.63	0.861	3.081
Malay	-1.178	0.651	0.07	0.31	0.086	1.103
Other ancestry	0.626	0.981	0.52	1.87	0.273	12.799
Height, cm	0.040	0.017	0.02	1.04	1.007	1.075
Model 3						
Age, yr	0.474	0.083	<0.0001	1.61	1.366	1.888
NEA	0.349	0.313	0.26	1.42	0.768	2.618
Malay	-1.040	0.655	0.11	0.35	0.098	1.276
Other ancestry	0.357	0.959	0.71	1.43	0.218	9.363
BMI, kg/m ²	0.046	0.018	0.01	1.05	1.011	1.085

Table 3.34 Physical activity and dietary variables at exam 1 predicting premenarcheal girl's time to reach menarche in a COX regression model (n=116 and number of events=97)

<u>Independent variables</u>	<u>β</u>	<u>Error</u>	<u>p</u>	<u>Hazards Ratio</u>	<u>95% CI</u>	
Model 1						
Age, yr	0.643	0.110	<.0001	1.903	1.534	2.360
NEA	0.532	0.342	0.12	1.702	0.871	3.327
Malay	-1.540	0.719	0.03	0.214	0.052	0.877
Other ancestry	-0.412	1.090	0.71	0.663	0.078	5.612
Energy intake, kcal	0.000	0.0003	0.39	1.000	0.999	1.000
Physical activity, METs	-0.002	0.004	0.66	0.998	0.992	1.005
Model 2						
Age, yr	0.645	0.110	<.0001	1.91	1.536	2.364
NEA	0.577	0.327	0.08	1.78	0.939	3.379
Malay	-1.525	0.719	0.03	0.22	0.053	0.890
Other ancestry	-0.397	1.081	0.71	0.67	0.081	5.601
Energy intake, kcal	-0.0003	0.0003	0.33	1.00	0.999	1.000
Model 3						
Age, y	0.666	0.112	<.0001	1.946	1.564	2.422
NEA	0.575	0.325	0.08	1.776	0.940	3.357
Malay	-1.594	0.733	0.03	0.203	0.048	0.855
Other ancestry	-0.521	1.105	0.64	0.594	0.068	5.175
Energy intake, kcal	-0.001	0.000	0.14	0.999	0.998	1.000
Carbohydrates, g	0.003	0.003	0.26	1.003	0.997	1.009
Model 4						
Age, yr	0.650	0.110	<.0001	1.915	1.543	2.378
NEA	0.600	0.329	0.07	1.821	0.955	3.473
Malay	-1.539	0.724	0.03	0.215	0.052	0.888
Other ancestry	-0.390	1.087	0.72	0.677	0.080	5.698
Energy intake, kcal	0.000	0.000	0.76	1.000	0.999	1.001
Protein, g	-0.004	0.008	0.63	0.996	0.980	1.013
Model 5						
Age, yr	0.669	0.112	<.0001	1.953	1.568	2.432
NEA	0.532	0.326	0.10	1.703	0.898	3.228
Malay	-1.634	0.735	0.03	0.195	0.046	0.824
Other ancestry	-0.618	1.115	0.58	0.539	0.061	4.794
Energy intake, kcal	0.000	0.000	0.58	1.000	0.999	1.001
Dietary fat, g	-0.013	0.010	0.19	0.988	0.969	1.006

Table 3.35 Body size variables at exam 1 predicting premenarcheal girl's time to reach menarche in a COX regression model (n=122 and number of events=102)

Independent variables	β	Error	p	Hazards Ratio	95% CI	
Model 1						
Age, yr	0.305	0.115	0.01	1.36	1.082	1.700
NEA	0.470	0.328	0.15	1.60	0.841	3.045
Malay	-1.115	0.654	0.09	0.33	0.091	1.182
Other ancestry	0.462	0.977	0.64	1.59	0.234	10.784
Height, cm	0.024	0.021	0.26	1.02	0.982	1.068
Weight, kg	0.016	0.014	0.23	1.02	0.990	1.044
Model 2						
Age, yr	0.333	0.115	0.00	1.40	1.114	1.747
NEA	0.502	0.329	0.13	1.65	0.867	3.151
Malay	-1.119	0.656	0.09	0.33	0.090	1.183
Other ancestry	0.458	0.974	0.64	1.58	0.234	10.655
Height, cm	0.030	0.017	0.07	1.03	0.997	1.065
BMI	0.043	0.022	0.05	1.04	0.999	1.090
Model 3						
Age, yr	0.320	0.122	0.01	1.38	1.085	1.748
NEA	0.481	0.327	0.14	1.62	0.852	3.074
Malay	-1.178	0.652	0.07	0.31	0.086	1.106
Other ancestry	0.588	0.972	0.55	1.80	0.268	12.108
Height, cm	0.034	0.019	0.07	1.03	0.997	1.072
BMI percentile	0.003	0.004	0.39	1.00	0.996	1.010

Table 3.36 Skinfold measures at exam 1 predicting premenarcheal girl's time to reach menarche in a COX regression model (n=122 and number of events=102)

Independent variables	β	Error	p	Hazards Ratio	95% CI	
Model 1						
Age, yr	0.285	0.113	0.01	1.330	1.065	1.661
NEA	0.482	0.330	0.14	1.620	0.848	3.096
Malay	-1.168	0.662	0.08	0.311	0.085	1.138
Other ancestry	0.609	0.999	0.54	1.838	0.259	13.023
Height, cm	0.039	0.018	0.03	1.040	1.004	1.077
Trunk skinfold*, mm	0.001	0.010	0.93	1.001	0.982	1.020
Model 2						
Age, yr	0.281	0.113	0.01	1.325	1.062	1.653
NEA	0.492	0.326	0.13	1.636	0.863	3.099
Malay	-1.208	0.669	0.07	0.299	0.080	1.110
Other ancestry	0.650	0.992	0.51	1.916	0.274	13.381
Height, cm	0.041	0.018	0.02	1.042	1.006	1.078
Peripheral skinfold**, mm	-0.001	0.007	0.85	0.999	0.984	1.013
Model 3						
Age, yr	0.283	0.113	0.01	1.33	1.063	1.656
NEA	0.491	0.328	0.13	1.63	0.859	3.104
Malay	-1.189	0.666	0.07	0.31	0.083	1.125
Other ancestry	0.638	0.995	0.52	1.89	0.269	13.292
Height, cm	0.040	0.018	0.02	1.04	1.005	1.078
Sum of skinfolds, mm	0.000	0.004	0.94	1.00	0.991	1.008
Model 4						
Age, yr	0.316	0.114	0.01	1.37	1.096	1.716
NEA	0.324	0.344	0.35	1.38	0.704	2.715
Malay	-1.275	0.660	0.05	0.28	0.077	1.019
Other ancestry	0.428	0.986	0.66	1.54	0.222	10.594
Height, cm	0.033	0.018	0.06	1.03	0.998	1.070
Trunk to periphery fat skinfold ratio	-0.273	0.214	0.20	0.76	0.500	1.157

* Trunk skinfold = Subscapular + Iliac skinfolds

** Peripheral skinfold = Biceps + Triceps + Calf skinfolds

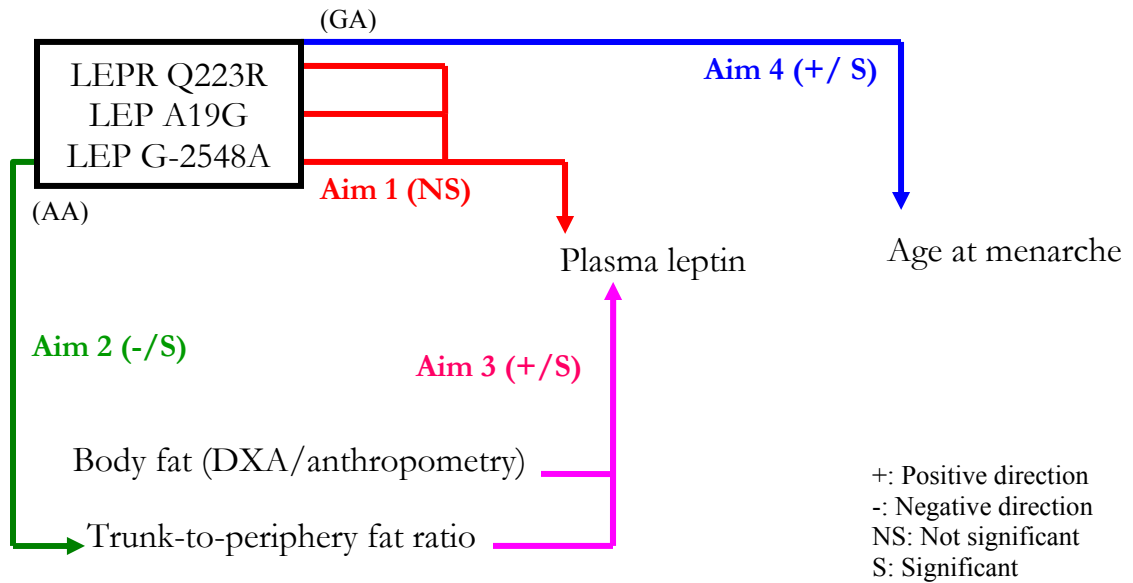
Summary of Aim 4 results

1. Birth weight and the *LEP* variants did not affect the girl's age at menarche.
2. Heterozygote (AG) genotype for *LEP G-2548A* had a hazards ratio of 1.4 to reach menarche early.
3. Taller and heavier girls at exam 1 reached menarche earlier.
4. Recorded physical activity, energy intake and macronutrient intake did not affect age at menarche.

Summary of all results

The results of the present study is summarized in Figure 4.1

Figure 4.1 Summary of associations found in the present study



White girls had a higher mean plasma leptin level than NEA or Malays before adjusting for DXA total body fat but the difference was not significant after adjusting for DXA total body fat. There was no association between plasma leptin level and *LEPR* variants. Girls with the heterozygote (AG) genotype for *LEP* G-2548A had 20% lower plasma leptin levels compared to the common homozygote (AA) ($p=0.048$). The Rarer homozygote for *LEPR* Q223R (AA) had lower DXA TPFR (adjusting for Tanner breast, physical activity and energy intake). The *LEP* A19G and G-2548A variants were not associated with waist, hip circumference, WHR or DXA body fat variables. Body fat variables measured by DXA (total body fat, trunk fat, peripheral fat and trunk-to-periphery fat ratio [TPFR]) and anthropometry (waist, hip circumferences, waist-to-hip ratio [WHR], skinfolds) were significant positive predictors of plasma leptin in our Asian-White population. Protein intake was negatively associated with plasma leptin (adjusted for BMI), but not after adjusting for DXA total body fat. Birth weight or the *LEP* variant did not affect the girl's time to reach menarche. Heterozygote (AG) genotype for *LEP* G-2548A had hazards ratio of 1.4 to reach menarche earlier. Taller and heavier girls at exam 1 reached menarche earlier in this population. Physical activity, energy intake and macronutrient intake did not affect age at menarche.

CHAPTER 4

DISCUSSION

4.1 Association of plasma leptin level with LEP and LEPR polymorphisms

There was no association between *LEP/LEPR* variants and mean plasma leptin in this study. Similarly, there was no significant difference in mean plasma leptin level and the homozygote genotypes, after adjusting for DXA total body fat. However, *LEP* G-2548A heterozygote genotype (AG) had 20% lower plasma leptin levels compared to the common homozygote (AA) ($p=0.048$) (Table 3.15a).

In previous studies, the reported association between *LEP/LEPR* and serum leptin level was conflicting (169, 176, 179-181). Potential limitations of previous studies include, their moderate sample size and lack of ethnic diversity (included predominantly White) populations. Previous studies did not explore associations between body composition variables and polymorphisms separately in subgroups divided by gender and BMI because of the small size of the subgroups, although they did adjust for gender and BMI (considered as both a dichotomous and a continuous variable) in the statistical analysis. The discordant observations in previous studies could be attributed to age-related and/or gender-specific effects of the polymorphisms (171, 172). Several detailed statistical analyses using different genotype models were performed leading to a possibility of inflated type one error.

The present study had statistical power of 80% to detect an effect of plasma leptin difference of 0.5 ng/ml between the two homozygotes. Nonetheless, the lack of

association between genetic variants and plasma leptin could be due to fact that the soluble leptin receptor (sOB-R) (the main leptin binding protein in plasma and determinant of free leptin), which is needed for leptin action, was not measured in this study. Thus, sOB-R may confound or modify the association between leptin and the polymorphism of interest (168). Leptin circulates both bound to sOB-R and as free/total leptin. Leptin also varies according to gender and adiposity (60, 64, 202). Yiannakouris (173) reported a significant interaction of adiposity and genotype (*LEP* G-2548A) in predicting free leptin index (leptin/sOB-R), when considered in addition to gender in healthy subjects. In this present study no such interaction was shown.

Another possible explanation for lack of association between genetic variants and plasma leptin could be that genetic factors, not examined in this study, may influence the effect of *LEP/LEPR* variants. For example, a previous study suggested that the interaction of *LEPR* Q223R with the tyrosine phosphatase 1B (*PTP1B*) gene polymorphism affected the BMI in Finnish subjects (203).

Difference in allele frequencies could explain why our results differed from the previous studies. This study's White subjects had higher A allele frequency compared to the HapMap data for *LEPR* Q223R and NEA had A allele frequency similar to Chinese and Japanese from the HapMap data. For *LEP* G-2548A variant, White and NEA subjects in our population had lower A allele frequency compared to the HapMap data. For the *LEP* A19G variant, both White and NEA had higher A allele frequency compared to the HapMap data.

In this present study a fasting blood sample for plasma leptin was drawn during the luteal phase (20-24th day) of the girl's menstrual cycles for leptin analysis. The variation in leptin levels throughout the menstrual cycle has been intensely studied, but the data are conflicting, possibly because of differences in methodology (especially the number of included subjects, and temporality of sample collections). A number of studies have shown considerable variation in leptin levels during the menstrual cycle, with higher levels in the midluteal rather than the follicular phase (204, 205). However, other studies have shown either small but insignificant trends toward higher serum levels at the end of the cycle or no variability at all (206). A previous study (206) reported that leptin levels were lowest on the first and the last day of the cycle and reached their maximum in a the midluteal phase. Thus, the leptin level during the luteal phase measured by the FAM study is expected to be higher than on other days of menstrual cycle.

North East Asians and Malays had lower plasma leptin levels compared to Whites, after adjusting for *LEP/LEPR* variants in the model. However, these differences were not significantly attenuated by adjusting for DXA total body fat. Since few other studies have measured DXA body fat, this is an important new finding.

A unique way to capture ancestry in a highly mixed population such as the population in Hawaii is presented in this study. In order to obtain the most accuracy, each parent was asked to detail their ancestral background and percentage of it (207). For the purpose of this genetically focused analysis, we grouped ancestry based on geographical approximation (NEA, SEA, Malay and White). In a recent study, adjusted (BMI) mean (95% CI) serum leptin was significantly higher in South Asians (India, Pakistan, Sri

Lanka, or Bangladesh) [11.82 (10.72-13.04)] compared to Europeans [9.21 (8.38-10.12)] (208) but the difference between Europeans and Chinese was not significant (Chinese: 8.25 [7.48-9.10]). However the study by Mente et al., (208) had an overall sample size of 1,176. Our sample was smaller and was not powered to detect differences among ancestral groups.

4.2 Association of LEP and LEPR polymorphisms with total body fat and body fat distribution

In the present study, girls of North East Asian ancestry had higher body fat measures in most of the models compared to Whites. The *LEP/LEPR* variants were not associated with waist, hip circumference, waist-to-hip ratio (WHR) or BMI percentile. However, some trends were observed for the distribution of body fat. For example the rarer homozygote genotype (AA) for *LEPR* Q223R was negatively associated with DXA TFFR (p=0.028) This means that these girls carry proportionately less body fat in their trunk region, or the girls with common homozygote genotype (GG) carry more fat in their trunk region.

The association of the variants p.Q223R with obesity was extensively studied in three meta-analyses (209, 210). The first two reviews (209, 211) selected 20 studies of different populations including African Americans, Pima Indians, and subjects from Canada, Japan, Denmark, Finland, and England. No statistically significant association was found between *LEPR* polymorphism and anthropometric variables, such as BMI and waist circumference. The third systematic review (210) analyzed the raw pooled data

from 18 studies of American, European, Asian, and Oceanian populations. They showed no relationship between the *LEPR* variants and BMI and other anthropometric measurements related to overweight and obesity. The allelic frequency in the 18 studies included in the meta-analysis varied significantly across different countries and ethnic groups. In particular, the frequency of the G allele for Asians was significantly higher than for other ethnicities. On the other hand, the G allele frequency in the White population varied from 0.32–0.58, with an overall rate of 0.45. The authors of these meta-analyses pointed out that the lack of association between *LEPR* polymorphisms and obesity could be due to the complex pathogenesis of obesity, which involves a large number of both genetic and environmental factors (Paracchini et al., 2005). The results were based on studies that used BMI as a marker for the obesity phenotype, while several other methods of defining this condition are available (210). In other non-White populations, such as Japanese and Pima Indians, an association of the leptin receptor gene and obesity was not found (212, 213).

In this present study, *LEP* G-2548A, *LEP* A19G and *LEPR* Q223R variants were not related to any anthropometric measures or with the DXA measured fat variables, except for *LEPR* Q223R and DXA trunk-to-periphery fat ratio (central body fat distribution), which was not measured in the other studies. The present study and also the previous studies were carried out in populations with background variation in other genes, which may mask the effects of the *LEP/LEPR* genes (214). Our sample was composed of multiethnic subjects living in Hawaii. The general population of Hawaii constitutes an admixture of populations arising from different ancestral groups. This population differs,

with respect to other populations of previous studies (210, 211), not only in genetic background, but also in culture, traditions, climate, type of diet, lifestyle, and prevalence of exposure to common environmental risk factors for obesity and related disorders.

In epidemiological studies, upper body rather than lower body obesity has been associated with an increased risk of diabetes and cardiovascular disease. Abdominal fat (central adiposity) has been linked to metabolic disturbances that contribute to the coronary risk (215). Previous studies analyzed three groups according to BMI (lean, overweight, and obese individuals) and found a significant association between intra-abdominal adipose tissue area and levels of fasting blood glucose, HDL cholesterol, and serum triglyceride only for the overweight and obese (215, 216). Waist circumference was shown to be significantly correlated with subcutaneous fat and visceral fat (measured by ultrasonography, 1cm above umbilicus) in normal subjects and therefore with central obesity (98, 217). Harmelen et al., (98) found a strong positive correlation between the secretion rate of leptin in subcutaneous and omental fat tissue, which in turn could be due to increased cell size and leptin gene expression. The present study did not show any association between *LEP/LEPR* variants and waist circumference.

Because there is a strong gender difference in fat distribution and because leptin expression is lower in visceral compared with subcutaneous fat (98), fat distribution has been suggested as a potential mechanism for the sexual dimorphism in serum leptin levels. The study by Thomas et al., (218) observed comparable relationships between serum leptin and trunkal or peripheral fat content among adults, and total fat mass was a

better predictor than any specific fat content site. Although trunkal fat measured by DXA is strongly correlated with intra-abdominal adipose tissue (219) this method does not differentiate subcutaneous and visceral fat and has been considered only a surrogate for the latter.

Biologic plausibility

Fat cells in the abdominal region have been thought to be more sensitive to nutritional and/or hormonal factors than fat cells in other regions (220). In the presence of sex steroid hormones, a normal gynoid distribution of body fat exists in females. With a decrease in sex steroid hormones, as occurs with aging, there is a tendency to increase central obesity (221, 222), as is seen in post-menopausal women. Further, women given exogenous androgens, or suffering from virilizing tumors, develop a more central adipose tissue distribution. Thus the lower TPF_R in girls with rarer homozygote (AA) for *LEPR* Q223R compared to common homozygote (GG) suggests that the *LEPR* variant at the hypothalamus binds with free leptin to activate the HPG axis differently, leading to increased sex steroid hormones and resulting in lower central adiposity.

The present study result was similar to that of a study by Wauters et al. (179) where, they found that the carriers of the rare allele (heterozygote {AG} and rarer homozygote {AA}) for *LEPR* had lower total abdominal fat (cm²) measured by CT scan, compared to the non-carriers (common homozygote {GG}) in postmenopausal women. In the present study, replicating the analysis as mentioned above did not reveal any significant difference in TPF_R. The Wauters et al. study also found significant positive association

of subcutaneous fat depot (measured by CT scan) with different *LEPR* polymorphism (Lys656Asn) and suggested that these leptin receptors present on adipose tissue could mediate an autocrine or paracrine function of leptin to influence fat distribution.

An association between blood leptin levels and subcutaneous fat distribution was reported previously, a phenomenon that is thought to be related to the higher leptin secretion by subcutaneous fat compared to visceral fat (97, 179). Also, subcutaneous gluteal fat contains more leptin mRNA than does abdominal fat (visceral fat). In this study, lower TPIR (lower central adiposity) in girls with AA genotype for *LEPR* Q223R versus GG genotype for *LEPR* Q223R could be caused, as suggested by Wauter et al, by leptin binding to the leptin receptor and activating signal transduction which results in different downstream effects between the genotypes in the trunk region.

4.3 Association of total body fat and body fat distribution with plasma leptin level

As mentioned earlier, a number of studies have addressed the association of leptin with body fat measures, and there are differences among these studies in the conclusions reached (Tables 1.3 & 1.4). Most studies, both longitudinal and cross sectional, show a positive association between BMI, or change in BMI, and leptin level in the adolescent age group (Tables 1.3 & 1.4) in White populations; the present study confirmed this association in Asian-White population. The present study also showed a positive association between leptin and all of the DXA measured body fat variables total body fat, trunk fat, peripheral fat and trunk-to-periphery fat ratio, the gold standard measurement of body fat not available in any other study.

Individuals with similar levels of body mass index (BMI) have very different leptin levels (223). The data from clinical studies examining whether body fat distribution plays a role in determining serum leptin concentrations have given conflicting results (Tables 1.3 & 1.4). The present study finding, with respect to leptin and BMI, is consistent with previous studies among Asian and White populations (Tables 1.3 & 1.4). On the other hand, a study by Hu et al., (126) found that leptin concentration was strongly correlated with overall adiposity as determined by BMI in both men and women in a Chinese population. Specifically, central obesity, as measured by waist to hip ratio and abdominal skinfold thickness, significantly predicted leptin levels in both men and women after adjustment for BMI in the multivariate models. In our sample eligibility, which required 0.5 proportion of Asians (NEA, SEA, Malay), demonstrated an association between central obesity measures (DXA trunk fat, DXA trunk-to-periphery fat ratio, hip circumference, waist-to-hip ratio) and plasma leptin level. Plasma leptin in the present study was non-normally distributed but exponential models were used. Normality of leptin distribution was not mentioned in the previous studies.

Association of plasma leptin and dietary factors

The acceptable macronutrient distribution range (AMDR) for percent calories from protein (15.46 ± 3.39) was within the recommended AMDR of 10-30; AMDR for percent calories from carbohydrate (51 ± 8.26) was within the recommended range of 45-65 and AMDR for percent calories from total dietary fat (34.5 ± 6.52) was just within the recommended range of 25-35 (201). Total fiber intake was lower (11.88 g/d) compare to the adequate intake (AI) recommendation of 25-26 g/d (224).

As mentioned earlier, plasma leptin levels showed individual variation for a given level of adiposity, indicating the likely effect of variables other than adipose mass, such as dietary habits. Little is known about the effect of specific dietary factors on plasma leptin (225). This present study demonstrated a negative association between protein intake and plasma leptin level, independently of BMI, but not of DXA total body fat. There was no association between of leptin level and carbohydrates, dietary fat or fiber intake.

A few studies documented the role of diet and nutrients in modulating circulating leptin concentrations. Havel et al., (226) reported decreased leptin concentration after ingestion of high fat, low-carbohydrate diet. Murukami et al., (227) observed an inverse association of dietary fiber with serum leptin concentration in a group of young (18-22y) Japanese women. Murukami's study also showed an inverse association of protein with serum leptin concentration independently of several potentially confounding factors, which included BMI, smoking, alcohol, physical activity; energy intake, rate of eating, residential block and size of residential area, but the association was not independent of other nutrient intake, which may be due to relatively strong positive correlations of intakes of protein with dietary fiber intake. Given that their subjects were female dietetic students, rather than a random sample of Japanese women, these results might not be extrapolated to the general Japanese population. In a different study, no association between macronutrient intake and circulating leptin level (adjusted for BMI) was demonstrated in 32 American men and women aged 20–31 y (228) and 114 Greek men and women aged 14–26 y (229).

Replacing BMI with DXA total body fat in the model, protein intake was not associated with the plasma leptin. DXA has the advantage of showing a three-dimensional model of body composition and taking into account bone-free lean mass (230, 231) and hence making it a valid and reliable reference measure of body composition. Body mass index (BMI) is a routinely used indirect measure for body fatness, specifically obesity, in epidemiological research. The CDC suggests that BMI is more highly correlated with body fat than height and weight (232). In previous studies, BMI was proven an inappropriate measure for athletes, the elderly, obese children, and children with disease states (233-235) and is unable to track body fat content.

Failure to show association between other dietary factors (carbohydrates, fat and fiber) and plasma leptin could be due to: first, the cross-sectional nature of the study which does not permit the assessment of causality, owing to the uncertain temporality of the association; second, a single measurement of plasma leptin level may represent short-term status only and introduce random errors. Nonetheless, this kind of error would tend to bias toward attenuating rather than enhancing the relation, and multiple or serial leptin measurements would have only increased the precision of the results; finally, leptin levels change during the menstrual cycle (236), although measures were taken during the luteal phase of menstruation, when girls had reached menarche. Also these dietary intakes did not include dietary supplements, which would confound the association.

Biological plausibility

To compare with Murukami's study (227), the present study also adjusted for BMI. It is difficult to explain the inverse association of dietary protein and plasma leptin because Murukami's study found a similar relationship adjusting for BMI and explained it was due to strong positive correlations of protein intake with dietary fiber intake. However, in our study no such correlation was found. Correlation between protein intake and BMI was checked and it was not significant.

It remains unknown whether dietary factors influence circulating leptin levels directly or indirectly. One possibility is that dietary factors are associated with a decrease in serum leptin concentrations through decreased leptin production. Alternatively, they may increase leptin sensitivity, leading, in turn, to a subsequent decline in leptin production through unknown feedback mechanisms, a possibility suggested by an observational study (237). But, this biologic explanation should be considered cautiously because after adjusting for DXA total body fat, there was no association with dietary variables in the present study.

4.4 Association of LEP and LEPR polymorphisms with age at menarche (AAM)

While there have been a number of studies showing association between *LEP/LEPR* polymorphisms and obesity, there has only been one study of the potential association between the *LEP* gene and AAM and no studies between *LEPR* gene and AAM.

Based on our knowledge of the role of leptin in initiating or at least permitting the onset of puberty, it would be anticipated that there would be an association between *LEP/LEPR* polymorphisms and AAM.

The present study did not show an association between *LEPR* polymorphisms with AAM. The heterozygote genotype for *LEP G-2548A* showed a hazards ratio of 1.4 to reach menarche (40% more likely to reach menarche earlier). But the study was not powered of comparison between heterozygote and homozygote. This variant, as expected, did not show any variation in the plasma leptin level, which is known to affect the girl's age at menarche (131).

One other study (184) examined genetic variations at *LEP* genes and AAM, and no association was found. It has also been proposed that the trend for an earlier age of onset of menarche is associated with the trend for increased obesity (185), and leptin is involved in both menarche and obesity, suggesting that the *LEP/LEPR* genes could play a role in early menarche. However the association between *LEPR/LEP* variants and AAM was not demonstrated in this present study.

A study by Commings et al., (184), mostly on obese adult females, reported that the variants at the *LEP* gene may have a significant effect on age at menarche. The mean age at menarche was 12.3y for those with the *LEP1875<208/<208* genotype (D7S1875 is a dinucleotide repeat), 12.4y for heterozygotes, and 12.1y for those with the *LEP1875 ≥208/≥208* genotype among white females, but the values were not significantly different

(F ratio = 0.72, P = 0.48). Recall bias for AAM should be considered, although in a study 66% and 45% of women recalled their age at menarche accurately with a mean interval of 323 days in a previous study (39, 186).

Other determinants (body size, dietary and physical activity) of AAM

Birth weight alone was not associated with the girl's event of reaching menarche. A study by Adair (238) also reported no association between birth weight and AAM. However, girls who were thin and long at birth (<3kg, >49cm) attained menarche approximately 6 months earlier than girls who were light and short (<3kg, >49cm).

In the present study girls who were heavier (weight, lb) and taller (height, cm) (in separate models) reached menarche earlier, after adjusting for the potential confounders of age and ancestral groups. In separate models, body fat variables were tested and body mass index (BMI) was the only variable that had significantly higher HR, of 1.04.

The present study suggests that height, weight, and BMI, but not BMI percentile (data not shown) are associated with age at menarche (adjusted for age, height and ethnicity). The relationship between height and early menarche are consistent with those observed in other studies (27, 28, 49) except that of Adair study, with short birth length being associated with later menarche. A number of investigators have proposed that AAM is closely related to skeletal maturity (239, 240).

An association between BMI and menarche has been observed in several studies (27, 28, 241). BMI is assumed to be a measure of weight uncorrelated with height and, as such, to be proportional to the amount of body fat (i.e. a higher body mass index is indicative of a larger amount of body fat), but the validity of this assumption is untested in premenarcheal girls. Our studies, on the other hand, measured actual body fat, using DXA.

Physical activity, energy intake and macronutrient intake did not affect menarche. The present study found no evidence to support an independent role of dietary macronutrients in determining menarche. The present study also did not find significant association between higher energy intake and delay in AAM.

Although nutritional status may be important compared to anthropometric measures of nutritional status, diet may not add more information. No specific relationship between diet and age at menarche has been clearly established. Among those studies that have controlled for other factors associated with onset of menarche, some have found an association between higher total energy intake and earlier AAM (26, 39, 241), whereas others have not (27, 28, 49). Studies that looked at specific components of diet are similarly inconclusive (27, 49, 241). Differences in study design and methodology make comparisons of studies difficult. For example, measurement of dietary intake has varied from observation of children's eating habits to a combination of food records and food frequency questionnaires (28).

Under-reporting of dietary intake by overweight or obese subjects has been documented previously (50-53). Also, timing of dietary measurements in relation to the onset of first menses may have negatively influenced our results as well as those of other investigators (27, 28, 49, 241). It may be that diet during early childhood, diet six months prior to menarche or diet during some other time frame is more critical.

The role of ancestry in the timing of AAM within a population has not been well studied. Our results did not demonstrate ancestral differences in age at menarche for a predominately Asian and White population in Hawaii. In another study, Hispanic, Asian/Pacific Islander and African-American girls achieved menarche earlier than non-Hispanic white girls (39). While the results of this study do not indicate relationship between *LEP/LEPR* variants, dietary nutrients and AAM, they further support previous finding that body size, independently of age, is a predictor of AAM in Asian-White populations. Since ancestral differences in AAM were not observed in this population; hereditary factors did not influence age at menarche.

4.5 Strength and weaknesses

The strength of this study is its longitudinal design in an understudied age group and ancestral population with repeated measurement of both anthropometric, DXA and dietary data and the ability to adjust for major potential confounders. However the study was initially designed to investigate dietary and physical activity factors influencing maturation and future breast cancer and osteoporosis risk, not ancestral, hormonal or genetic factors. The present study is primarily on healthy subjects and was not designed

to study the effect of polymorphisms on body weight. The design would be strengthened by a larger study population with a wider BMI range.

DXA became available as an additional body fat measure, which is considered the gold standard, for exams 2 and 3 only. Funding for blood measures became available at exam 3 and thus plasma leptin level was not measured before the onset of pubertal maturation in most cases. Hence, longitudinal analysis, with plasma leptin predicting age at menarche, was not possible.

Relative to waist circumference, DXA predicts fat mass with greater accuracy and reproducibility. However, a disadvantage of DXA is that it does not separate visceral (intra-abdominal) and subcutaneous fat tissues (242).

The biologic action of leptin is controlled by its soluble leptin receptor depending on certain metabolic or developmental conditions (Zastrow et al., 2003). It is possible that soluble leptin receptor, not measured in our study, may confound or modify the association between leptin and the selected SNPs. A single measurement of plasma leptin level may represent short-term status only and introduce random errors. Nonetheless, this kind of error would tend to bias toward attenuating rather than enhancing the relation, and multiple or serial leptin measurements would have increased the precision of the results.

There is good statistical power to demonstrate statistically significant associations that could be of importance. Although our outcome variable, age at menarche, is self reported, it has been shown that 66% and 45% of girls can recall their age at menarche accurately with a mean interval of 323 and 649 days respectively (186).

4.6 Contribution to science

Methodologically, the study used a unique measure of ethnic ancestry by geographic approximation, which has not been attempted before. The study was the first to use DXA body fat and DXA body fat distribution in relation to leptin and its polymorphisms. DXA is gold standard methodology for measuring body fat, presenting an advance from previous research that relied on BMI. And body fat distribution is an important risk factor for chronic disease, independently of BMI or overall obesity.

The present study demonstrated that girls with the rarer homozygous (AA) genotype for *LEPR* Q223R had lower central adiposity (lower DXA trunk-to-periphery fat ratio). The relationship between *LEP* and *LEPR* variants and DXA body fat and body fat distribution was confirmed in an Asian White population. The study also demonstrated that girls with the heterozygous (AG) genotype for *LEP* G-2548A had a hazards ratio of 1.4 (40% more likely) to reach menarche earlier than the common homozygous (AA) genotype. This study confirmed the association of *LEP/LEPR* variants and the body fat variables, especially DXA body fat, in the Asian White population of Hawaii.

4.7 Public health implications

This present study provides new information with respect to *LEP/LEPR* genetic variants, body fat and age at menarche which is mentioned in section 4.6. The study contributed to our current knowledge of the complex association of how carrying a certain polymorphism can affect the girl's age at reaching menarche, which affects the risk of breast cancer, osteoporosis, and obesity. Recommendations with respect to modifiable factors might be made during the pre-adolescent growth that would allow girls to reach menarche at healthy age after, substantiating our findings with future studies. More research is needed to determine the mechanisms of pubertal timing and the reasons for its alterations and understanding the role of genotypes and other modifiable factors, such as diet and physical activity in understudied Asian White populations. This understanding will ultimately provide tools for improved children's health risk assessment.

4.8 Future studies

Carefully designed longitudinal studies, with exposures related to age at menarche, such as body fat and plasma leptin levels, measured before the onset of maturation will further advance this field of study. A detailed leptin profile, which includes measuring total leptin, free leptin, and bound leptin along with measures of soluble leptin receptors in both normal weight and overweight, is needed to fully understand the complex association of plasma leptin, genotypes and age at menarche. Multiple dietary records in preadolescent age group should be considered when planning studies to demonstrate association between dietary factors and age at menarche. Genotypes and distribution of body fat in populations of mixed ancestries that include Pacific Islanders, where obesity

is more common, and with larger study samples with a wider range in body fat would be valuable to further advance research in this area.

4.9 Conclusions

The present study provides findings on leptin and body fat among North East Asian, Malay, White and other ancestral mixes of adolescent girls. We have grouped the ancestries in a unique way based on the geographical approximation, which has not been attempted before. The present study examined the association between *LEP/LEPR* variants and age at menarche.

The present study did not show association between plasma leptin and *LEP/LEPR* variants. The study also showed association between plasma leptin and body fat measures in this Asian White population. An association between plasma leptin and dietary protein intake was demonstrated, which was attenuated after adjusting for DXA total body fat.

The study also showed the association between the *LEPR* rarer homozygous (AA) genotype and lower trunk-to-periphery body fat (lower central adiposity). The study also showed earlier menarche with *LEP* heterozygous (AG) genotype in this Asian White population.

Appendix A

Lori A Jennings/Hi/KAIPERM
10/14/2009 08:42 AM

Subject Communication from the KPHI IRB
This is your official IRB letter.

Principal

Investigator: Novotny, Rachel PhD

LeMarchand, Loic MD

Re: Nutritional and Genetic Determinants of Early Puberty

Study ID: HI-04TVogt-01

Date: 10/14/2009

IRB Expiration Date: 10/13/2010

Dear Dr Novotny:

On 10/14/2009, the Kaiser Permanente Hawaii Institutional Review Board (IRB) reviewed by expedited review and reapproved your research application to conduct the referenced study. This study is reapproved for one year. It is renewable (upon your request for continuing review by the IRB) before this period is expired.

Federal regulations require that all studies be reviewed at least annually. It is your responsibility to ensure that you apply for reapproval at least one month prior to this study's expiration date. You will be reminded of the deadline for submitting your reapproval request.

You are required to be in compliance with all Kaiser Permanente Hawaii Standard Operating Procedures (SOPs). The SOPs are found on the following internal website, available to all Kaiser Permanente investigators named on your application: <http://web.hi.kp.org/irb/sop.asp>.

If your study or study-related documents require modification, you must seek IRB approval for these changes before they are implemented. You must promptly notify the IRB of any unanticipated problems, i.e., unforeseen events that involve potential or actual physical, social, psychological, financial, or legal risks to research participants or others. You must also report serious adverse events (SAEs) affecting research participants or controls as well as any complications that occur during any experimental procedure associated with this study. For SAE reporting requirements, please refer to Standard Operating Procedure KP-028.

Please note, it is your responsibility as KP- investigator to inform all study staff (specifically outside investigators) of all IRB actions.

Sincerely,

Lori Jennings, CIP, CHRC

Institutional Review Board

Administrator

[NEW ADDRESS and PHONE NUMBER 5/2009](#)

"Advancing knowledge to improve health"

Lori Jennings, CIP, IRB Administrator, Kaiser Permanente, Center for Health Research, Hawai'i, 711 Kapiolani Blvd.,
2nd floor Honolulu, HI 96813

E-Mail: Lori.A.Jennings@KP.org

Office: (808) 432-5411

Recruitment letter for Female Adolescent Maturation Study 1

October 11, 2000

Name
Address

Dear XX:

Kaiser Permanente and the University of Hawaii would like to invite your child to participate in an important new study. The study is called the "Female Adolescent Maturation Study", and is looking at how diet and exercise are related to the size and strength of girls' bones. Kaiser Permanente is sending this invitation to the parents or guardians of girls between 9 and 13 years old. We are focusing on ethnic groups known to be at risk for osteoporosis (Asians and Caucasians). The study requires the completion of a questionnaire and a regular physical exam, which can take the place of your child's annual school physical. Your names and address will not be shared with the University of Hawaii researchers unless you and your child agree to participate in this study. Children who have severe asthma, smoke, or have a medical condition that requires medication are not eligible for participation. This important study, which may influence future dietary and exercise recommendations for youth, is described more fully in the enclosed study summary.

All information about your child will be confidential and stored in locked files. Your child can refuse or withdraw from the study if you or she chooses, and this will not affect any of the services she would normally receive from Kaiser. For participating in this study, reimbursement of costs totaling \$15.00 will be made. If you would like to participate or learn more about this program, please return the enclosed form with your name, telephone number and the best times to call. We will call to answer your questions and, if you are interested, schedule an appointment for your child.

Mahalo,



Thomas M. Vogt, M.D., M.P.H.
Director, Kaiser Permanente Center for Health Research

Female Adolescent Maturation Study 1 Summary

The purpose of this study is to identify dietary and physical activity patterns that are associated with menstruation onset and patterns and with size and denseness of bone tissue. Menstrual patterns may be related to future risk of breast cancer. If your child normally receives an annual physical for school, measurements for this study can be conducted at that time. If not, we can schedule an appointment for her participation.

If you and your child agree to participate, we will mail the following to her before her visit to the Kaiser clinic:

- 3-day diet record,
- a physical activity questionnaire, and a
- menstrual questionnaire

(Please assist your child in filling out these forms)

You, the Parent or Guardian, will also be mailed a questionnaire on medical and infant feeding histories.

Please complete these forms and bring them to her visit.

At the visit to the Kaiser clinic, your child will be measured for height, weight and other dimensions of her body; her bone mass will be measured with a heel measure. All examinations are the same as a regular school physical except for the addition of several anthropometric (body shape/size) measures (for example heel measurements).

If you are interested in learning more about this study, please complete the enclosed form and mail it back to us in the enclosed envelope at:

Kaiser Permanente Center for Health Research
531 Ohohia Street
Honolulu, Hawaii 95819

Female Adolescent Maturation Study 1

Parent's Name: _____
Child's Name: _____
Telephone Number: _____
Best times to contact us: _____
Email address (only if you prefer to be contacted via electronic mail): _____ _____

- Yes we are interested in participating in this study. Please call us to set up an appointment.
- No, we are not interested.
- We may be interested, but would like more information. Please call us.

Agreement to Participate

Parent/Guardian

“Female Adolescent Maturation Study”
Rachel Novotny PhD, RD, Principal Investigator
University of Hawaii at Manoa
FSHN/CTAHR 1955 East West Road
Ag Sci 302 I
Honolulu, HI 96822

Project Description

The purpose of this study is to identify dietary and physical activity patterns that are associated with bone mass and with menstruation. Building bone in adolescence is important to prevent osteoporosis in elder years. Menstrual patterns are related to future risk of breast cancer. Before your child’s visit, you will be mailed a 3-day diet record, a physical activity questionnaire, and a menstrual questionnaire to bring to the visit, completed. At the visit at Kaiser, your child will be measured for height, weight and other dimensions of her body; her bone mass will be measured with a heel measure. Measurements will be performed by trained staff. Parents/Guardians will be mailed one questionnaire on medical and infant feeding history to complete, and will be asked to assist with the child with the completion of the other questionnaires. All information on your child will be confidential, as part of her Kaiser record and in locked files of investigators. Your child can withdraw from the study if you or she chooses without affecting the services normally received by Kaiser. Your participation is important to determine future dietary and physical activity recommendations for youth. For participating, a reimbursement of costs totaling \$15.00 will be made when she completes all measurements.

Certification

I have read and I understand the above; I have been given satisfactory answers to my inquiries concerning project procedures and other matters; I am free to withdraw my child from the study at any time.

I herewith give my consent for my child to participate in this project with the understanding that such consent does not waive any of my legal rights, nor release the Principal Investigator or the institution or any employees or agent thereof from liability for negligence.

Signature

Adult Parent/Guardian

Date

(If you cannot obtain satisfactory answers to your questions or have comments or complaints about your treatment in this study, contact Committee on Human Studies, University of Hawaii, 2540 Maile Way, Honolulu, Hawaii 96822. Ph. 808-956-5007).

If you have questions about your rights as a research participant, you may contact: J. Marc Rosen MD, Chair, Institutional Review Board, Kaiser Permanente, 3288 Moanalua Road, Honolulu HI 96819. Phone (808)-441-3500.

KAISER PERMANENTE – Hawaii Region
Institutional Review Board

Consent Form – Approved 7 July 1999

Agreement to Participate

Adolescent Minor

“Female Adolescent Maturation Study”

Rachel Novotny PhD, RD, Principal Investigator

University of Hawaii at Manoa

FSHN/CTAHR 1955 East West Road

Ag Sci 302 I

Honolulu, HI 96822

Project Description

The purpose of this study is to identify dietary and physical activity patterns that build bone and help youth grow and develop. Building bone in the pre-teen and teen years is important to prevent bone disease in later years. The pattern of your monthly period is related to your future risk of breast cancer. Before your visit you will be mailed a diet questionnaire, a physical activity questionnaire, and a questionnaire about your pattern of menstrual periods, to bring to your visit, completed. At the visit to your Kaiser clinic, you will be measured for height, weight and other dimensions of your body; your bone mass will be measured with a heel measure. Your parents or guardians will be mailed a questionnaire on medical and infant feeding history to complete, and will be asked to help you with completing the other questionnaires. All information on you will be confidential and you can withdraw from the study if you choose. Your participation is important to determine future dietary and physical activity recommendations for youth.

Certification

I have read and I understand the above; I have been given satisfactory answers to my inquiries concerning project procedures and other matters; I am free to withdraw from the study at any time.

I consent to participate in this project with the understanding that such consent does not waive any of my legal rights, nor release the Principal Investigator or the institution or any employees or agent thereof from liability for negligence.

Signature

Adolescent Minor

Date

(If you cannot obtain satisfactory answers to your questions or have comments or complaints about your treatment in this study, contact Committee on Human Studies, University of Hawaii, 2540 Maile Way, Honolulu, Hawaii 96822. Ph. 808-956-5007).

If you have questions about your rights as a research participant, you may contact: J. Marc Rosen MD, Chair, Institutional Review Board, Kaiser Permanente, 3288 Moanalua Road, Honolulu HI 96819. Phone (808)-441-3500.

KAISER PERMANENTE – Hawaii Region
Institutional Review Board

Consent Form – Approved 7 July 1999

Recruitment letter for Female Adolescent Maturation Study 2

[Month dayth, year]

Dear [Parent and subject]

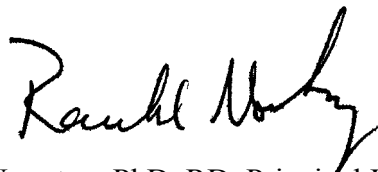
The University of Hawaii and Kapiolani Health Clinical Research Center invite your child to participate in the follow-up study of the Female Adolescent Maturation Study (FAM). Asian and Caucasian girls who participated in the US study are asked to participate in this study. We were seeing 349 Asian and Caucasian girls from the FAM study at the Kapiolani Clinical Research Center.

Her involvement will expose her to professionals in health and research. Many of the project staff are women working in the health arena. We have enclosed a short description of each staff member involved in this project. This is a wonderful opportunity to learn and be part of a research study that may contribute to future women's health.

Your child's second visit will be at Kapiolani Health Clinical Research Center. It will be similar to the first visit and will help to further track her development. This study requires the completion of a Health Questionnaire, a Family Background Questionnaire, a Physical Activity Questionnaire and a 3-day dietary record. At the visit her height, weight and other measurements of her body will be taken. Her bone mass and density will be measured with a heel measure and a body scan. She will also be asked to give a urine sample to screen for pregnancy and two mouthwashes to collect loose cells from her mouth. Your child's second visit will be scheduled within 2 years and 1 month from her first visit. Her appointment will be after school hours or on Saturday and the visit will take about an hour. Free validated parking is available at Kapiolani.

All information about your child will be confidential and stored in locked files. Your daughter's participation in this study is completely voluntary and she will have the right to withdraw from any one test at any time without explanation or penalty. For participating in this study, a payment of \$15.00 will be made. If you would like to participate or learn more about this program, please visit our web site or email us (see next page), or return the enclosed form with your name, telephone number and the best times to call. We will call to answer your questions and, if you are interested, schedule an appointment for your child.

Mahalo,



Rachel Novotny, PhD, RD, Principal Investigator
Professor, University of Hawaii

Female Adolescent Maturation 2

Study Summary

The purpose of this study is to identify dietary, physical activity and genes that are associated with menstruation onset and patterns, and with size and denseness of bone tissue. Menstrual patterns may be related to future risk of breast cancer.

If you and your child agree to participate, we will mail the following to her before her visit to Kapiolani Health Clinical Research Center:

1. Health Questionnaire
2. Family Background Questionnaire
3. Physical Activity Questionnaire
4. Dietary Records
5. Cup and spoon

Please complete the Family Background Questionnaire, and assist your daughter in completing the rest of the questionnaires. Bring completed forms to your daughter's visit. Consent and assent forms will also be included for your review prior to the visit.

At the visit to the Kapiolani Clinical Research Center, your child will be measured for height, weight and other dimensions of her body; her bone mass and density will be measured with a heel measure and a body scan. She will also be asked to give a urine sample to screen for pregnancy and two mouthwashes to collect loose cells from her mouth.

If you are interested in participating or learning more about this study, please do one of the following:

1. Visit our web site at <http://www2.ctahr.hawaii.edu/ctahr2001/NAP/FAM/index.html>. You can sign up online to participate in the study. You can also read up on the study and updated FAM results as they become available. We will call/email you to set up an appointment.
2. Email the study at famstudy@hawaii.edu or call Samantha Stavar at 956-3838. If you have any questions want to sign up for the study, you can email or call us. You can also email us and request that we give you a call (please indicate best times).
3. Complete the enclosed form and mail it back to us in the self addressed & stamped envelope at:
Attn: Yihe Daida
University of Hawaii
Dept of Human Nutrition Food and Animal Science
Ag Sci Bldg Room 216
Honolulu, HI 96822

Female Adolescent Maturation 2 Study

Parent's Name: _____
Child's Name: _____
Telephone Number: _____
Best times to contact us: _____
Email address (only if you prefer to be contacted via electronic mail): _____ _____

- Yes we are interested in participating in this study. Please call us to set up an appointment.
- No, we are not interested.
- We may be interested, but would like more information. Please call us.

Assent To Participate in a Research Study

Female Adolescent Maturation 2

Principal Investigator: Rachel Novotny PhD RD
University of Hawaii at Manoa
1955 East West Road, Ag Sci Bldg 302 I
Honolulu, HI 96822
Phone: (808) 956-3848

Sponsor: USDA – National Research Initiative
Competitive Grants Program

Purpose

We are asking you to participate in a follow-up study of the Female Adolescent Maturation Study (FAM). This follow-up study will study your diet, physical activity patterns and genetics. These factors influence how teenagers grow and develop. Some examples are when you start your menstruation, and the size and quality of your bone. The pattern of your monthly period may influence your future risk of breast cancer. Asian and Caucasian girls who participated in the USDA FAM study were asked to participate in this study. We will be seeing 349 Asian and Caucasian girls from the FAM study at the Kapiolani Clinical Research Center.

Summary

Your second visit will be scheduled within 2 years and 1 month from your first visit. Your appointment will be after school hours and the visit will take about an hour.

Your second visit to Kapiolani Health Clinical Research Center will help to further track your development. Before your visit your parent/guardian will be mailed a Health Questionnaire, a Family Background Questionnaire, a Physical Activity Questionnaire and a 3-day Dietary Records. Your parents/guardians will be asked to help you with completing the questionnaires, and bring them to your visit. At the visit, your height, weight and other measurements of your body will be taken. Your bone mass and density will be measured with a heel measure and a body scan. You will also be asked to give a urine sample to screen for pregnancy and two mouthwashes to collect loose cells from your mouth. The information and the cells from inside of your mouth will allow us to study how genes affect one's maturation and growth. Some of the cells will be stored for use in future research studies (see "Future Research Studies" section).

Description of Specific Procedures

- Questionnaires:
 1. Health Questionnaire
 2. Family Background Questionnaire
 3. Physical Activity Questionnaire
 4. 3-day Dietary Records

- Urine Sample:

You will be asked to provide urine in a cup to check if you are pregnant. The body scan will not be done if you are pregnant.

- Mouthwash:
You will be asked to brush your teeth at the beginning of your visit. You will also be asked to complete a mouthwash at the beginning and end of your visit. Mouthwash collection is a painless procedure, done by swishing Scope® inside your mouth for about one minute. The mouthwash is then spit into a small container.
- Bone Densitometry Scan:
You will receive a scan of your bones during the visit. The scan is a painless test that involves lying on a table for about 20 minutes while your bones are being scanned.
- Measurements:
 - a. Weight
 - b. Standing and sitting height
 - c. Shoulder and hip width
 - d. Size of waist, hip and calf
 - e. Fatfolds
 - f. Stage of maturation

Possible risks or discomforts

There is possible loss of privacy, confidentiality and small exposure to radiation associated with this study. The investigators and staff will be careful about your privacy and confidentiality while taking the measures. Bone densitometry scans involve some exposure to radiation. The safety guideline for radiation control is 500 mRem. For comparison, a chest x-ray is 20-50 mRem and a full x-ray of your teeth involves 50-300 mRem. In this study, the radiation for a bone scan is 0.04 mRem.

Future Research Studies

Some of the cells from the mouthwash will be stored for use in future research studies. Although the exact tests that will be done are not known at this time, researchers may use them to further study: 1) how diet, lifestyle, environment, race/ethnicity, age and other factors are related to growth and maturation; 2) how differences in genes affects ability to process hormones or substances that cause growth and maturation. These studies will follow the guidelines of the Committee on Human Studies, University of Hawaii, and laws for the protection of human subjects.

I agree to have my mouthwash sample stored for use in future research studies.

_____ (X): ___ Yes ___ No
Signature of Participant Date

Benefits to be expected from the research

Your participation is important to help determine future dietary and physical activity recommendations for youth.

Informed Consent To Participate in a Research Study

Female Adolescent Maturation 2

Principal Investigator: Rachel Novotny PhD RD
University of Hawaii at Manoa
1955 East West Road, Ag Sci Bldg 302 I
Honolulu, HI 96822
Phone: (808) 956-3848

Sponsor: USDA – National Research Initiative
Competitive Grants Program

Purpose

We are asking your child to participate in a follow-up study of the Female Adolescent Maturation Study (FAM). This follow-up study will study her diet, physical activity patterns and genetics. These factors influence how teenagers grow and develop. Some examples are when she starts her menstruation, and the size and quality of her bone. The pattern of her monthly period may influence her future risk of breast cancer. Asian and Caucasian girls who participated in the USDA FAM study were asked to participate in this study. We will be seeing 349 Asian and Caucasian girls from the FAM study at the Kapiolani Clinical Research Center.

Summary

Your child's second visit will be scheduled within 2 years and 1 month from her first visit. Her appointment will be after school hours and the visit will take about an hour.

Her second visit to Kapiolani Health Clinical Research Center will help to further track her development. Before her visit, you will be mailed a Health Questionnaire, a Family Background Questionnaire, a Physical Activity Questionnaire and a 3-day Dietary Records. Please help her with completing the questionnaires, and bring them to her visit. At the visit, her height, weight and other measurements of her body will be taken. Her bone mass and density will be measured with a heel measure and a body scan. She will also be asked to give a urine sample to screen for pregnancy and two mouthwashes to collect loose cells from her mouth. The information and the cells from inside of her mouth will allow us to study how genes affect one's maturation and growth. Some of the cells will be stored for use in future research studies (see "Future Research Studies" section).

Description of Specific Procedures

- Questionnaires:
 1. Health Questionnaire
 2. Family Background Questionnaire
 3. Physical Activity Questionnaire
 4. Dietary Records
- Urine Sample:

Your daughter will be asked to provide a urine sample to check if she is pregnant. The body scan will not be done if she is pregnant.
- Mouthwash:

Your child will be asked to brush her teeth at the beginning of her visit. She will also be asked to complete a mouthwash at the beginning and end of her visit.

Mouthwash collection is a painless procedure, done by swishing Scope® inside her mouth for about one minute. The mouthwash is then spit into a small container.

- Bone Densitometry Scan:
Your child will receive a scan of her bones during the visit. The scan is a painless test that involves lying on a table for about 20 minutes while her bones are being scanned.
- Measurements:
 - g. Weight
 - h. Standing and sitting height
 - i. Shoulder and hip width
 - j. Size of waist, hip and calf
 - k. Fatfolds
 - l. Stage of maturation

Possible risks or discomforts

There is possible loss of privacy, confidentiality and small exposure to radiation associated with this study. The investigators and staff will be careful about your child’s privacy and confidentiality while taking the measures. Bone densitometry scans involve some exposure to radiation. The safety guideline for radiation control is 500 mRem. For comparison, a chest x-ray is 20-50 mRem and a full x-ray of teeth involves 50-300 mRem. In this study, the radiation for a bone scan is 0.04 mRem.

Future Research Studies

Some of the cells from the mouthwash will be stored for use in future research studies. Although the exact tests that will be done are not known at this time, researchers may them to further study: 1) how diet, lifestyle, environment, race/ethnicity, age and other factors are related to growth and maturation; 2) how differences in genes affects ability to process hormones or substances that cause growth and maturation. These studies will follow the guidelines of the Committee on Human Studies, University of Hawaii, and laws for the protection of human subjects.

I agree to have my child’s mouthwash sample stored for use in future research studies.

Signature Relationship to child (X): ___ Yes ___ No
Date

Signature Relationship to child (X): ___ Yes ___ No
Date

Benefits to be expected from the research

Your child’s participation is important to determine future dietary and physical activity recommendations for youth.

Payment to Participants

For participating, your child will be paid \$15.00.

Confidentiality

The information collected on your child will be locked in a filing cabinet. We will use a code system instead of her name to be private. Once the code numbers have been matched, any list with names will be destroyed. Also, she will not be identified in any publication or in the final report to the funding agency.

Compensation for Injury

We do not expect risk of injury as a result of participation. In the unlikely event of physical injury or illness from the study, no additional money will be paid to you or her.

Voluntary Participation Statement

Your daughter's participation in this study is completely voluntary and she will have the right to withdraw from any one test at any without explanation or penalty.

If You Have Questions

If you have any questions about this research project, please contact the Principal Investigator, Dr. Rachel Novotny at (808) 956-3848. Any other questions that you may have regarding your child's rights as a research participant should be directed to Raul Rudoy, M.D., Chair, Kapi'olani Health Institutional Review Board at (808) 535-7500 or the Committee on Human Studies, University of Hawaii, 2540 Maile Way, Honolulu, HI 96822. Ph. 808-956-5007.

VOLUNTARY STATEMENT

My signature below indicates that I have read and that I understand the procedures described above and give my informed and voluntary consent to participate in this study. I have been given satisfactory answers to my inquiries concerning project procedures and other matters; I am free to withdraw my child from the study at any time. I understand that I will receive a signed copy of this consent form.

CHILD'S NAME: _____

PARENTS:

NAME: _____
(Print or Type)

NAME: _____
(Print or Type)

SIGNATURE: _____

SIGNATURE: _____

RELATIONSHIP: _____

RELATIONSHIP: _____

Signature of Witness

Date

Signature of Investigator

Date

If consent has not been signed by both parents, please state reason:

Hawaii Pacific Health

Authorization to Use and Disclose Protected Health Information for Research

A federal law known as the Health Insurance Portability and Accountability Act (HIPAA) sets new rules for protecting the privacy of patient's photograph or images. HIPAA now requires researchers, except in specific circumstances, to get written permission from study participants before using or disclosing their existing photograph, slides or video obtained for a research study.

This authorization allows the research team to use and disclose my child's photograph/images associated with the study entitled Female Adolescent Maturation II.

Purpose of this disclosure:

1. Information collected for this research study is intended to determine the effectiveness of a school curriculum intervention.
2. The photographs/images taken from your child might also be used for educational purposes such as lecture illustrations, presentations and/or public relational presentations such as brochures, press release etc, but your child will not be identified.

Description of the information

My authorization covers all information about my child that is collected for this study. It includes:

- Photographs
- Slides
- Video

Who may use and disclose my child's photographs or images?

I authorize the following persons or groups to use and disclose my child's study-related photographs or images:

- Rachel Novotny, PhD, RD, Co-Investigators and other research personnel on her research team
- Clinical Research Center personnel
- University of Hawaii, Department of Human Nutrition, Food and Animal Sciences research personnel
- Hawaii Pacific Health Research Institute

Who may receive my child's photographs or images?

I authorize the following persons or groups to receive study-related photographs/images about my child:

University of Hawaii, Cancer Research Center of Hawai'i, United States Department of

Agriculture (USDA), Officials at Hawaii Pacific Health, Hawaii Pacific Health Institutional

Review Board.

Expiration of the authorization

This authorization will not expire for the purpose of this research.

Canceling the authorization

I have the right to cancel this authorization at any time. My cancellation must be in writing, addressed to: Rachel Novotny, PhD, RD, University of Hawaii at Manoa, Department of Human Nutrition, Food and Animal Sciences, 1955 East-West Road, Agricultural Science 216, Honolulu, HI, 96822.

Disclosures outside the research study

Some of the persons or groups that receive my child's photographs or images may not be required to comply with HIPAA privacy laws, and my child's photographs or images may lose its federal protection if those persons or groups disclose it. The Confidentiality section of the consent form describes other ways in which my child's photographs or images will be protected.

Right to refuse to sign the authorization

I have the right to refuse to sign this authorization. This would prevent those listed above from having access to or using any of my child's photographs or images for the purpose stated. I understand that refusal to sign the authorization means that my child cannot participate in this research study.

Written Permission

I hereby authorize the release of my child's photographs or images in connection with the "Female Adolescent Maturation II" research study, which has been approved by the HPH Institutional Review Board (IRB). I understand that this authorization is voluntary. I will receive a signed copy of this authorization.

Signature of Research Participant's Parent/Guardian

Date

Printed Name of Research Participant

If signed by someone other than the patient's Parent/Guardian, please describe your authority to act on behalf of the Patient:

If you have any questions about HIPAA privacy protections, you may call the Hawaii Pacific Health Institutional Review Board at (808) 535-7500.

Recruitment letter for Female Adolescent Maturation Study 3

Dear Kaiser Member,

Aloha. Kaiser Permanente is participating with the University of Hawaii and Kapiolani Clinical Research Center in an important new research study that is examining how diet, exercise and genetics affect the rate of maturation among girls 13-17. The speed of maturation may be related to future risk of breast cancer, osteoporosis and other health conditions. This study will help to determine whether it may be possible to identify such risks early in life and apply preventive measures to reduce these risks. Girls who participate will need to complete a questionnaire, a physical exam and bone scan at the Kapiolani Clinical Research Center, and provide small samples of urine and blood. The study focuses on ethnic groups known to be at particular risk of osteoporosis and breast cancer (Asians, Caucasians). Kaiser Permanente is sending this invitation to the parents of age-eligible health plan members. If your child is not a severe asthmatic, does not smoke, is not taking oral contraceptive and does not have any pre-existing medical condition that requires medication, we are writing to ask for her participation in this important study. The results may influence future dietary and physical activity recommendations for youth.

All information collected in this study will be confidential, and will be stored in locked files. Your child can refuse or withdraw from the study if you or she chooses without affecting any of the services she would normally receive from Kaiser Permanente. For participating in this study, compensation totaling \$80.00 in gift certificates will be made. If you would like to participate or would like to learn more about this program, return the enclosed form with your telephone number and the best times to call. A study staff from Kaiser Permanente will contact you for screening and scheduling. Your name and address will not be shared with the University of Hawaii researchers if you return the attached form declining participation. If you return the attached form declining participation, no one will contact you. If you do not respond, someone from Kaiser Permanente will call you.

Mahalo.

Thomas M. Vogt, M.D., M.P.H.
Director, Kaiser Permanente Center for Health Research

Female Adolescent Maturation Study 3

Parent's Name: _____ _____
Child's Name: _____ _____
Telephone Number: _____ _____
Best times to contact us: _____ _____
Email address (only if you prefer to be contacted via electronic mail): _____ _____

- Yes we are interested in participating in this study. Please call us to set up an appointment.
- We may be interested, but would like more information. Please call us.
- No, we are not interested.

Assent To Participate in a Research Study

Female Adolescent Maturation 3

Principal Investigators: Loïc Le Marchand, M.D., Ph.D.
Cancer Research Center of Hawaii
University of Hawaii at Manoa
1236 Lauhala Street, Suite 407
Honolulu, HI 96813
Phone: (808) 586-2988

Rachel Novotny, Ph.D., R.D.
Dept. of Human Nutrition & Food
and Animal Science
University of Hawaii at Manoa
1955 East West Road,
Ag Sci Bldg 216
Honolulu, HI 96822
Phone: (808) 956-3848

Study Title: “Nutritional and Genetic Determinants of Early Puberty”

Sponsor: Department of Defense Breast Cancer Research Program

Purpose

We are asking you to take part in a follow-up of the “Female Adolescent Maturation Study” (FAM), a study involving research. This study investigates nutrition, physical activity, hormones (natural substance circulating in your blood) and genes (your body characteristics passed down from your mother or father from birth). These factors influence how teenagers grow and develop, including when they start their first periods, and the size and quality of their bone. The pattern of your monthly period may influence your chance of having breast cancer in the future. Asian and Caucasian girls, especially those who took part in the USDA FAM study, are asked to be part of this study. We will be seeing about 300 Asian and Caucasian girls at the Kapiolani Clinical Research Center.

Eligibility

All FAM girls in the original study are invited to take part. If you were not part of the original study, you will be asked on the phone to take part in the study if you are 50% or more White or Asian; non smoker; not using or have used certain medication; and have no preexisting medical conditions requiring medication.

Summary

You will be asked to visit Kapiolani Clinical Research Center (CRC) twice. . (If you were part of the FAM study, these visits will be scheduled approximately 2 years from your last examination). Your visits will be after school hours or on a state holiday or a Saturday; the first visit will take about 2 hours and the second visit will take about ½ hour. At CRC, you will be asked some questions about your general health, and your height, weight and other measurements of your body will be taken. Your bone mass and density will be measured with a heel measure and a body scan and two mouth washes to collect loose cells from your mouth. You will also be asked to give a urine sample to screen for pregnancy and two mouth washes to collect loose cells from your mouth. You will be taught how to record your diet for 3 days, how to record your period on a menstrual calendar (for 3 cycles of your period), and will be given a take-home questionnaire to complete before your 2nd visit.

The information from the study and the cells from inside of your mouth will allow us to study how nutrition and genes affect one’s growth and development. Some of the cells will be stored for use in future research studies (see “Future Research Studies” section).

If you already have had your first periods, you will be asked to call the study staff on the first day of your next period to schedule a blood and urine collection. The study MLT will schedule a home blood collection in the morning between the 20-24th day after your period (if you have your period). You will be asked to fast for at least 8 hours and collect your urine during the night before your blood draw. A small blood

sample (2 tablespoons) will be collected. The blood and urine will be used to measure several hormones related to growth and development.

You will also be asked to collect your urine overnight, 3 additional times during the 6th to 9th and 20th to 24th day after your period. Study staff will arrange to pick-up the urine at your house or at another convenient location.

Description of Specific Procedures

- Questionnaires:
 1. Family Background Questionnaire
 2. Health Questionnaire
 3. Physical Activity Questionnaires
 4. 3-day Dietary Records
 5. Menstrual Calendar
- Spot Urine Sample:

You will be asked to provide urine in a cup before the body scan to check if you are pregnant. The body scan, and the blood and urine samples will not be collected if you are pregnant.
- Mouthwash:

You will be asked to brush your teeth at the beginning of your visit. You will also be asked to do a mouthwash at the beginning and end of your visit. Mouthwash collection is a painless procedure, done by swishing Scope[®] inside your mouth for about one minute. Then, you are asked to spit into a small container.
- Measurement of your bones strength :

You will receive a scan of your bones during the visit. You will be asked to change into a hospital “scrub” suit. The scan is a painless test that involves lying on a table for about 20 minutes while your bones are being measured for size and quality.
- Measurements:
 - m. Weight
 - n. Standing and sitting height
 - o. Shoulder and hip width
 - p. Size of waist, hip and calf
 - q. Fatfolds
 - r. Stage of your body growing and developing
 - s. Skin color of the outer forearm, inner upper arm and forehead
- Blood sample:

A small blood sample will be collected from a vein in your arm, just like it would be done at your doctor’s office.
- Overnight urine samples:

You will be provided with a urine container and cooler for overnight urine collections. An overnight urine collection includes the first morning urine and any urine passed during the night (if you use the bathroom during the night). You will be asked to do 4 overnight urine collections and a blood collection timed with your periods.

Possible risks or discomforts

You might feel some loss of privacy, and you may receive limited radiation (energy in the form of rays or waves). The investigators and staff will be careful about your privacy while taking the measures. Bone measurements cause some radiation. For comparison, a chest x-ray is 5 mRem and one year of background radiation is 300 mRem. In this study, the radiation for a bone scan is less than 1 mRem, comparable to what

you receive during a round-trip flight from Hawaii to the mainland, which is 6 mRem. You may feel embarrassed or uncomfortable answering some of the questions or during the physical exam. You may refuse to answer any questions that you find embarrassing. You may experience pain, discomfort or faintness while the blood is being drawn but the blood will be collected by an experienced blood drawer. You may also decide not to take part in any part of the study without explanation or consequences. And you may continue with the rest of the study.

Future Research Studies

During this study, you will be asked to provide 4 urine samples, one blood sample and two mouthwash samples. There is a chance that the samples that you are donating under this study may be used in other related research studies and may have some commercial value. Should your donated sample(s) lead to the development of a commercial product, the University of Hawaii will own it and may take action to patent and license the product. The University of Hawaii does not intend to provide you with any compensation for your participation in this study nor for any future value that the sample you have given may be found to have. You will not receive any notice of future uses of your sample(s)

(You can consent to participation in the research protocol, but refuse to consent to the future use of her biological samples). I agree to have my samples stored for use in future research studies.

(X): Yes No

Signature of Participant

Date

Benefits to be expected from the research

You will have a chance to observe and participate in the scientific process, research and observe role models of researchers and health professionals. You will be mailed some materials to help you better understand the measurements taken and enhance your awareness about your health. Included in will be a Measurements Summary Table listing your height, weight, and body mass index and bone scans. Periodically, a newsletter explaining the study progress will be sent to you in the mail.

Results from Research tests

If you are under 18 years old, a positive pregnancy test result will be given to you and your parents. If you are 18 or over, the result will be given only to you. The results of other tests done on your samples will not be returned to you as they will not be helpful. However, the findings for the research will be mailed to you in a newsletter.

Compensation to Participants

For your trouble, your total compensation for your time and travel to take part in this study is \$80.00. You will receive two \$30.00 gift certificates, one at each CRC visit. The last gift certificate (\$20.00) will be given to you by the CRCH Medical Laboratory Technician during your scheduled blood draw.

Confidentiality (Keeping your records safe)

All information about you will remain strictly confidential to the extent allowable by law. You will be assigned a code number and all study forms and questionnaires will be stored in locked files. Subject information will be stored at the University of Hawaii, protected by user passwords and a computer firewall. All questionnaires, data collection form and samples will only be identified with ID numbers. Data will be entered and analyzed only with ID numbers and no identifying information will be used in publications.

Compensation for Injury

We do not expect risk of injury as a result of participation. In the unlikely event of physical injury or illness from the study, no additional money will be paid to you. Other than medical care that may be provided and any other payment specifically stated in the consent form, there is no other compensation available for your participation in this research. This does not mean that you are giving up any legal rights that you may have nor will there be additional costs to you for participating in the study.

Cost for participating

There will be no cost to you for being part of this study.

Voluntary Participation Statement

You can choose whether to take part in this study. If you decide not to be part of this study, you can stop at any time without loss of any benefits. Please notify in writing, Drs. Loic Le Marchand and Rachel Novotny (University of Hawaii at Manoa, Department of Human Nutrition, Food and Animal Sciences, 1955 East West Road, Agricultural Science 216, Honolulu, HI 96822) if you wish to withdraw from the study. Data and samples collected prior to your withdrawal may be used by researchers to preserve the integrity of the study.

Contact In The Event Of Research-Related Injury

Please report any health problem that could be related to your taking part in this study to the Clinical Research Center at 983-6242.

Rights of Research Subjects

Investigators do not foresee any circumstances under which they will terminate your participation early. You may withdraw your consent at any time and stop participation. No one will be mad and there will be no consequences. You are not giving up any legal claims, rights or remedies (what is lawfully yours) because of your participation in this research study. If you have any questions about this research project, please contact the Principal Investigator, Dr. Loic Le Marchand or Dr. Rachel Novotny at (808) 956-3848. If you have questions regarding your rights as a person who takes part in a study, you may contact: Raul Rudoy, M.D., Chair, Hawaii Pacific Health Institutional Review Board at (808) 535-7500, the Committee on Human Studies, University of Hawaii, 2540 Maile Way, Honolulu, HI 96822. Ph. (808)956-5007, or Kaiser Permanente Hawaii IRB, Center for Health Research Hawaii, 501 Alakawa Street, 2nd floor, Honolulu, HI 96817, Ph: (808) 432-4757, or the Department of Clinical Investigations at the Tripler Army Medical Center at (808) 433-6709.

SIGNATURE OF RESEARCH SUBJECT

I have read the information provided above. I have been given an opportunity to ask questions and all of my questions have been answered to my satisfaction. I understand that I will receive a copy of this form.

NAME of PARTICIPANT: _____
(Print or Type)

Signature of Participant Date

Permanent address of participant:

NAME _____ OF _____ WITNESS:

SIGNATURE OF WITNESS

My signature as witness certifies that the subject signed this consent form in my presence as his/her voluntary act and deed

Signature of Witness/Study Staff Date

Informed Consent To Participate in a Research Study

Female Adolescent Maturation 3

Principal Investigators:	Loïc Le Marchand, M.D., Ph.D. Cancer Research Center of Hawaii University of Hawaii at Manoa 1236 Lauhala Street, Suite 407 Honolulu, HI 96813 Phone: (808) 586-2988	Rachel Novotny, Ph.D., R.D. Dept. of Human Nutrition & Food and Animal Science University of Hawaii at Manoa 1955 East West Road, Ag Sci Bldg 216 Honolulu, HI 96822 Phone: (808) 956-3848
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Study Title: “Nutritional and Genetic Determinants of Early Puberty”

Sponsor: Department of Defense Breast Cancer Research Program

* If you are an 18-year old consenting to this study, the term “your child” in this document refers to you.

Purpose

We are asking your child to participate in a follow-up study of the Female Adolescent Maturation Study (FAM), a study involving research. This follow-up study will investigate her diet, physical activity patterns, hormones and genetics. These factors influence how teenagers grow and develop, including the time when they start to menstruate and the size and quality of their bone. The pattern of her monthly period may influence her future risk of breast cancer. Asian and Caucasian girls, especially those who participated in the USDA FAM study are asked to participate in this study. We will be seeing about 300 Asian and Caucasian girls at the Kapiolani Clinical Research Center.

Eligibility

All FAM girls in the original cohort have been screened for eligibility, and will not be screened again. If your child was not part of the original cohort, she was screened for the following eligibility criteria during the recruitment phone call: for ethnicity (50% or more White or Asian); non smokers; not using or have used oral corticosteroids, oral contraceptives or other steroidal hormones; and have no preexisting medical conditions requiring medication. An appointment at CRC was made if your daughter was eligible.

Summary

Your child will be asked to visit Kapiolani Health Clinical Research Center (CRC) twice to track her development. (If she was part of the FAM Study, this follow-up will be scheduled approximately 2 years from her last examination).

Your child’s appointments will be after school hours or on a state holiday or Saturday. The first visit will take about 2 hours and the second visit will take ½ hour. At the visits, she will be asked some questions about her general health, and her height, weight and other measurements of her body will be taken. Her bone mass and density will be measured with a heel measure and a body scan. She will also be asked to give a urine sample to screen for pregnancy, taught how to record her diet for 3 days, how to record her period on a menstrual calendar for 3 cycles, and will be given a questionnaire to complete before her second visit. Two mouthwash samples will also be collected

The information from the study and the cells from inside of her mouth will allow us to study how nutrition and genes affect one’s maturation and growth. Some of the cells will be stored for use in future research studies (see “Future Research Studies” section).

If your child has already had her first periods, she will be asked to call the study Medical Laboratory Technician (MLT) on the first day of her next period to schedule a blood and urine collection. The MLT will schedule a home blood collection in the morning between the 20-24th day after the beginning of her period. She will be asked to fast for at least 8 hours and collect her urine during the night before her visit. At the visit, a small blood sample (2 tablespoons) will be collected. The blood and urine will be used to measure several hormones related to growth and maturation.

If your child has already had her first periods, she will also be asked to collect her urine overnight, 3 additional times, during the first or second half of her menstrual cycle. Study staff will arrange to pick-up the urine at your house or at another convenient location.

Description of Specific Procedures

- Questionnaires:
 1. Family Background Questionnaire
 2. Health Questionnaire
 3. Physical Activity Questionnaire
 4. Dietary Records
 5. Menstrual Calendar

- Spot Urine Sample:

Your daughter will be asked to provide a urine sample before the body scan to check if she is pregnant. The body scan, and the blood and urine samples will not be collected if she is pregnant.

- Mouthwash:

Your child will be asked to brush her teeth at the beginning of her visit. She will also be asked to complete a mouthwash at the beginning and end of her visit. Mouthwash collection is a painless procedure, done by swishing Scope[®] inside her mouth for about one minute. The mouthwash is then spit into a small container.

- Bone Densitometry Scan:

Your child will receive a scan of her bones during the visit. She will be asked to change into a hospital “scrub” suit. The scan is a painless test that involves lying on a table for about 20 minutes while her bones are being scanned.

- Measurements:
 - t. Weight
 - u. Standing and sitting height
 - v. Shoulder and hip width
 - w. Size of waist, hip and calf
 - x. Fatfolds
 - y. Stage of maturation
 - z. Skin color of the outer forearm, inner upper arm and forehead

- Blood sample:
A small blood sample will be collected from a vein in your child's arm the way it would be done for a blood test in your doctor's office.
- Overnight urine samples:
Your child will be provided with urine containers and coolers with blue ice to do several overnight urine collections. An overnight urine collection includes the first morning urine and any urine passed during the preceding night after going to bed. She will be asked to do 4 overnight urine collections and a blood collection timed with her periods

Possible risks or discomforts

There is possible loss of privacy, confidentiality and small exposure to radiation associated with this study. Bone densitometry scans involve some exposure to radiation. For comparison, a chest x-ray is 5 mRem and one year of background radiation exposure is 300 mRem In this study, the radiation for a bone scan is less than 1 mRem, comparable to the exposure received during a round-trip flight from Hawaii to the mainland, which is 6 mRem.

There may also be possible discomfort from answering some of the sensitive questions on the questionnaires and she may feel embarrassed or uncomfortable during the physical exam. The investigators and staff will be careful about your child's privacy while taking the measures and your child is free to not answer any questions that cause discomfort. Your child may experience pain, discomfort or faintness while the blood is drawn but the sample will be collected by an experienced phlebotomist. Your child may decline to participate in this or any part of the study without explanation or penalty and she may continue with the remainder of the study.

Future Research Studies

During this study, your child will be asked to provide 4 urine samples, one blood sample and 2 mouthwash samples. These samples will be used for understanding 1) how diet, lifestyle, environment, race/ethnicity, age and other factors are related to growth and maturation; 2) how differences in genes affects ability to process hormones or substances that cause growth and maturation. They may also be used for purposes that are currently unknown. There is a chance that the samples that your daughter is donating under this study may be used in other research studies and may have some commercial value. Should your daughter's donated sample(s) lead to the development of a commercial product, the University of Hawaii will own it and may take action to patent and license the product. The University of Hawaii does not intend to provide your daughter with any compensation for her participation in this study nor for any future value that the sample your daughter has given may be found to have. Your daughter will not receive any notice of future uses of her sample(s)

You and your child can consent to participation in the research protocol, but refuse to consent to the future use of her biological samples. I agree to have my child's samples stored for use in future research studies.

_____ (X):
 Yes No
 _____ Signature _____ Relationship to child _____ Date
 Date signed: _____

Benefits to be expected from the research

Your child will have a chance to observe and participate in the scientific process, research and observe role models of researchers and health professionals. She will be mailed some materials to help better understand the measurements taken and enhance her awareness about her body. Included will be a Measurements Summary Table listing girl's height, weight, and body mass index and bone scan images. Periodically, a newsletter will be sent to you and your child in the mail.

Results from

Positive pregnancy test results for girls eighteen years of age and older will be given to girls participating in the study but not to their parents. For girls under 18, positive results of the pregnancy test will be given to the girls and their parents. The results of other tests done on your child's samples will not be returned to her as they would not be helpful. However, the results of the research will be mailed to you and your daughter in a newsletter.

Compensation to Participants

For your child's trouble, she will receive \$80.00 total compensation for her time and travel to participate in this study. She will receive two \$30.00 gift certificates, one at the first visit (examination and bone scan) and one at the second visit (mouthwash and urine collection). The last gift certificate (\$20.00) will be given to her by the CRCH Medical Laboratory Technician during her scheduled blood draw.

Confidentiality

All information will remain strictly confidential to the extent allowable by law. Your child will be assigned a code number and all study forms and questionnaires will be stored in locked files. Subject information will be stored at the University of Hawaii, protected by user passwords and a computer firewall. All questionnaires, data collection forms and samples will only be identified with ID numbers. Data will be entered and analyzed only with ID numbers and no identifying information will be used in publications to preserve the confidentiality your child.

Compensation for Injury

We do not expect risk of injury as a result of participation. In the unlikely event of physical injury or illness from the study, no additional money will be paid to you or her. Other than medical care that may be provided and any other payment specifically stated in the consent form, there is no other compensation available for your daughter's participation in this research. This does not mean that she is giving up any legal rights that she may have.

Cost for Participating

There will be no cost to you or your child for participating in this study.

Voluntary Participation Statement

Your daughter's participation in this study is completely voluntary and she will have the right to withdraw from any one test at any time without explanation or penalty. Please notify in writing, Drs. Loïc Le Marchand and Rachel Novotny (University of Hawaii at Manoa, Department of Human Nutrition, Food and Animal Sciences, 1955 East West Road, Agricultural Science 216, Honolulu, HI 96822) if your daughter wishes to withdraw from the study. Data collected prior to your daughter's withdrawal may be used by researchers to preserve the integrity of the study.

Contact In The Event Of Research-Related Injury

Please report any health problem that could be related to your daughter's taking part in this study to the Clinical Research Center at 983-6242.

Rights of Research Subjects

Investigators do not foresee any circumstances under which they will terminate your child's participation early.

You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights or remedies because of your child's participation in this research study. If you have any questions about this research project, please contact the Principal Investigator, Dr. Loïc Le Marchand or Dr. Rachel Novotny at (808) 956-3848. If you have questions regarding your child's rights as a research participant you may contact: Raul Rudoy, M.D., Chair, Hawaii Pacific Health Institutional Review Board at (808) 535-7500, the Committee on Human Studies, University of Hawaii, 2540 Maile Way, Honolulu, HI 96822. Ph. (808) 956-5007, or Kaiser Permanente Hawaii IRB, Center for Health Research Hawaii, 501 Alakawa Street, 2nd floor, Honolulu, HI 96817, Ph: (808) 432-4757 or the Department of Clinical Investigations at the Tripler Army Medical Center at (808) 433-6709.

VOLUNTARY STATEMENT

SIGNATURE OF PARENT/GUARDIAN

My signature below indicates that I have read and that I understand the procedures described above and give my informed and voluntary consent to participate in this study. I have been given satisfactory answers to my inquiries concerning project procedures and other matters; I am free to withdraw my child from the study at any time. I understand that I will receive a signed copy of this consent form.

- Sign here if you are a **PARENT/GUARDIAN** consenting for your child's participation.

CHILD'S NAME: _____

PARENT/GUARDIAN'S NAME: _____
(Print or Type) Relationship to Child

Signature Date

- Sign here if you are **18 YEARS OLD** consenting for your own participation.

PARTICIPANT'S NAME:

(Print or Type)

Permanent address of participant:

NAME OF WITNESS:

SIGNATURE OF WITNESS:

My signature as witness certifies that the subject signed this consent form in my presence as his/her voluntary act and deed.

Signature of Witness/Study Staff Date

Addendum to Consent Form

Authorization to Use and Disclose Protected Health Information for Research

Principal Investigators: Loïc Le Marchand, M.D., Ph.D. & Rachel Novotny, Ph.D., R.D., University of Hawaii

Study Title: “Nutritional and Genetic Determinants of Early Puberty” (FAM III)

A federal law known as the Health Insurance Portability and Accountability Act (HIPAA) sets new rules for protecting the privacy of patients’ health care records. HIPAA now requires researchers, except in specific circumstances, to get written permission from study participants before using or disclosing their existing health information or new information obtained for a research study.

This authorization allows the research team to use and disclose my child’s health information associated with the study.

Purpose of this disclosure

We are asking your child to participate in a follow-up study of the Female Adolescent Maturation Study (FAM). This follow-up study will study your child’s diet, physical activity patterns, hormones and genetics. These factors influence how teenagers grow and develop, for example, when your child starts her menstruation, and the size and quality of your child’s bone. The pattern of her monthly period may influence her future risk of breast cancer. Asian and Caucasian girls, especially those who participated in the USDA FAM study, are asked to participate in this study.

The study sponsor may use your health information to analyze and make conclusions about the results of the study. The study results might also be published in medical journals. If the study results are published, your child will not be identified.

Description of the information

My authorization covers all information about my child that is collected for this study. It includes:

- Information obtained from the procedures used to determine my child’s eligibility for the study and information used to contact me regarding my child’s participation in the study, which may include my name, address, zip code, telephone number(s), email address, and my child’s name, birth date, age, ethnicity
- Questionnaires:
 1. Family Background Questionnaire
 2. Health Questionnaire
 3. Physical Activity Questionnaires
 4. 3-day Dietary Records
 5. Menstrual Questionnaire and Calendar
- Spot Urine Sample
- Mouthwash Specimen
- Bone Densitometry Scan and ultrasound
- Measurements
 1. Weight
 2. Standing and sitting height
 3. Shoulder and hip width
 4. Size of waist, hip and calf
 5. Fatfolds
 6. Stage of maturation
 7. Skin color
- Blood and Overnight Urine Samples

Who may use and disclose my child's information?

I authorize the following persons or groups to use and disclose my child's study-related health information:

- Loïc Le Marchand, M.D., Ph.D., Rachel Novotny, PhD, RD, David Easa, MD, Thomas Vogt, MD, Co-Investigators and other research personnel on their research team
- Clinical Research Center personnel
- University of Hawaii, Department of Human Nutrition, Food and Animal Sciences research personnel

Who may receive my child's information?

I authorize the following persons or groups to receive study-related health information about my child:

- University of Hawaii, Cancer Research Center of Hawai'i (recipient of research funds to conduct this study)
- United States Department of Agriculture (USDA) (sponsor of prior FAM studies)
- Officials at Hawaii Pacific Health, Hawaii Pacific Health Research Institute (location of research study)
- Hawaii Pacific Health and Kaiser Permanente Institutional Review Boards (as part of their responsibility to protect human subjects in research)
- Representatives of the Department of Defense
- Kapiolani Clinical Research Center

Expiration of the authorization

This authorization will not expire for the purpose of this research.

Canceling the authorization

I have the right to cancel this authorization at any time. My cancellation must be in writing, addressed to. Drs. Loïc Le Marchand and Rachel Novotny, University of Hawaii at Manoa, Department of Human Nutrition, Food and Animal Sciences, 1955 East-West Road, Agricultural Science 216, Honolulu, HI, 96822. After cancellation, researchers may continue to use data collected only to preserve the integrity of the study.

Disclosures outside the research study

Some of the persons or groups that receive my child's study information may not be required to comply with HIPAA privacy laws, and my child's information may lose its federal protection if those persons or groups disclose it. The Confidentiality section of the consent form describes other ways in which my child's information will be protected.

Use of information for other research activities

Biospecimens

The research team would like to store samples collected from your child in this study with information that will make it possible to identify your child for future research. The samples consist of mouthwash, blood, and urine samples. The samples will be stored at the Cancer Research Center of Hawaii.

Storing your child's extra samples is not required for your child to take part in this research study. If you do not want your child's samples to be used in this manner, do not initial these options.

(Please indicate your approval of any or all of the following statements by writing your initials in the space provided.)

_____ My child's samples may be stored with identifiers in the above named sample bank for future analysis related to this study.

_____ My study samples may be stored with identifiers in the above named sample bank for future analysis not related to this study.

Right to refuse to sign the authorization

I have the right to refuse to sign this authorization. This would prevent those listed above from having access to or using any of my child's health information for the purpose stated. I understand that refusal to sign the authorization means that my child cannot participate in this research study.

Written Permission

I hereby authorize the release of my child's health information in connection with the research study, which has been approved by the Hawaii Pacific Health Institutional Review Board (IRB). I understand that this authorization is voluntary. I will receive a signed copy of this authorization.

Signature of Research Participant's Parent/Guardian

Date

Printed Name of Research Participant

If signed by someone other than the patient's Parent/Guardian, please describe your authority to act on behalf of the Patient:

If you have any questions about HIPAA privacy protections, you may call the Hawaii Pacific Health Institutional Review Board at (808) 535-7500 or the Kaiser Permanente IRB at (808) 432-4757.

Appendix B

Background Questionnaire

Name of Parent/Guardian: _____

Relationship to Child: _____

Today's date: _____/_____/_____
(mth) (day) (yr)

Child's Full Name: _____ (First) _____ (Middle) _____ (Last)

Home Phone Number: _____ Business Number: _____

Pager: _____ Cell Phone: _____

Email address: _____

INFANT BACKGROUND

1. What is the birth date of this child? _____/_____/_____
(mth) (day) (yr)

2. What was the birth weight of this child?
_____ lbs _____ oz (or _____ grams)

3. How was this child fed when she left the hospital (or at 3 days old)? Please check one.

Breast _____

Bottle _____ (go to question 5a)

Breast and Bottle _____

Other _____ (go to question 5a)

4. If breastfed, how many weeks of months was this child breastfed?

Number of **WEEKS** breastfed _____

OR

Number of **MONTHS** breastfed _____

5a. If ever bottle-fed, what type of formula was **mostly** used? (Please check one)

_____ Milk-based infant formula

_____ Soy-based infant formula

Other (please specify): _____

5b. When was the formula introduced?

Infant's age in **WEEKS** _____

OR

Infant's age on **MONTHS** _____

5c. How long was this formula (in question 5a) used?

_____ **WEEKS** or _____ **MONTHS**

5d. Were any other formula used?

YES / **NO** / **Don't know**

(if *NO* or *Don't know*, go to question 6a)

5e. If yes, which type? _____

5f. How long was the other formula used?

_____ **WEEKS** OR _____ **MONTHS**

6a. Has this child ever taken Prednisone, Azmacort, oral contraceptives or any oral medication with steroid content for asthma?

YES / **NO** / **Don't know**

(If *NO* or *Don't know*, go to question 7)

6b. If yes, about how many courses of treatment has the child had in her lifetime?

_____ courses

7. Does this child take any vitamin or mineral supplement in pill or liquid form?

YES / **NO** / **Don't know**

(If *NO* or *Don't know*, go to question 10)

8. How often does she take them?

____ Everyday or almost every day (5 to 7 days a week)

____ Every so often (1 to 4 days a week)

9a. Which of the following does she take?

____ Multivitamin **Brand** _____

____ Multivitamin with iron or other minerals **Brand** _____

____ Single Vitamin/Mineral (please check)

Brand

____ Vitamin A _____

____ Vitamin B/B complex _____

____ Vitamin C _____

____ Vitamin D _____

____ Vitamin E _____

____ Folic acid/Folate _____

____ Calcium _____

____ Iron _____

____ Zinc _____

____ Fluoride _____

____ Chromium _____

____ Selenium _____

____ Something else (please specify):

____ Don't know

____ Don't know

10. What is the ethnicity of each biologic parent of this child? (Estimate percent ethnicity of each parent).

	Father	Mother
	%	%
Japanese	_____	_____
White	_____	_____
Hawaiian	_____	_____
Filipino	_____	_____
Chinese	_____	_____
Other (please specify) :	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

11. Have female family members had breast cancer? Please circle one

YES / NO / Don't know

(if *NO* or *Don't know*, go to question 12)

11a. If yes, what is the relationship of the family member to this child? (Check all that apply and indicate ethnicity).

	Ethnicity
Sister	_____
Mother	_____
Grandmother	_____
Aunt	_____

12. At what age did the mother of this child begin to menstruate?

_____ Years-old

13. At what age did this child begin to menstruate?

_____/_____(if known)
(mth) (yr)

OR

_____ Years-old

Menstrual Questionnaire

Today's date: _____ / _____ / _____
(*month*) (day) (yr)

1. Have you begun to menstruate (had your first period)?

YES / NO (If NO, do not need to complete the rest)

2. If yes, what was the date when you **FIRST** saw menstrual blood?

_____ / _____ OR _____ Years-old
(*month*) (yr)

3. In how many of the last 12 months did you menstruate (seen some blood)?

_____ months

4. How many pads/tampons do you usually use during the **FIRST DAY** (24 hours)?

_____ PADS

OR

_____ TAMPONS

Female Adolescent Maturation Study 1
University of Hawaii-Kaiser Permanente

ID# _____

Physical Activity Questionnaire

Date: _____

1. How many times in the past 14 days have you done at least 20 minutes of exercise hard enough to make you breathe heavily and make your heart beat fast? (Hard exercise includes, for example, playing basketball, jogging or fast bicycling; include time in physical education class).

None	_____	6 to 8 days	_____
1 to 2 days	_____	9 or more days	_____
3 to 5 days	_____		

2. How many times in the past 14 days have you done at least 20 minutes of light exercise that was not hard enough to make you breathe heavily and make your heart beat fast? (Light exercise includes walking or slow bicycling; include time in physical education class).

None	_____	6 to 8 days	_____
1 to 2 days	_____	9 or more days	_____
3 to 5 days	_____		

3. During a normal week how many hours a day do you watch television and videos, or play computer or video games?

None	_____	4 to 5 hours	_____
1 hour or less	_____	6 or more hours	_____
2 to 3 hours	_____		

4. During the past 12 months, how many team or individual sports or activities did you participate in on a competitive level, such as varsity or junior varsity sports, intramurals, or out-of-school programs.

None	_____	3 activities	_____
1 activity	_____	4 or more activities	_____
2 activities	_____		

Female Adolescent Maturation Study 1

University of Hawaii-Kaiser Permanente

ID #: _____

Past Year Physical Activity

Date: _____

1. Please check all activities that you did at least 10 times in the PAST YEAR. Include time spent in school physical education classes. Make sure you include all sport teams that you participated in during the LAST YEAR.

- | | | | | | |
|--|------------------------------------|---|---|-------------------------------------|--|
| <input type="checkbox"/> Aerobics | <input type="checkbox"/> Bicycling | <input type="checkbox"/> Ice Skating | <input type="checkbox"/> Running for Exercise | <input type="checkbox"/> Surfing | <input type="checkbox"/> Weight Training |
| <input type="checkbox"/> Cheerleading | <input type="checkbox"/> Bowling | <input type="checkbox"/> Garden/Yard work | <input type="checkbox"/> Scuba-diving | <input type="checkbox"/> Swimming | <input type="checkbox"/> Wrestling |
| <input type="checkbox"/> Band/Drill Team | <input type="checkbox"/> Canoeing | <input type="checkbox"/> Gymnastics | <input type="checkbox"/> Snorkeling | <input type="checkbox"/> Tennis | <input type="checkbox"/> Baseball |
| <input type="checkbox"/> Dance | <input type="checkbox"/> Hiking | <input type="checkbox"/> Soccer | <input type="checkbox"/> Volleyball | <input type="checkbox"/> Basketball | <input type="checkbox"/> Football |
| <input type="checkbox"/> Roller Skating | <input type="checkbox"/> Softball | Others: _____ | | | |

2. List each activity that you checked above in the "Activity" box below.
3. Check the months that you did each activity.
4. Estimate the amount of time spent in each activity.
5. Use the next page if you run out of space.
6. Please sign at the bottom of page.
7. Ask your Parent/Guardian if you need help filling this out.

ACTIVITY	MONTHS SPENT IN ACTIVITY												TIME SPENT IN ACTIVITY		
	(check boxes)												(WRITE IN NUMBER)		
	Jan	Feb	Mar	Apr	May	Jun	Jly	Aug	Sep	Oct	Nov	Dec	Months per year	Days per wk	Minutes per day

Data Sheet

Date: _____

CHECKLIST

Form	Brought	Reviewed
Menstrual Questionnaire		
Physical Activity Questionnaire		
Diet Record Form		
Background Questionnaire		

FOOT MEASUREMENT
3

Measurement 1 Measurement 2 Measurement 3

Foot Length			
Foot Length to Ball of Foot			

SHIM

DK _____ MD _____ LT _____

ULTRASOUND MEASUREMENT **Measurement 1** **Measurement 2** **Measurement 3**

BUA			
SOS			
SI			

Comments: _____

ANTHROPOMETRY**Measurement 1****Measurement 2****Measurement 3**

Weight (kg)			
Height (cm)			
Sitting height (cm)			
Biacromial breadth (cm)			
Biiliac breadth (cm)			
Arm circumference (cm)			
Abdomen circumference (cm)			
Buttock circumference (cm)			
Calf circumference (cm)			
Subscapular skinfold thickness(mm)			
Tricep skinfold thickness (mm)			
Biceps Skinfold thickness (mm)			
Iliac skinfold thickness (mm)			
Calf skinfold thickness (mm)			

Tanner Staging

Breast

Pubic hair

Comments: _____

Female Adolescent Maturation Study 1

ID#: _____

University of Hawaii – Kaiser Permanente

Diet Record Form

Date: _____ **DAY OF WEEK:** _____

Who is this for?	✓ You! Please complete this Diet Record. You may ask your parent or guardian for help, or call Yihe with questions at 581-1242.
When & Where?	✓ List foods immediately after you eat them for the dates shown above. ✓ Keep this form with you at all times so that you can record your intake whenever you eat. ✓ Record one food item per line.
What did you eat?	✓ Write down everything that you eat and drink throughout your assigned days and nights (4 a.m. to 4 a.m. the next day).
Describe it accurately.	✓ Be as specific as possible. ✓ Include cooking method used to prepare food e.g. baked, broiled, fried, canned, fresh, frozen. ✓ Include brand names whenever possible. ✓ Describe liquid included in canned foods e.g. tuna in water, sliced peaches in heavy syrup. ✓ Include additions such as sugar drink or milk.
How much did you eat?	✓ Record only the portions that you ate. ✓ Estimate amount using collapsible cup and ruler attached to the back of the Diet Record Forms. ✓ Record the amount of oil added in cooking.

We are interested in finding out what you normally eat. Please do not change your eating habits during the diet recording period.

Example:

TIME	PLACE	WHAT YOU ATE	DESCRIPTIONS OF WHAT YOU ATE	AMOUNT
8:30 am	Kitchen	Scrambled eggs	2 large, white eggs, 1 tsp canola oil, 1/8 cup onions and 1 Tbsp ketchup	¼1/2 ½¼ cup
8:30 am	Kitchen	Banana	Apple banana, 7 inches	1 banana
8:30 am	Kitchen	Milk	Skim milk, Viva brand, Vitamin A fortified	½ 1 cup
12:30 pm	School cafeteria	Rice	White, medium-grain rice	1/3 cup
12:30 pm	School cafeteria	Chicken stir fry	¼ ¼1/4 cup chicken breast, skinless, 1/8 cup fresh onions, 1/8 cup cabbage (canned with no salt added), 1 tbsp canola oil	1 cup
12:30 pm	School cafeteria	Soup	1 cup chicken broth (canned, 1/3 less fat), 1/4¼ ¼cup fresh carrots, ¼ 1/8 cup onions	½1/2 cup
12:30 pm	School cafeteria	Milk	Chocolate, 2% fat, Viva brand	1 cup
12:30 pm	School cafeteria	Water	Plain	1 cup
12:30 pm	School cafeteria	Oranges	Canned, heavy syrup	¼1 cup
3.00 pm	Friend's house	Chips	Lay's brand. Regular, sour cream and onion flavor	1 cup
3.00 pm	Friend's house	Soda	Mountain Dew, regular one 12-oz can, ¼ ¼ ¼ cup ice crushed ice	12 oz
3.00 pm	Friend's house	Chocolate bar	Snickers's bar, regular	1 6-inch bar
5:30 pm	Living room	Sandwich	Love's brand wheat bread 2 slices, 1 tbsp Best Foods regular mayonnaise, ½ 3 leaves iceberg lettuce and 1/4 ¼¼ cup roast beef (deli, thin sliced)	1 sandwich
5:30 pm	Living room	Soda	Diet Coke, regular, chilled	1 12-oz can
7:00 pm	Dining room	Pizza	Pizza Hut, large pizza, 11 inch long, 5 inch wide, triangle-shaped, thick crust, 3 slices pepperoni, 1 tbsp black olives	2 slices
7:00 pm	Dining room	Garlic bread	2 pieces french bread 5-inch X 2-inch, white bread, 2 tbsp butter, 1 tsp garlic	2 pieces
7:00 pm	Dining room	Water	Plain	2 cups
7:00 pm	Dining room	Ice-cream	Dreyer's brand, reduced fat, no sugar added, chocolate flavor	½1 cup

Date: _____

Day : _____

ID #: _____

Family Background Questionnaire

Please complete the information according to the following instructions:

1. Use black pen only
2. Keep the numbers/ letters within the boxes provided
3. Fill the bubble completely
4. If a mistake should occur, mark a wrong answer with an X on it and fill the correct one.

1. What is the birth date of each biologic parent of this child?

a. Father

M	M	D	D	Y	Y

b. Mother

M	M	D	D	Y	Y

2. How many years of education did the **FATHER** of this child complete?

		Years
--	--	-------

3. What is the last level of education obtained by the **FATHER** of this child?

- 00 Did not complete high school
- 10 Complete high school
- 20 Completed post high school training, excluding college (trade school or business school)
- 30 Completed some college/community college
- 40 Graduated from a four-year college or university
- 50 Attended and/or completed graduate school
- 60 Other (specify):

4. How many years of education did the **MOTHER** of this child complete?

		Years
--	--	-------

5. What is the last level of education obtained by the **MOTHER** of this child?

00 Did not complete high school

10 Complete high school

20 Completed post high school training, excluding college (trade school or business school)

30 Completed some college/community college

40 Graduated from a four-year college or university

50 Attended and/or completed graduate school

60 Other (specify):

6. What is the current weight and height of the *biological* parents of this child?

Parent	Weight	Height
a. Father	1 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Lbs	3 <input type="text"/> <input type="text"/> Ft <input type="text"/> <input type="text"/> In
	OR	OR
	2 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Kg	4 <input type="text"/> <input type="text"/> Mt <input type="text"/> <input type="text"/> Cm
b. Mother	1 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Lbs	3 <input type="text"/> <input type="text"/> Ft <input type="text"/> <input type="text"/> In
	OR	OR
	2 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Kg	4 <input type="text"/> <input type="text"/> Mt <input type="text"/> <input type="text"/> Cm

7. Is there any family history of diabetes? (If no or don't know, go to question 9)

⁰O YES

¹O NO

²O DON'T KNOW

8. If yes, what is the relationship of the family member to this child? Check all that apply.

- ⁰ Father
- ¹ Mother
- ² Brother
- ³ Sister
- ⁴ Paternal Grandfather
- ⁵ Maternal Grandfather
- ⁶ Paternal Grandmother
- ⁷ Maternal grandmother
- ⁸ Paternal Uncle
- ⁹ Maternal Uncle
- ¹⁰ Paternal Aunt
- ¹¹ Maternal Aunt

9. Have female family members had breast cancer *since your child's last visit?* (If no or don't know, go to question 11).

- ^A YES ^B NO ^C DON'T KNOW

10. If yes, what is the relationship of the family member to this child? (Check all that apply and indicate ethnicity).

Ethnicity

- | | |
|--|----------------------|
| ¹ <input type="checkbox"/> Sister | <input type="text"/> |
| ² <input type="checkbox"/> Mother | <input type="text"/> |
| ³ <input type="checkbox"/> Maternal Aunt | <input type="text"/> |
| ⁴ <input type="checkbox"/> Paternal Aunt | <input type="text"/> |
| ⁵ <input type="checkbox"/> Maternal Grandmother | <input type="text"/> |
| ⁶ <input type="checkbox"/> Paternal Grandmother | <input type="text"/> |

The following questions are about the biological mother when she was pregnant with this child in the study:

11. Did you experience morning sickness (nausea during pregnancy) when you were pregnant with her?

(If no, go to question 14)

- ⁰ YES ¹ NO

12. If yes, was your morning sickness

- ¹ Mild ² Moderate ³ Severe

13. Did you have morning sickness? (Circle all that apply).
- a. During the first trimester of your pregnancy? ⁰O YES ¹O NO
- b. During the second trimester? ⁰O YES ¹O NO
- c. During the third trimester? ⁰O YES ¹O NO
14. Did your doctor tell you that you had pregnancy-induced hypertension (e.g. high blood pressure, hypertension, pre-eclampsia, eclampsia or toxemia)? (If no, go to question 16)
- ⁰O YES ¹O NO
15. If yes, did you have to be hospitalized?
- ⁰O YES ¹O NO

The following questions refer to your daughter in the study.

16. Was your daughter born
- ⁰O On your due date
- ¹O Early. If early, how many weeks and days early weeks days
- ²O Late. If late, how many weeks and days late weeks days
17. What was your daughter's length at birth?
- A in |
- OR
- B Cm |

Health Questionnaire

Please complete the information according to the following instructions:
 5. Use black pen only
 6. Keep the numbers/ letters within the boxes provided
 7. Fill the bubble completely
 If a mistake should occur, mark a wrong answer with an X on it and fill the correct one.

1. What is the name of the school you attend? *(If you are home schooled, please write "home schooled")*.

2. Have you begun to menstruate (had your first period)? *(If no or don't know, go to question 8)*

⁰ YES ¹ NO ² DON'T KNOW

3. If yes, what was the date when you **FIRST** saw menstrual blood?

⁰ OR ¹
 a M M Y Y b Years-old

4. How many pads/tampons do you usually use during the **FIRST DAY** (24 hours)?

⁰ OR ¹
 a PADS b TAMPON

5. What is the usual length of time from the **FIRST** day of one period to the **FIRST** day of the next period?

a ⁰ Days
 b ¹ Don't know

6. Did your period ever become regular (that is, you could usually predict about when they would start)? *(If no or don't know, go to question 8)*

⁰ YES ¹ NO ² DON'T KNOW

7. If yes, at what age did your menstrual periods become regular?

⁰ OR ¹
 a M M Y Y b Years-old

8. Are you currently taking birth control pills? *(If no, go to question 13)*

⁰ YES ¹ NO

9. How long have you been taking birth control pills?

⁰ OR ¹
a M M Y Y b Years-old

10. What brand of birth control pills are you currently using?

11. Have you taken any other brand? (If NO, go to question 11)

⁰ YES ¹ NO

12. If so, name the other brand:

13. If you do **NOT** take birth control pills:

a) How would you describe your menstrual periods?

- ⁰ Very regular (you could always predict when they would start within 3 days)
- ¹ Regular
- ² Irregular
- ³ No periods

If you are **CURRENTLY** taking birth control pills:

b) How would you describe your menstrual periods 12 months before taking birth control pills?

- ⁰ Very regular (you could always predict when they would start within 3 days)
- ¹ Regular
- ² Irregular
- ³ No periods

14. Do you smoke? (If NO, go to question 17)

⁰ YES ¹ NO

15. How long have you been smoking?

M M Y Y

16. How many cigarettes do you smoke in a day?

Cigarettes

17. Do you take any medication for asthma? (If no, go to question 19)

⁰ YES ¹ NO

18. If **YES**, please describe the medicines you take for asthma.

	Medication Name: ^A	Medication Name: ^B	Medication Name: ^C	Medication Name: ^D
Times per Day ^A				
OR				
Times per Week ^B				
TOTAL LENGTH OF TIME IN THIS PAST YEAR				
Days ^C				
OR				
Weeks ^D				
OR				
Month ^E				
OR				
Year ^F				

19. Have you ever taken medication for acne that was prescribed by a doctor?

⁰ YES

¹ NO

20. Have you ever broken any bones?

⁰ YES

¹ NO

21. When and which bone(s) have you broken?

Bone	Month and Year It was Broken						
A	<table data-bbox="1024 401 1479 499"><tr><td data-bbox="1024 401 1084 499">□</td><td data-bbox="1084 401 1149 499">□</td><td data-bbox="1159 401 1224 499">Mont h</td><td data-bbox="1279 401 1344 499">□</td><td data-bbox="1344 401 1409 499">□</td><td data-bbox="1419 401 1479 499">Year</td></tr></table>	□	□	Mont h	□	□	Year
□	□	Mont h	□	□	Year		
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□	□	Mont h	□	□	Year		
C	<table data-bbox="1024 821 1479 919"><tr><td data-bbox="1024 821 1084 919">□</td><td data-bbox="1084 821 1149 919">□</td><td data-bbox="1159 821 1224 919">Mont h</td><td data-bbox="1279 821 1344 919">□</td><td data-bbox="1344 821 1409 919">□</td><td data-bbox="1419 821 1479 919">Year</td></tr></table>	□	□	Mont h	□	□	Year
□	□	Mont h	□	□	Year		
D	<table data-bbox="1024 1031 1479 1129"><tr><td data-bbox="1024 1031 1084 1129">□</td><td data-bbox="1084 1031 1149 1129">□</td><td data-bbox="1159 1031 1224 1129">Mont h</td><td data-bbox="1279 1031 1344 1129">□</td><td data-bbox="1344 1031 1409 1129">□</td><td data-bbox="1419 1031 1479 1129">Year</td></tr></table>	□	□	Mont h	□	□	Year
□	□	Mont h	□	□	Year		

Physical Activity Questionnaire

Please complete the information according to the following instructions:
 1. Use black pen only
 2. Keep the numbers/ letters within the boxes provided
 3. Fill the bubble completely ○ ○ ○ ○ ●

If a mistake should occur, mark a wrong answer with an X on it and fill the correct one.

○ ○ ○ ● ●

1. How many times in the past 14 days have you done at least 20 minutes of exercise hard enough to make you breathe heavily and make your heart beat fast? (Hard exercise includes, for example, playing basketball, jogging or fast bicycling; include time in physical education class).

- | | | | |
|-------------|-----------------------|----------------|-----------------------|
| None | <input type="radio"/> | 6 to 8 days | <input type="radio"/> |
| 1 to 2 days | <input type="radio"/> | 9 or more days | <input type="radio"/> |
| 3 to 5 days | <input type="radio"/> | | |

2. How many times in the past 14 days have you done at least 20 minutes of light exercise that was not hard enough to make you breathe heavily and make your heart beat fast? (Light exercise includes walking or slow bicycling; include time in physical education class).

- | | | | |
|-------------|-----------------------|----------------|-----------------------|
| None | <input type="radio"/> | 6 to 8 days | <input type="radio"/> |
| 1 to 2 days | <input type="radio"/> | 9 or more days | <input type="radio"/> |
| 3 to 5 days | <input type="radio"/> | | |

3. During a normal week how many hours a day do you watch television and videos, or play computer or video games?

- | | | | |
|----------------|-----------------------|-----------------|-----------------------|
| None | <input type="radio"/> | 4 to 5 hours | <input type="radio"/> |
| 1 hour of less | <input type="radio"/> | 6 or more hours | <input type="radio"/> |
| 2 to 3 hours | <input type="radio"/> | | |

4. During the past 12 months, how many team or individual sports or activities did you participate in on a competitive level, such as varsity or junior varsity sports, intramurals, or out-of-school programs.

- | | | | |
|--------------|-----------------------|----------------------|-----------------------|
| None | <input type="radio"/> | 3 activities | <input type="radio"/> |
| 1 activity | <input type="radio"/> | 4 or more activities | <input type="radio"/> |
| 2 activities | <input type="radio"/> | | |

5. What activities did you compete in? (fill in the numbers corresponding to the activity, according to the list on the **Past Year Physical Activity** questionnaire)

1. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	5. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	9. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	13. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>
2. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	6. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	10. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	14. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>
3. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	7. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	11. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	15. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>
4. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	8. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	12. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>	16. <table border="1" style="display: inline-table; width: 40px; height: 20px; vertical-align: middle;"></table>

Female Adolescent Maturation Study 2

University of Hawaii-Kapiolani Clinical Research Center

ID #: _____

Past Year Physical Activity

Date: _____

1. Please check all activities that you did at least 10 times in the PAST YEAR. Include time spent in school physical education classes. Make sure you include all sport teams that you participated in during the LAST YEAR.

- | | | | | | |
|--|------------------------------------|---|---|-------------------------------------|--|
| <input type="checkbox"/> Aerobics | <input type="checkbox"/> Bicycling | <input type="checkbox"/> Ice Skating | <input type="checkbox"/> Running for Exercise | <input type="checkbox"/> Surfing | <input type="checkbox"/> Weight Training |
| <input type="checkbox"/> Cheerleading | <input type="checkbox"/> Bowling | <input type="checkbox"/> Garden/Yard work | <input type="checkbox"/> Scuba-diving | <input type="checkbox"/> Swimming | <input type="checkbox"/> Wrestling |
| <input type="checkbox"/> Band/Drill Team | <input type="checkbox"/> Canoeing | <input type="checkbox"/> Gymnastics | <input type="checkbox"/> Snorkeling | <input type="checkbox"/> Tennis | <input type="checkbox"/> Baseball |
| <input type="checkbox"/> Dance | <input type="checkbox"/> Hiking | <input type="checkbox"/> Soccer | <input type="checkbox"/> Volleyball | <input type="checkbox"/> Basketball | <input type="checkbox"/> Football |
| <input type="checkbox"/> Roller Skating | <input type="checkbox"/> Softball | Others: _____ | | | |

2. List each activity that you checked above in the "Activity" box below.
3. Check the months that you did each activity.
4. Estimate the amount of time spent in each activity.
5. Use the next page if you run out of space.
6. Please sign at the bottom of page.
7. Ask your Parent/Guardian if you need help filling this out.

ACTIVITY	MONTHS SPENT IN ACTIVITY												TIME SPENT IN ACTIVITY		
	(check boxes)												(WRITE IN NUMBER)		
	Jan	Feb	Mar	Apr	May	Jun	Jly	Aug	Sep	Oct	Nov	Dec	Months per year	Days per wk	Minutes per day

Data Sheet

1. CHECKLIST

Form	Brought	Reviewed by (initials)			
^a CONSENT	⁰ O YES ¹ O NO	²			
^b Health Questionnaire	⁰ O YES ¹ O NO	²			
^c Family Background Questionnaire	⁰ O YES ¹ O NO	²			
^d Physical Activity Questionnaire	⁰ O YES ¹ O NO	²			
^e Diet Record Forms	⁰ O YES ¹ O NO	²			

2. ^aPREGNANCY TEST

⁰O Positive

¹O Negative

^bComments

--

--

3. What have you drunk in the past 12 hours? (Include any liquid you drank, such as water, milk and soda).

⁰ TIME	¹ PLACE	² WHAT DID YOU DRINK? (ml)	³ AMOUNT
^a			
^b			
^c			
^d			
^e			
^f			

4. MOUTHWASH

^AVial 1 ID#:

--	--	--	--

--	--	--

--	--	--

^B Completed 1st mouthwash:

⁰O YES

¹O NO

^CIf no, state reason:

--

5. ANTHROPOMETRY	Measurement 1	Measurement 2	Measurement 3
^A Weight (lbs)	0	1	2
^B Height (cm)	0	1	2
^C Sitting height (cm)	0	1	2
^D Ulna length (cm)	0	1	2
^E Biacromial breadth (cm)	0	1	2
^F Biiliac breadth (cm)	0	1	2
^G Arm circumference (cm)	0	1	2
^H Abdomen circumference (cm)	0	1	2
Buttock circumference (cm)	0	1	2
^J Calf circumference (cm)	0	1	2
^K Subscapular skinfold thickness(mm)	0	1	2
^L Tricep skinfold thickness (mm)	0	1	2
^M Biceps Skinfold thickness (mm)	0	1	2
^N Iliac skinfold thickness (mm)	0	1	2
^O Calf skinfold thickness (mm)	0	1	2

^PInitials Of Person Who Took Anthropometry:

--	--	--

^QComments:

--

6. FOOT MEASUREMENT	Measurement 1	Measurement 2
^A Foot Length	0	1
^B Foot Width	0	1

7. TANNER STAGING

Tanner Staging	1	2	3	4	5
^A Breast	1	2	3	4	5
^B Pubic hair	1	2	3	4	5

^CInitials Of Person Who Staged:

^DComments:

8. ULTRASOUND MEASUREMENT

^ASHIM

⁰DK

¹MD

²LT

	Measurement 1	Measurement 2	Measurement 3
^B BUA	1	2	3
^C SOS	1	2	3
^D SI	1	2	3

^EInitials Of Person Who Took Ultrasound Measurement:

^FComments:

9. MOUTHWASH

^AVial 2 ID#:

^BCompleted 2nd mouthwash: ⁰O YES ¹O NO

^CIf no, state reason:

Female Adolescent Maturation Study 2

ID#: _____

University of Hawaii-Kapiolani Clinical Research Center

Diet Record Form

Date: _____

Day : _____

ID #: _____

TIME	<i>PLACE</i>	WHAT YOU ATE	DESCRIPTIONS OF WHAT YOU ATE	AMOUNT

ID#: _____

University of Hawaii-Kapiolani Clinical Research Center
Family Background Questionnaire

Please complete the information according to the following instructions:
 1. Use black pen only
 2. Keep the numbers/ letters within the boxes provided
 3. Fill the bubble completely
 4. If a mistake should occur, mark a wrong answer with an X on it and fill the correct one.

1. What is the birth date of each biologic parent of this child?

a. Father
 M M D D Y Y

b. Mother
 M M D D Y Y

2. How many years of education did the **FATHER** of this child complete?

Years

3. What is the last level of education obtained by the **FATHER** of this child?

- 00 Did not complete high school
- 10 Complete high school
- 20 Completed post high school training, excluding college (trade school or business school)
- 30 Completed some college/community college
- 40 Graduated from a four-year college or university
- 50 Attended and/or completed graduate school
- 60 Other (specify):

4. How many years of education did the **MOTHER** of this child complete?

Years

5. What is the last level of education obtained by the **MOTHER** of this child?

- 00 Did not complete high school
- 10 Complete high school
- 20 Completed post high school training, excluding college (trade school or business school)
- 30 Completed some college/community college

40 Graduated from a four-year college or university

50 Attended and/or completed graduate school

60 Other (specify):

6. What is the current weight and height of the *biological* parents of this child?

Parent	Weight	Height
a. Father	1 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Lbs OR	3 <input type="text"/> <input type="text"/> Ft <input type="text"/> <input type="text"/> In OR
	2 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Kg	4 <input type="text"/> <input type="text"/> Mt <input type="text"/> <input type="text"/> C m
b. Mother	1 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Lbs OR	3 <input type="text"/> <input type="text"/> Ft <input type="text"/> <input type="text"/> In OR
	2 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Kg	4 <input type="text"/> <input type="text"/> Mt <input type="text"/> <input type="text"/> C m

7. Is there any family history of diabetes? (If no or don't know, go to question 9)

⁰O YES

¹O NO

²O DON'T KNOW

8. If yes, what is the relationship of the family member to this child? Check all that apply.

⁰ O Father

¹ O Mother

² O Brother

³ O Sister

⁴ O Paternal Grandfather

⁵ O Maternal Grandfather

⁶ O Paternal Grandmother

⁷ O Maternal grandmother

⁸ O Paternal Uncle

⁹ O Maternal Uncle

¹⁰ O Paternal Aunt

¹¹ O Maternal Aunt

9. Have female family members had breast cancer *since your child's last visit?* (If no or don't know, go to question 11).

^AO YES

^BO NO

^CO DON'T KNOW

10. If yes, what is the relationship of the family member to this child? (*Check all that apply and indicate ethnicity*).

	Ethnicity
¹ O Sister	<input type="text"/>
² O Mother	<input type="text"/>
³ O Maternal Aunt	<input type="text"/>
⁴ O Paternal Aunt	<input type="text"/>
⁵ O Maternal Grandmother	<input type="text"/>
⁶ O Paternal Grandmother	<input type="text"/>

11. What is the ethnicity of each of this child's grandparents? (Estimate percent ethnicity of each grandparent).

	Paternal Grandfather %	Paternal Grandmother %	Maternal Grandfather %	Maternal Grandmother %
Japanese	_____	_____	_____	_____
White	_____	_____	_____	_____
Hawaiian	_____	_____	_____	_____
Filipino	_____	_____	_____	_____
Chinese	_____	_____	_____	_____
Other (please specify):	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

The following questions are about the biological mother when she was pregnant with this child in the study:

12. Did you experience morning sickness (nausea during pregnancy) when you were pregnant with her?

(If no, go to question 14)

⁰O YES

¹O NO

13. If yes, was your morning sickness

¹O Mild

²O Moderate

³O Severe

14. Did you have morning sickness? (Circle all that apply).
- | | | |
|--|--------------------|-------------------|
| a. During the first trimester of your pregnancy? | ⁰ O YES | ¹ O NO |
| b. During the second trimester? | ⁰ O YES | ¹ O NO |
| c. During the third trimester? | ⁰ O YES | ¹ O NO |

15. Did your doctor tell you that you had pregnancy-induced hypertension (e.g. high blood pressure, hypertension, pre-eclampsia, eclampsia or toxemia)? (If no, go to question 17)

⁰O YES ¹O NO

16. If yes, did you have to be hospitalized?

⁰O YES ¹O NO

The following questions refer to your daughter in the study.

17. Was your daughter born

⁰O On your due date

¹O Early. If early, how many weeks and days early weeks days

²O Late. If late, how many weeks and days late weeks days

18. What was your daughter's length at birth?

A in |

OR

B Cm |

ID#: _____

University of Hawaii-Kapiolani Clinical Research Center
Family Background Questionnaire

Please complete the information according to the following instructions:

1. Use black pen only
2. Keep the numbers/ letters within the boxes provided
3. Fill the bubble completely
4. If a mistake should occur, mark a wrong answer with an X on it and fill the correct one.

1. What is the birth date of each biologic parent of this child?

a. Father

M	M	D	D	Y	Y

b. Mother

M	M	D	D	Y	Y

2. How many years of education did the **FATHER** of this child complete?

		Years
--	--	-------

3. What is the last level of education obtained by the **FATHER** of this child?

- 00 Did not complete high school
- 10 Complete high school
- 20 Completed post high school training, excluding college (trade school or business school)
- 30 Completed some college/community college
- 40 Graduated from a four-year college or university
- 50 Attended and/or completed graduate school
- 60 Other (specify):

4. How many years of education did the **MOTHER** of this child complete?

		Years
--	--	-------

5. What is the last level of education obtained by the **MOTHER** of this child?

00 Did not complete high school

10 Complete high school

20 Completed post high school training, excluding college (trade school or business school)

30 Completed some college/community college

40 Graduated from a four-year college or university

50 Attended and/or completed graduate school

60 Other (specify):

6. What is the current weight and height of the *biological* parents of this child?

Parent	Weight	Height
a. Father	1 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Lbs	3 <input type="text"/> <input type="text"/> Ft <input type="text"/> <input type="text"/> In
	OR	OR
	2 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Kg	4 <input type="text"/> <input type="text"/> Mt <input type="text"/> <input type="text"/> Cm
b. Mother	1 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Lbs	3 <input type="text"/> <input type="text"/> Ft <input type="text"/> <input type="text"/> In
	OR	OR
	2 <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Kg	4 <input type="text"/> <input type="text"/> Mt <input type="text"/> <input type="text"/> Cm

7 Is there any family history of diabetes? (If no or don't know, go to question 9)

⁰O YES

¹O NO

²O DON'T KNOW

8 If yes, what is the relationship of the family member to this child? Check all that apply.

- ⁰ Father
- ¹ Mother
- ² Brother
- ³ Sister
- ⁴ Paternal Grandfather
- ⁵ Maternal Grandfather
- ⁶ Paternal Grandmother
- ⁷ Maternal grandmother
- ⁸ Paternal Uncle
- ⁹ Maternal Uncle
- ¹⁰ Paternal Aunt
- ¹¹ Maternal Aunt

9 Have female family members had breast cancer? (If no or don't know, go to question 11).

^A YES

^B NO

^C DON'T KNOW

10. If yes, what is the relationship of the family member to this child? (Check all that apply and indicate ethnicity).

Ethnicity

- ¹ Sister
- ² Mother
- ³ Maternal Aunt
- ⁴ Paternal Aunt
- ⁵ Maternal Grandmother
- ⁶ Paternal Grandmother

The following questions are about the biological mother when she was pregnant with this child in the study:

11 Did you experience morning sickness (nausea during pregnancy) when you were pregnant with her?

(If no, go to question 14)

⁰O YES

¹O NO

12 If yes, was your morning sickness

¹O Mild

²O Moderate

³O Severe

13 Did you have morning sickness? (Circle all that apply).

a. During the first trimester of your pregnancy?

⁰O YES

¹O NO

b. During the second trimester?

⁰O YES

¹O NO

c. During the third trimester?

⁰O YES

¹O NO

14 Did your doctor tell you that you had pregnancy-induced hypertension (e.g. high blood pressure, hypertension, pre-eclampsia, eclampsia or toxemia)? (If no, go to question 16)

⁰O YES

¹O NO

15 If yes, did you have to be hospitalized?

⁰O YES

¹O NO

The following questions refer to your daughter in the study.

16 Has your daughter born

⁰O On your due date

¹O Early. If early, how many weeks and days early

weeks

days

²O Late. If late, how many weeks and days late

weeks

days

17. What is the birth date of this child? _____ / _____ / _____
(mth) (day) (yr)

18. What was the birth weight of this child?

_____ lbs _____ oz (or _____ grams)

19. What was your daughter's length at birth?

A in

OR

B

--	--

 Cm |

20. How was this child fed when she left the hospital (or at 3 days old)? Please check one.

- Breast _____
- Bottle _____
- Breast and Bottle _____
- Other _____

21. If breastfed, how many weeks or months was this child breastfed?

Number of WEEKS breastfed _____
OR
Number of MONTHS breastfed _____

22a. If ever bottle-fed, what type of formula was **mostly** used? (Please check one)

- _____ Milk-based infant formula
- _____ Soy-based infant formula
- Other (please specify): _____

22b. When was the formula introduced?

Infant's age in WEEKS _____
OR
Infant's age on MONTHS _____

22c. How long was this formula (in question 22a) used?

_____ **WEEKS** or _____ **MONTHS**

22d. Were any other formula used?

YES / NO / Don't know

(if *NO* or *Don't know*, go to question 6a)

22e. If yes, which type? _____

22f. How long was the other formula used?

_____ **WEEKS** **OR** _____ **MONTHS**

23a. Has this child ever taken Prednisone, Azmacort, oral contraceptives or any oral medication with steroid content for asthma?

YES / **NO** / **Don't know**

(If *NO* or *Don't know*, go to question 24)

23b. If yes, about how many courses of treatment has the child had in her lifetime?

_____ courses

24. Does this child take any vitamin or mineral supplement in pill or liquid form?

YES / **NO** / **Don't know**

(If *NO* or *Don't know*, go to question 26)

25. How often does she take them?

____ Everyday or almost every day (5 to 7 days a week)

____ Every so often (1 to 4 days a week)

FAMILY BACKGROUND

26. What is the ethnicity of each biologic parent of this child? (Estimate percent ethnicity of each parent).

	Father	Mother
	%	%
Japanese	_____	_____
White	_____	_____
Hawaiian	_____	_____
Filipino	_____	_____
Chinese	_____	_____
Other (please specify) :	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

27. What is the ethnicity of each of this child's grandparents? (Estimate percent ethnicity of each grandparent).

	Paternal Grandfather %	Paternal Grandmother %	Maternal Grandfather %	Maternal Grandmother %
Japanese	_____	_____	_____	_____
White	_____	_____	_____	_____
Hawaiian	_____	_____	_____	_____
Filipino	_____	_____	_____	_____
Chinese	_____	_____	_____	_____
Other (please specify) :				
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

28. At what age did the mother of this child begin to menstruate?

_____ Years-old

ID#: _____

Health Questionnaire

Please complete the information according to the following instructions:

1. Use black pen only
2. Keep the numbers/ letters within the boxes provided
3. Fill the bubble completely

If a mistake should occur, mark a wrong answer with an X on it and fill the correct one.

1. What is the name of the school you attend? *(If you are home schooled, please write "home schooled")*.

2. Have you begun to menstruate (had your first period)? *(If no or don't know, go to question 8)*

- ⁰ YES ¹ NO ² DON'T KNOW

3. If yes, what was the date when you **FIRST** saw menstrual blood?

⁰ OR ¹
a M M Y Y b Years-old

4. How many pads/tampons do you usually use during the **FIRST DAY** (24 hours) of your periods?

⁰ OR ¹
a PADS b TAMPON

5. What is the usual length of time from the **FIRST** day of one period to the **FIRST** day of the next period?

⁰ Days
¹ Don't know

6. Did your period ever become regular (that is, you could usually predict about when they would start)? *(If no or don't know, go to question 8)*

- ⁰ YES ¹ NO ² DON'T KNOW

7. If yes, at what age did your menstrual periods become regular?

⁰ OR ¹
a M M Y Y b Years-old

8. .Are you currently on female hormonal contraceptives? (if no, go to the question 9)

⁰ YES

¹ NO

a) How long have you been taking female hormonal contraceptives?

⁰ OR ¹
a M M Y Y b Years-old

b) What brand of female hormonal contraceptives are you currently using?

c) Have you taken any other brand? (If NO, go to question 13)

⁰ YES

¹ NO

d) If so, name the other brand:

9. If no, have you used any female hormonal contraceptives within the past 6 months?

⁰ YES

¹ NO (if no, go to the question 10)

a) When was the last time you use female hormonal contraceptives ?

⁰ OR ¹
a M M Y Y b Years-old

b) What brand of female hormonal contraceptives did you use?

10. If you have not, currently or within the past 6 months, used female hormonal contraceptives,

a) How would you describe your menstrual periods?

⁰ Very regular (you could always predict when they would start within 3 days)

¹ Regular

² Irregular

³ No periods

If you are **CURRENTLY** using female hormonal contraceptives:

b) How would you describe your menstrual periods 12 months before taking female hormonal contraceptives?

⁰ Very regular (you could always predict when they would start within 3 days)

¹ Regular

² Irregular

³ No periods

11. Do you smoke? (If NO, go to question 17)

⁰ YES

¹ NO

12. How long have you been smoking?

M	M

Y	Y

13. How many cigarettes do you smoke in a day?

--	--

Cigarettes

14. Do you take any medication for asthma? (If no, go to question 19)

⁰ YES

¹ NO

15. If YES, please describe the medicines you take for asthma.

	Medication Name: ^A	Medication Name: ^B	Medication Name: ^C	Medication Name: ^D
Times per Day ^A				
OR				
Times per Week ^B				
TOTAL LENGTH OF TIME IN THIS PAST YEAR				
Days ^C				
OR				
Weeks ^D				
OR				
Month ^E				
OR				
Year ^F				

16. Do you take any medication for diabetes? (If no, go to question 21)

⁰ YES

¹ NO

17. If **YES**, please describe the medicines you take for diabetes.

	Medication Name: ^A	Medication Name: ^B	Medication Name: ^C	Medication Name: ^D
Times per Day ^A				
OR				
Times per Week ^B				
TOTAL LENGTH OF TIME IN THIS PAST YEAR				
Weeks ^C				
OR				
Months ^D				

18. Have you ever taken medication for acne that was prescribed by a doctor?

⁰ YES

¹ NO

19. Have you ever broken any bones?

⁰ YES

¹ NO

20. When and which bone(s) have you broken?

Bone	Month and Year It was Broken
A	<input type="text"/> <input type="text"/> Month <input type="text"/> <input type="text"/> Year
B	<input type="text"/> <input type="text"/> Month <input type="text"/> <input type="text"/> Year
C	<input type="text"/> <input type="text"/> Month <input type="text"/> <input type="text"/> Year
D	<input type="text"/> <input type="text"/> Month <input type="text"/> <input type="text"/> Year

ID#: _____

University of Hawaii-Kapiolani Clinical Research Center
Physical Activity Questionnaire

Please complete the information according to the following instructions:
 1. Use black pen only
 2. Keep the numbers/ letters within the boxes provided
 3. Fill the bubble completely ○ ○ ○ ○ ●

If a mistake should occur, mark a wrong answer with an X on it and fill the correct one.

○ ○ ○ ✕ ●

1. How many times in the past 14 days have you done at least 20 minutes of exercise hard enough to make you breathe heavily and make your heart beat fast? (Hard exercise includes, for example, playing basketball, jogging or fast bicycling; include time in physical education class).

- | | | | |
|-------------|---|----------------|---|
| None | ○ | 6 to 8 days | ○ |
| 1 to 2 days | ○ | 9 or more days | ○ |
| 3 to 5 days | ○ | | |

2. How many times in the past 14 days have you done at least 20 minutes of light exercise that was not hard enough to make you breathe heavily and make your heart beat fast? (Light exercise includes walking or slow bicycling; include time in physical education class).

- | | | | |
|-------------|---|----------------|---|
| None | ○ | 6 to 8 days | ○ |
| 1 to 2 days | ○ | 9 or more days | ○ |
| 3 to 5 days | ○ | | |

3. During a normal week how many hours a day do you watch television and videos, or play computer or video games?

- | | | | |
|----------------|---|-----------------|---|
| None | ○ | 4 to 5 hours | ○ |
| 1 hour of less | ○ | 6 or more hours | ○ |
| 2 to 3 hours | ○ | | |

4. During the past 12 months, how many team or individual sports or activities did you participate in on a competitive level, such as varsity or junior varsity sports, intramurals, or out-of-school programs.

- | | | | |
|--------------|---|----------------------|---|
| None | ○ | 3 activities | ○ |
| 1 activity | ○ | 4 or more activities | ○ |
| 2 activities | ○ | | |

5. What activities did you compete in? (fill in the numbers corresponding to the activity, according to the list on the **Past Year Physical Activity** questionnaire)

1.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	5.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	9.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	13.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>
2.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	6.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	10.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	14.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>
3.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	7.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	11.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	15.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>
4.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	8.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	12.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>	16.	<table border="1" style="display: inline-table; width: 40px; height: 20px;"></table>

Female Adolescent Maturation Study 3

All Girls

University of Hawaii-Kapiolani Clinical Research Center

ID #: _____

Past Year Physical Activity

Date: _____

1. Please check all activities that you did at least 10 times in the PAST YEAR. Include time spent in school physical education classes. Make sure you include all sport teams that you participated in during the LAST YEAR.

- | | | | | | |
|--|------------------------------------|---|---|-------------------------------------|--|
| <input type="checkbox"/> Aerobics | <input type="checkbox"/> Bicycling | <input type="checkbox"/> Ice Skating | <input type="checkbox"/> Running for Exercise | <input type="checkbox"/> Surfing | <input type="checkbox"/> Weight Training |
| <input type="checkbox"/> Cheerleading | <input type="checkbox"/> Bowling | <input type="checkbox"/> Garden/Yard work | <input type="checkbox"/> Scuba-diving | <input type="checkbox"/> Swimming | <input type="checkbox"/> Wrestling |
| <input type="checkbox"/> Band/Drill Team | <input type="checkbox"/> Canoeing | <input type="checkbox"/> Gymnastics | <input type="checkbox"/> Snorkeling | <input type="checkbox"/> Tennis | <input type="checkbox"/> Baseball |
| <input type="checkbox"/> Dance | <input type="checkbox"/> Hiking | <input type="checkbox"/> Soccer | <input type="checkbox"/> Volleyball | <input type="checkbox"/> Basketball | <input type="checkbox"/> Football |
| <input type="checkbox"/> Roller Skating | <input type="checkbox"/> Softball | Others: _____ | | | |

2. List each activity that you checked above in the "Activity" box below.
3. Check the months that you did each activity.
4. Estimate the amount of time spent in each activity.
5. Use the next page if you run out of space.
6. Please sign at the bottom of page.
7. Ask your Parent/Guardian if you need help filling this out.

ACTIVITY	MONTHS SPENT IN ACTIVITY												TIME SPENT IN ACTIVITY		
	(check boxes)												(WRITE IN NUMBER)		
	Jan	Feb	Mar	Apr	May	Jun	Jly	Aug	Sep	Oct	Nov	Dec	Months per year	Days per wk	Minutes per day

Female Adolescent Maturation Study 3

All Girls

University of Hawaii-Kapiolani Clinical Research Center

ID#: _____

Data Sheet

10. CHECKLIST

Form	Brought	Reviewed by (initials)			
^a CONSENT	⁰ O YES ¹ O NO	2			
^b Health Questionnaire	⁰ O YES ¹ O NO				
^c Family Background Questionnaire	⁰ O YES ¹ O NO				
^d Physical Activity Questionnaire	⁰ O YES ¹ O NO				
^e Diet Record Forms	⁰ O YES ¹ O NO				

11. ^a PREGNANCY TEST

⁰O Positive

¹O Negative

^bComments

--

12. What have you drunk in the past 12 hours? (Include any liquid you drank, such as water, milk and soda).

⁰ TIME	¹ PLACE	² WHAT DID YOU DRINK? (ml)	³ AMOUNT
A			
b			
c			
d			
e			
f			

13. MOUTHWASH

^AVial 1 ID#:

--	--	--	--	--	--	--	--	--	--

^B Completed 1st mouthwash:

⁰O YES

¹O NO

^CIf no, state reason:

14. ANTHROPOMETRY	Measurement 1	Measurement 2	Measurement 3
^A Weight (lbs)	0	1	2
^B Height (cm)	0	1	2
^C Sitting height (cm)	0	1	2
^D Ulna length (cm)	0	1	2
^E Biacromial breadth (cm)	0	1	2
^F Biiliac breadth (cm)	0	1	2
^G Arm circumference (cm)	0	1	2
^H Abdomen circumference (cm)	0	1	2
^I Buttock circumference (cm)	0	1	2
^J Calf circumference (cm)	0	1	2
^K Subscapular skinfold thickness(mm)	0	1	2
^L Tricep skinfold thickness (mm)	0	1	2
^M Biceps Skinfold thickness (mm)	0	1	2
^N Iliac skinfold thickness (mm)	0	1	2
^O Calf skinfold thickness (mm)	0	1	2

^PInitials Of Person Who Took Anthropometry:

--	--	--

^QComments:

--

15. FOOT MEASUREMENT	Measurement 1	Measurement 2
^A Foot Length	0	1
^B Foot Width	0	1

16. TANNER STAGING

Tanner Staging	1	2	3	4	5
^A Breast	1	2	3	4	5
^B Pubic hair	1	2	3	4	5

^CInitials Of Person Who Staged:

^DComments:

17. ULTRASOUND MEASUREMENT

^ASHIM

⁰DK

¹MD

²LT

	Measurement 1	Measurement 2	Measurement 3
^B BUA	1	2	3
^C SOS	1	2	3
^D SI	1	2	3

^EInitials Of Person Who Took Ultrasound Measurement:

^FComments:

18. MOUTHWASH

^AVial 2 ID#:

^BCompleted 2nd mouthwash:

⁰O YES

¹O NO

^CIf no, state reason:

**Female Adolescent Maturation Study 3
Menstrual Calendar**

All Girls
ID# _____

University of Hawaii-Kaiser Permanente-Kapiolani Clinical Research center

Please put a \checkmark on the days you menstruated for the month.
e.g. if your period **started on April 3 and ended April 9**, it
should look like this:

Month : April Year: 2005

1	2	3 \checkmark	4 \checkmark	5 \checkmark	6 \checkmark	7 \checkmark
8 \checkmark	9 \checkmark	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

Month: _____ Year: _____

1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

Female Adolescent Maturation Study 3
University of Hawaii-Kapiolani Clinical Research Center

All Girls

ID#: _____

Diet Record Form

Date: _____ Day : _____ ID #: _____

TIME	<i>PLACE</i>	WHAT YOU ATE	DESCRIPTIONS OF WHAT YOU ATE	AMOUNT

Appendix C

Table below details the physical activities reported in exam 2, the code used in the analysis and the MET values assigned. When the activity reported was not listed in the reference list, a mean of the closest activities was assigned.

Physical Activities, MET values assigned and source of the MET value

Activity	Met value	Ainsworth's Code
Aerobics	5.5	Lee et al., 1992
Cheerleading	5	Lee et al., 1992
Band/drill Team	5	Lee et al., 1992
Baseball	5	Lee et al., 1992
Basketball	5	Lee et al., 1992
Bicycling	5.5	Lee et al., 1992
Bowling	3	Lee et al., 1992
Canoeing	4.5	Lee et al., 1992
Dance	5	Lee et al., 1992
Football	7	Lee et al., 1992
Garden/Yard work	4	Lee et al., 1992
Gymnastics	5	Lee et al., 1992
Hiking	6	Lee et al., 1992
Ice Skating	7	Lee et al., 1992
Roller Skating	7	Lee et al., 1992
Running for Exercise	8.5	Ainsworth et al. Average of act 12140 and 12150
Scotering	2.5	Lee et al., 1992
Scuba-diving	7	Lee et al., 1992
Snorkeling	4.5	Lee et al., 1992
Soccer	7	Lee et al., 1992
Softball	5	Lee et al., 1992
Surfing	3	Lee et al., 1992
Swimming	3	Lee et al., 1992
Tennis	7	Lee et al., 1992
Volleyball	4	Lee et al., 1992
Wrestling	8	Lee et al., 1992
Weight training	4.5	Lee et al., 1992
Aikido	8	Lee et al., 1992
Air riflery	3.5	Ainsworth et al. Considered as 15010
Badmington	4.5	Lee et al., 1992
Bodyboarding	5.5	Lee et al., 1992
Canine agility	3.5	From FAM1 dog agility = walking dog
Equestrian	4	Ainsworth et al. Considered as 15370
Farm chores	4.75	Ainsworth et al. Average of 11180, 11190, 11191 and 11192
Fencing	6	Ainsworth et al. Considered as 15200
Frisbee	5.5	Ainsworth et al. Average of 15240 and 15250
Golf	4	Lee et al., 1992
Handball	10	Ainsworth et al. Average of 15320 and 15330
Horseback riding	4.5	Lee et al., 1992
Intramural	5.5	Lee et al., 1992
Judo	8	Lee et al., 1992
Juggling	4	Ainsworth et al. 15440

Karate	10	Ainsworth et al. 15430
Kickball	7	Lee et al., 1992
Kung fu	10	Ainsworth et al. 15430
Lion dancing	8	Ainsworth et al. Considered as judo
Martial arts	10	Ainsworth et al. 15430
Modeling	3.5	Ainsworth et al. Considered as walking. MET used in FAM1
PE	5.5	Lee et al., 1992
Pilates	4	Ainsworth et al. Considered as yoga, METs for yoga in FAM1
Punching bag	6	Lee et al., 1992
Raquetball	7	Ainsworth et al. 15530
Situps and leg lifts	8	Ainsworth et al. 02020
Snowboarding	3	Ainsworth et al. Considered as 18220 (bodyboarding/surfing) Snowboarding not listed.
Track	8	Lee et al., 1992
Walk the dog	3.5	Lee et al., 1992
Walking	3.5	Lee et al., 1992
Waterpolo	10	Lee et al., 1992

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