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Calciotropic Hormonal Influence on Energy Homeostasis

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by

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ABSTRACT

Energy balance exists when intake is equivalent to expenditure. It has become evident that beyond quantitative aspects of intake, dietary components also have directive impact. On the expenditure side, an underappreciated yet key contributor is resting energy expenditure (REE). As the largest constituent of overall energy output, REE encompasses physiologic, dietary and genetic influence on body composition. While cellular and overall systemic contribution cannot be ignored, the relative proportion of adipose, bone and lean body tissue (i.e., body composition) represents the primary determinant underlying REE. It is possible that the mineral calcium is a common denominator encompassing energy balance influenced by diet while influencing tissue maintenance, with an active role in regulating tissue metabolism, as well as in cellular and systemic function comprising REE. Despite the substantial contribution of REE to overall energy balance, underlying factors which alter energy utilization pathways at rest remain relatively unexplored, with investigations centered on growth and development even more limited, a time particularly influential in terms of long-term body composition trajectory. In a multi-ethnic sample of peri-pubertal children (n=315), three specific aims were investigated. Investigation of the first specific aim to determine associations among calcium intake, REE and body fat indicated (as opposed in part to that which was

hypothesized) that REE mediated a positive relationship between calcium intake and body fat, providing support for effects of calcium intake on body composition. The second specific aim to identify associations between calciotropic hormonal factors and body composition with REE indicated increased intake of calcium-related nutrients and decreased circulating calciotropic hormone PTH were associated with higher REE. The third specific aim to evaluate relationships of REE with genetic polymorphisms having been shown to influence calcium regulation, indicating adiposity- and ethnic-specific associations with vitamin D receptor genotype, whereas sex and calcium intake seem to influence that between REE and calcium-sensing receptor genotype. Taken together, regulatory factors exerting effects on the calciotropic network are associated REE and may manifest as alterations in body composition. These findings support the importance of dietary nutrient adequacy for optimal allocation of energy for growth with potential long-term implications.

Keywords: calcium homeostasis, resting energy expenditure, parathyroid hormone, vitamin D receptor, calcium-sensing receptor, body composition

DEDICATION

To Evelyn Weaver Jernigan, “Big Mama”

Your love and strength carries on.

“Evelyn Jernigan, Big Mama to me.

Whatever you call her, she’s the best that can be.

We call her Big Mama ‘cause she has a big heart.

She’s been giving right from the start.

She’s raised children once, she’s raised children twice.

All she’s put up with, and she’s still so nice.

A very smart woman. A very good cook.

A very nice grandma, with very good looks.

She’s good on the inside. She’s good on the out.

No matter how you say it, she’s the best no doubt.”

-Lynae Hanks, Age 11

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And lastly (but certainly not least), I have to extend my gratitude to the Andre corporation which has provided champagne by the $5 bottle. Tropicana, particularly the Trop50 selection, has never partnered so well to suit my own personal preference for stress-relief delight. My mind and palate thank you for the needed rest and delight!

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INTRODUCTION

Energy balance, encompassing the integrated effects of energy intake and expenditure, influences body weight and composition over the life course (*1*). Although the degree to which physiologic or behavioral processes affect the state of energy (im)balance are not known, it is becoming increasingly clear that foundations of many of the underlying metabolic pathways are established early in life (*2-4*). Periods during the lifespan representing rapid growth and physiologic adaptations may serve as a sensitive window in which perturbations in energy balance may have the greatest impact on long-term health. Thus, a comprehensive understanding of factors contributing to this balance during puberty may help us develop targeted approaches for optimal body composition trajectory.

Resting Energy Expenditure and Energy Balance

Conceptually, the state of energy (im)balance is driven by energy intake and energy output, with quantitative caloric consumption and physical activity energy expenditure the most commonly cited contributors to each side of the equation, respectively. Despite such attribution, a clear role for either as the primary contributor underlying energy balance shifts has not been established (*5;6*). In the context of the obesity epidemic, consideration of other factors which may be driving the secular trend towards positive energy imbalance are emerging (*7-9*), both invoking physiologic and environmental factors. One physiological factor representing a promising avenue for investigation is resting energy expenditure (REE). REE is the largest constituent of energy output for most individuals. REE represents the amount of energy required for obligatory physi-

ologic maintenance, and it has been estimated that approximately 65% of total energy expenditure in individuals corresponds with REE.

Regulation of REE occurs at the cellular, tissue and systemic level. REE is influenced at the cellular level by numerous catabolic and anabolic processes involving second messenger signaling, at the tissue level by variable energy demand of body compartments, and at the systemic level by metabolic activity of organ function. While contribution at the cellular and systemic level cannot be ignored, large variability in REE is represented by the relative proportion of adipose, bone and lean body tissue that comprise body composition. Undoubtedly, as body mass increases, so too does absolute resting energy requirements; however, the degree to which energy requirements increase (*10*) is determined by the type of tissue accrued. Adipose tissue is less metabolically active relative to lean and bone, accounting for a lower proportion of REE (*11*). Conversely, lean and/or bone tissue gain, each energetically more demanding to accrue and maintain, would lend to a greater need for energy supply (thereby positively influencing REE). As growth occurrence during pubertal years provides the foundation for establishing body composition patterns, it is reasonable to assume that intrinsic aspects of REE are also established during this time. An understanding of influential factors underlying REE, particularly those contributing to the relative proportion of body tissue compartments during the peri-pubertal stage, is ideal for targeting intervention efforts which may contribute to long-term energy balance attainment.

Calcium Homeostasis and REE

The initiation of puberty, marked by a spurt in linear growth, is represented by a highly energy-dependent series of events. Rapid mineral deposition and long bone lengthening during the adolescent period accounts for up to half of adult peak bone mass (*12*). Calcium comprises almost one-third of the skeleton, which serves as a readily exchangeable source for calcium levels in circulation. As calcium plays an active role in regulating tissue metabolism, indirectly calcium may likely substantially influence REE.

The typical ascribed function of calcium is its role in skeletal and teeth maintenance through its functional form as hydroxyapatite, which accounts for 99% of the body's calcium. However, the remaining calcium in circulation serves as an essential component of cellular physiology, where movement into and out of the cytoplasm functions as a signal for vital cellular processes. Various energy- and cofactor-dependent mechanisms are in place to ensure that calcium concentration is kept within a tight range (between 4.5 and 5.6 mg/dl), comprising what can be termed the "calciotropic" network. The stringent regulation of this network is highly energy-dependent, and in turn influences REE.

Regulation of Calcium Homeostasis

The key regulators of the calciotropic network are parathyroid hormone (PTH) and 25-hydroxy vitamin D (25OHD), which respond to fluctuation of calcium levels in circulation. In response to lowered serum calcium, PTH is rapidly released into circulation, which subsequently stimulates the conversion of 25OHD to its 'active form,' $1,25(OH)_2D$, via renal hydroxylation. These actions initiate release of calcium from the skeleton and enhance calcium absorption at the intestine to restore serum calcium con-

centrations. Frequent occurrence of these actions in response to serum calcium decrements may impede maximal linear growth, and consequentially may ultimately result in lowered overall resting energy requirements. Additionally, vitamin D activation transduces a rapid intracellular serum calcium response, which stimulates lipogenesis and suppresses lipolysis, leading to increased energy stored as adipose tissue (*13;14*). As there has been a reciprocal relationship reported between adipose tissue and bone mass accrual, energy stored as adipose may be at the expense of the more highly energy-demanding bone (*15*). Taken together, physiologic mechanisms directed toward the normalization of serum calcium may impact overall energy resource disposal and requirements.

Diet and Calcium Homeostasis

Dietary calcium availability is crucial for adequate bone mineralization; however beyond intake of calcium, dietary bioavailability of various micronutrients also involved in the calciotropic network plays a role in metabolic processes underlying energy allocation (*16*). Dietary calcium, along with vitamin D (also obtained via sun exposure), are central nutrients to calcium homeostasis through skeletal maintenance and necessary metabolic signaling. Calcium requirements have been principally established on the basis of skeletal growth criteria (*17-19*). It is considered that by meeting the requirements of bone growth, the extra- and intra- cellular needs of other tissues will also be covered. Although variation according to race, sex and body habitus is probable, it is suggested that 1300 mg of dietary calcium is optimal for maximal absorption among adolescents (*20*). Although source-dependent, approximately 23-37% of dietary calcium is absorbed (*21*), which was taken into account during recommendation establishment. Vitamin D

has a key role in calcium uptake, as its primary physiologic function is serum calcium maintenance. Daily nutritional recommendations for vitamin D have been difficult to establish with precision given source complexity, however, it is currently suggested that 600 IU of dietary vitamin D is necessary to meet the needs of most adolescents (*20;22*). Although skin pigmentation and exposure to sunlight represent the greatest variation in vitamin D status, dietary intake has a substantial, direct impact (*23*). Additionally, because of its intricate involvement in growth (i.e. skeletal maturation), its essentiality is well-recognized in childhood and adolescence. Vitamin D is involved in activating calcium binding proteins for saturable calcium absorption, indicating the importance of adequate vitamin D intake for maximal calcium absorptive capacity. Adequate intake and absorption of calcium and vitamin D allows for normalization of extra- and intra-cellular calcium levels. As dietary intake is consistently documented to be at levels below requirements, particularly in this age group, profound consequences may manifest in terms of body composition patterning and ultimately REE.

Beyond calcium and vitamin D metabolism, ingestion and subsequent metabolism of other key nutrients may interfere with the calciotropic network (*24*). Vitamin K exerts influence on calcium homeostatic mechanisms mainly through calcium-to-bone binding capacity (*25-28*). Dietary adequacy of vitamin K is evidenced by its role as a cofactor for carboxylation of the osteoblast-derived hormone osteocalcin (OC), which influences calcium (de)mineralization for serum replenishment. Vitamin K supplementation (particularly in those with low intakes) has been associated with reductions in calcium excretion, bone resorption, and undercarboxylation of OC (*26*). The mineral phosphorus is also indicated in the calciotropic network. The majority of body phosphorus (~85%) is present

in bone (*29*), and a delicate balance between calcium and phosphorus exists. As calcium is liberated from bone and intestinal calcium absorption is augmented in order to restore its levels in the serum, there is a concurrent increase in phosphorus concentration via similar mechanisms. Phosphorus displays allosteric inhibitory properties on 25OHD, and the kidney serves to limit this interference by simultaneously upregulating phosphorus excretion. However, reduced serum phosphate concentration may lead to uncoupled work efficiency associated with bone remodeling (*24;30*). It is evident that dietary components necessary for maintenance of serum calcium cannot be considered in isolation. Dietary micronutrient adequacy of those involved (i.e., calcium, vitamin D, phosphorus and vitamin K) allows for optimal obligatory function, whereas insufficiency leads to reduced calcium absorption and a lower concentration of circulating ionized calcium. Through downstream effects on body composition, nutrients involved in the calciotropic network have the capacity to influence energy requirements at rest.

Calcium Retention Variation

Diet, the major factor affecting calcium homeostasis in adolescents, accounts for approximately 12.3 and 21.7% of the variation in calcium retention between girls and boys, respectively (*31;32*). Boys retain more calcium than girls, whereas 1140 mg is the level of dietary calcium intake in which absorption is maximized, whereas 1300 mg is the level in girls. Racial/ethnic variation in maximal calcium retention has been reported, with African Americans (AA) having higher rates than European American (EA) adolescent girls (*33*). In a study using calcium kinetics analysis, urinary calcium losses in AA were half that of EA girls (*34*), consistent with studies in children (*35-37*) and some studies in adults (*38;39*). Fecal calcium excretion was also lower in AA compared with EA

girls (*40*). Racial differences in both urinary and fecal calcium losses account for 57% greater calcium retention in AA than in EA girls. Sexual maturation has also been associated with calcium retention. A rapid decline has been reported with postmenarcheal age in females, paralleling the rapid decline in bone accrual rate after the peak (*41*), and explaining 10% of the variation in calcium retention (*42*). Adiposity has also recently been identified as a significant contributor to calcium retention in adolescents. Based on calcium balance studies, when intake is sufficiently adequate obese and overweight adolescents retain more skeletal calcium with increasing dietary calcium compared with healthy-weight peers (*43*). Further, the capacity for calcium absorption upon intake is greater for those with greater BMI (*4*). It is apparent that multiple factors contribute to variation in calcium retention, particularly in the pubertal stage, and must be taken into consideration when evaluating the interrelationships of calciotropic variables.

Genes and Calcium Homeostasis

Elucidation of dietary and physiologic influence by the calciotropic network on REE is limited without an understanding of inherent genetic contribution. Evidence for the importance of genetic factors in determining bone resorption and formation, calcium excretion, and the hormones regulating these processes has been demonstrated (*44*). However, there is a paucity of studies dealing with the extent to which genetic variation of calcium regulation may mediate physiologic function as a complex trait within the general population, with even fewer investigations focusing on the "healthy" pediatric population (*45*).

Most of the genetic variants that regulate the energy-dependent pathways within the calciotropic network remain to be identified. However, polymorphisms of the vita-

min D receptor (VDR) and calcium-sensing receptor (CASR) genes represent a promising starting point as variation within each gene has been consistently documented to influence calcium absorption and utilization (*46-48*). Although VDR is a widely studied candidate gene in relation to bone endpoints, emerging hypotheses suggest that it may be involved in various disease states related to energy balance (e.g. cancer, diabetes, atherosclerosis) (*49-52*). Expectedly, as CASR is responsible for mediating alterations in bone resorption and renal calcium reabsorption (*53*), the primary genetic associations involving CASR evolved from investigations centered on skeletal-related outcomes (*54*). It has been reported that VDR enhances vitamin D activity, which further increases CASR expression/activity, providing evidence of potential synergism between the two genes, and supporting the notion of a potential interaction among these two receptors. Although the genetic underpinnings of VDR and CASR are not completely understood, certain VDR and CASR genetic variants may influence disturbances in calcium metabolism in some individuals, thereby impacting REE in a manner that ultimately affects body composition.

Various SNPs across the VDR gene have been associated with phenotypes related to calcium regulation. The SNP rs15568820, located in the 1e promoter region at the 5'-terminal of the core sequence (5'-Ataaaaacttat-3') of the VDR gene, has been most consistently shown to influence inherent capacity for calcium utilization (*55*) through its role in nutrient absorption regulation (*56;57*). Alteration in transcriptional activity of the promoter region of VDR enhances calcium absorptive capacity (*58*) mainly due to the intestinal-specific transcription of the well-characterized caudal-related homeodomain transcription factor Cdx2 binding site located at the gene (*46*). As the intestine is the

predominant area for calcium absorption, the Cdx2 site is thought to influence vitamin D regulation of intestinal calcium absorption associated with dietary intake. The 'A' allele of the rs15568820 SNP has been found to have the ability to bind the Cdx2 transcription factor more strongly and manifesting as higher transcriptional activity (*46*), has been described as being a more "active" allele. Thus, among individuals with the 'A' allele, increased VDR expression can increase transcription of calcium transport proteins, leading to enhanced intestinal calcium absorption. As a consequence, serum calcium concentrations might be expected to be greater in those with the 'A' allele. We hypothesize that through this mechanism greater serum calcium concentrations could lead to increased REE via increased bone mineral apposition, particularly in the context of dietary sufficiency.

Polymorphisms in the CASR gene (*59*) have been shown to affect calcium regulation through signal transduction, intracellular trafficking and cell surface expression (*60*). CASR (locus 3q13) allows for the regulation of calcium homeostasis through close monitoring of serum calcium levels. Calcium 'sensed' by CASR leads to direct inhibition of PTH, and direct or indirect (PTH-mediated) inhibition of calcium reabsorption by the kidney, which together promote increased calcium excretion. The common A986S polymorphism at rs1801725 encodes a missense variant in exon 7 leading to a non-conservative amino acid change (serine substitution for alanine-986, A968S, corresponding to nucleotides 2956G>T). This polymorphism has been reported to be associated with the strongest signals influencing serum calcium levels, explaining 1.26% of the variance in serum calcium (*47*). Interestingly, the SNP had the strongest association in individuals of European descent, suggesting that population-based differences at this loci

may mediate inherent differences in calcium utilization capacity (*47*). Though contribution of CASR to regulation of bone remodeling and intestinal calcium absorption still remains unclear (*61*), it is known that CASR is expressed in the skeleton (*62*) and intestine (*63*). We contend that decreased calciotropic signaling responses in individuals with one or more copies of the 'A' allele at this locus within the CASR gene region will have an impact on REE.

Several polymorphisms in both the VDR and CASR gene have been examined in osteoporosis-related association studies, yielding inconclusive results (*64*). In these studies, Type I error rates may be inflated because of bone-related genetic heterogeneity within populations, a problem conceptualized by the term "population stratification (*65*)." An effective way to overcome that is utilization of genetic admixture estimates, which elucidates biological rather than environmental variances that may influence physiologic processes within individuals (*66;67*). Investigation into the calciotropic network with respect to admixture of populations is warranted to capture the complexity of population differences. Accordingly, genetic admixture has been included as a genomic control variable in statistical models representing population-based differences.

Summary of Introduction

Clearly, parameters surrounding energy balance are complex and interactive, having dietary, physiologic and genetic components. Insight into calcium homeostasis suggests the potential for mediation of energy regulation through influence on REE. The growing child may be an ideal model system in which to explore such relationships, particularly during the period of reproductive maturation, and studies investigating the interactive effects of dietary, physiologic and genetic factors involved in calcium homeostasis

and energy metabolism particularly in children is limited. Paucity in the literature exists regarding the extent to which the cascade of events involved in the calciotropic network contributes to REE. The objective of the proposed research is to a) gather evidence that these interrelationships exist during the peri-pubertal life stage and b) work towards designing and implementing protocols to optimize long-term metabolic health.

Specific Aims

The following distinct, yet overlapping experimental aims were designed to investigate the contribution of dietary, physiologic and genetic factors involved in calcium homeostasis to REE in a multi-ethnic cohort of peri-pubertal children.

Specific Aim 1

To determine associations among calcium intake, REE and body fat in a multiethnic sample of peri-pubertal children.

Hypothesis: Calcium intake would be positively associated with REE, and that both calcium intake and REE would be inversely associated with total body fat.

For this aim, we used indirect calorimetry, dual-energy x-ray absorptiometry (DXA), and 24-hour recall to assess REE, body composition, and dietary calcium, respectively, and evaluated the relationships among REE, calcium intake, and body fat using structural equations modeling, while accounting for differences in body composition, sex, pubertal status, socioeconomic status, ethnicity, total energy intake, as well as genetic admixture as a control for genetic variability (*68*).

Specific Aim 2

To investigate the association of subjective and objective measures reflecting dietary and hormonal factors involved in the regulation of calcium homeostasis with REE in peri-pubertal girls.

Hypothesis 1: Lean and bone mass would be positively associated with REE.

Hypothesis 2: Dietary calcium, vitamins D and K, and phosphorus, along with objective measure of calcium homeostasis (i.e., PTH, 25OHD and OC) would be associated with REE.

For this aim, we used indirect calorimetry, DXA, 24-hour recall, and serum assays to assess REE, body composition, dietary intake (calcium, vitamins D and K, phosphorus, and overall energy), and serum hormones (parathyroid hormone, PTH; osteocalcin, OC; and 25-hydroxy vitamin D; 25OHD), respectively. We used multiple linear regression to assess adiposity- and dietary adequacy-specific analyses, including relevant covariates (i.e., European genetic admixture, pubertal stage, and fat and lean mass).

Specific Aim 3

To evaluate the associations of vitamin D receptor and calcium-sensing receptor polymorphisms with REE in peri-pubertal males and females.

Hypothesis: Genetic polymorphisms implicated in the calciotropic network would be independently associated with REE.

For this aim, we obtained genetic measures using pyrosequencing technology (*69*) and indirect calorimetry to assess genotype and REE, respectively. Children were classified into a low body fat group or high body fat group based on their percent body fat. All children were categorized based on the criteria for normal (males with <25% and females

with <30% body fat) /excess body fat levels. Body composition was determined using DXA, whereas genetic admixture estimates were obtained using 140 genetic markers informative for European, African, and Amerindian ancestry. Using this information, we compared the classification of children of various sex, adiposity levels, and median calcium intake with REE based on genotype using multiple linear regression analysis.

Overall Perspective

Figure 1 is a representation of the integration of the specific aims and what each uniquely and interactively contributes to the overall objective. Each aim, centered on REE, is comprised of a specific aspect of the calciotropic network, and constructed to contribute to the guidance of continued investigations.

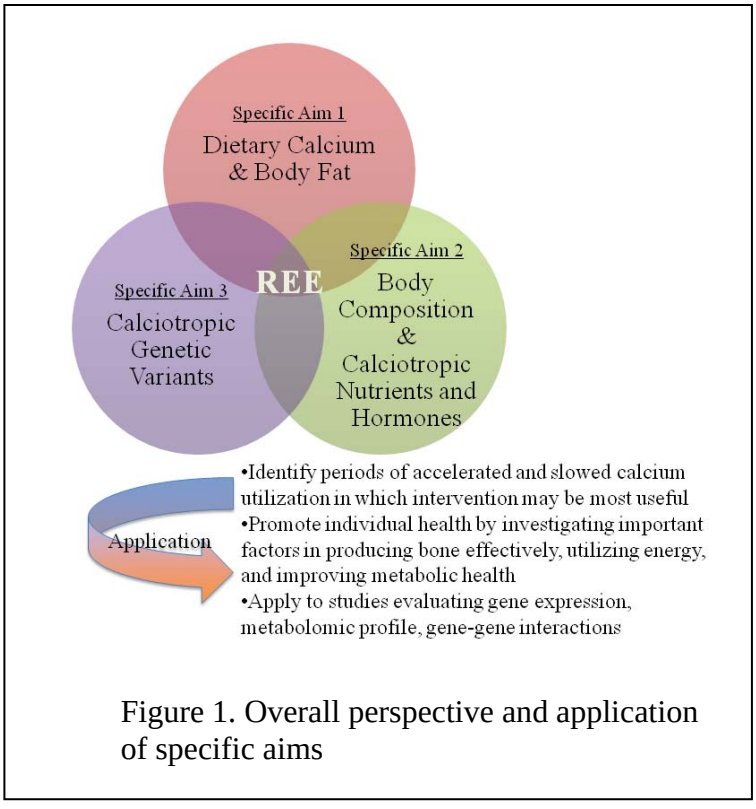

Figure 1. Overall perspective and application of specific aims

ASSOCIATIONS OF CALCIUM INTAKE, RESTING ENERGY EXPENDITURE, AND BODY FAT IN A MULTIETHNIC SAMPLE OF CHILDREN

by

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ABSTRACT

Background: Dietary calcium has been proposed to influence obesity by mediating the relationship of resting energy expenditure (REE) to body fat.

Objective: The objective was to determine if calcium intake was associated with REE and body fat in children, after accounting for ancestral genetic background.

Design: Participants included 315 children. REE, body composition, and dietary calcium were assessed by indirect calorimetry, dual energy x-ray absorptiometry (DXA), and 24-hour recalls, respectively. Structural equations modeling assessed the relationships among REE, calcium intake and body fat.

Results: There were positive associations between calcium intake and REE ($p<0.01$) and between REE and total body fat ($p<0.0001$). There was indirect effect of calcium intake on total body fat ($p<0.01$). There were positive associations between calcium intake and REE ($p<0.01$), and a trend towards an association of calcium intake and total body fat ($p=0.065$) among males only; whereas, the only significant relationship among females was an association of REE on total body fat ($p<0.0001$).

Conclusions: REE was associated with calcium intake and mediated a relationship between calcium intake and total body fat. These findings suggest calcium intake may play a role in fat accumulation and energy balance through its effects on REE, especially in males.

INTRODUCTION

Over the past three decades, the incidence of obesity in the US has steadily risen and continues to be a health concern among adults, adolescents, and children [1,2]. Pediatric obesity tracks into adulthood and has been linked to a number of chronic illnesses including type 2 diabetes and cardiovascular disease, as well as certain types of cancers. The prevention of obesity-related comorbidities implicates the necessity of identifying factors affecting adiposity, particularly in the pediatric population.

Efforts to combat obesity have led to the development of a variety of dietary management programs which generally incorporate a combination of macronutrient manipulation coupled with caloric restriction [3]. More recent investigations have explored how the consumption of specific dietary factors influence weight loss/maintenance and overall health, including the role of calcium as a functional micronutrient [4,5,6]. Calcium, a key regulator of metabolism, may influence body fat levels through its effects on resting energy expenditure (REE). The largest fraction of total daily energy expenditure is accounted for by REE [7], and alterations in energy expenditure can predict weight changes [8,9]. In the growing child, studies have indicated inadequate levels of dietary calcium can interfere with metabolism, possibly contributing to fat accumulation [4,10,11,12]. However, the underlying mechanism driving the relationship of dietary calcium and body fat is complex and has yet to be fully understood. The relationship between calcium and body fat is further complicated when considering inherent differences in physiology and metabolism observed between racial/ethnic groups.

Differences in body composition [13], REE [14,15,16] and dietary intake [17] have been previously observed, utilizing traditionally racial/ethnic classification as the unit of comparison. However, disentangling the etiology of these differences, particularly among

intermixed individuals, becomes challenging since race/ethnicity represents a unique social construct characterized by autochthonous cultural differences, behavioral practices, and dietary preferences. Genetic admixture elucidates biological rather than environmental variance within individuals, which may also have a mediating effect on metabolic pathways [18,19]. Thus, further investigation into the relationship of specific nutrients with REE that influence body composition, while taking factors depicting this admixture of populations into account, are warranted to capture the complex etiology of population differences.

Investigations including the associations of etiological factors may be particularly critical in childhood, as body fat trajectories are likely established during this period [20]. To our knowledge, no studies have evaluated the relationship of calcium intake and REE and its effects on body fat in children, nor have previous studies taken into account genetic admixture as a biological contributor to the relationship. This study was conducted to investigate relationships among calcium intake, REE, and body fat in peripubertal children, while accounting for differences in body composition, as well as using genetic admixture as a control for genetic variability.

METHODS

Subjects

A sample (n=315; 53% male) of European- (n=122) African- (n=107) and Hispanic-American (n=86) children, 7-12 years of age, were recruited to study the effects of genetic and environmental parameters on racial/ethnic differences in metabolic outcomes. The children were pubertal stage ≤3 as assessed by a pediatrician (according to Marshall and Tanner) [21], healthy, and not taking medications known to affect body composition. Parents and children provided consent/assent, respectively, after receiving the protocol by study personnel. The protocol was approved by the Institutional Review Board for human subjects at the University of Alabama at Birmingham (UAB). All measurements were performed between 2004 and 2008.

Protocol

Subjects participated in two visits. On the first visit, pubertal status, anthropometric assessment, and body composition, were measured and a 24-hour dietary recall was obtained. On the second visit, subjects were admitted for an overnight stay and a second 24-hour dietary recall was obtained. All participants received the same meal and snack foods. Only water and/or non-caloric, decaffeinated beverages were permitted after 2000h until after the morning testing.

Anthropometric Measures

Anthropometric measures were obtained by the same registered dietitian. Height (Heightronic 235; Measurement Concepts, Snoqualmie, WA) and weight (Scale-tronix 6702W; Scale-tronix, Carol Stream IL) was obtained in minimal clothing without shoes. BMI percentile was calculated using age- and sex-specific growth charts [22].

Dietary Assessment

Dietary composition was assessed using the average of the two 24-hour dietary recalls using the "multiple pass" method, providing cup and bowl sizes to help estimate portion sizes. Each recall was performed in the presence of at least one parent. A registered dietitian coded and entered the data into Nutrition Data System for Research version 2006 (Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN). Total energy (kcal/d) and calcium intake (g/d) were generated as variables from the analyses. Total energy intake was included since calcium intake most likely increases with increasing caloric intake, and positive energy balance is known to have an effect on body fat [10].

Body Composition Assessment

Body composition was measured by DXA using a GE Lunar Prodigy densitometer (DXA; GE Lunar Radiation corp., Madison, WI) with pediatric software (version 1.5e). Subjects were scanned in light clothing, lying flat on their back with arms at their sides.

Pubertal Status

The Tanner stages have been demonstrated as reliable indicators of pubertal development. Direct observation for the assessment of pubertal stage by a pediatrician, the 'gold standard' for differentiating among the five stages of maturity [23,24], was utilized. The staging based on the criteria of Marshall and Tanner [25,26] is according to both breast and pubic hair development in girls and genitalia and pubic hair development in boys. One composite number is assigned for Tanner staging, representing the higher of the two values defined by breast/genitalia and pubic hair [27].

REE

REE was measured in the morning immediately after awakening during the overnight visit. A computerized, open-circuit, indirect calorimetry system with a ventilated canopy (Delta Trac II; Sensor Medics, Yorba Linda, CA) was used. While lying supine on a bed, the head of the subject was enclosed in a plexiglass canopy. Subjects were instructed not to sleep and remain quiet and still, breathing normally. One-minute average intervals of oxygen uptake (VO_2) and carbon dioxide production (CO_2) were measured continuously for thirty minutes.

Race/Ethnicity

Parental self-report was used for classification of subjects into racial/ethnic categories. Scientific evaluation of the uniqueness of population-based differences is challenging, in particular because in many contexts, delineation between biology and environment in the variable "race/ethnicity" is not clearly defined. Further, race/ethnicity changes according to historical periods, social structure, and as individuals become more admixed. In our analysis, statistical models include race/ethnicity as a control variable for social and cultural characteristics. Although there is multi-colinearity between the admixture variables and race/ethnicity, it is accounted for using the structural equations modeling (SEM) approach.

Genetic Admixture Analysis

Genotyping of the ancestry informative markers (AIMs) for the measurement of genetic admixture was performed at Prevention Genetics using the Chemicon Amplifluor SNPs Genotyping System coupled with ArrayTape technology**.** as previously described [17]. A panel of 140 AIMs was used to estimate the genetic admixture proportion of each

subject. Molecular techniques for the allelic identification and methodology for genetic admixture application have been described elsewhere [28]. The information from the AIMs was translated into estimates of African, European, and Native American admixture for each subject using maximum likelihood estimation based on the maximum likelihood (ML) algorithm described by Hanis et al. [29].

Socioeconomic Status (SES)

Because SES has been reported as an environmental factor influencing dietary intake and adiposity [30,31], a measure of SES was included in analyses. SES was determined according to the Hollingshead four factor index of social status [32]. This scale (ranging from 8 to 66) combines the education level and occupational prestige for the working parents in each child's family with higher values representing higher SES.

Modeling and Statistical Methods

Descriptive statistics evaluating sex differences were determined using ANOVA (SAS version 9.2 software; SAS Institute, Cary, NC) with statistical significance level was set at α=0.05. Our objective of identifying the relationships among dependent and independent variables was evaluated using a SEM approach. Specifically, Mplus software (Muthen and Muthen, Los Angeles, CA) with ML estimation was employed to test models that describe the relationship between calcium intake and REE and how these measures predict total body fat. SEM allows for simultaneous evaluation of multiple regression equations with the inclusion of covariates, providing estimates of the direct and indirect effects, while accounting for colinearity among all variables. Specifically, the direct effects refer to paths, and statistical estimates representing path coefficients are interpreted as regression coefficients. The estimates control for correlations among mul-

tiple presumed causes of the same variable. Indirect effects are estimated statistically as the product of the direct effects which comprise them, and are also interpreted as path coefficients.

The following measures of fit for SEM [33] were employed: chi-square (χ^2) test of model fit, its p-value and degrees of freedom (df); CFI (comparative fit index); and RMSEA (root mean square error of approximation). The χ^2 test signified how well the models fit the data, whereas small, non-significant χ^2 values indicated little divergence between the structure of the observed data and hypothesized model. The CFI compared the hypothesized model with the null model, taking model complexity into account. A well-fitting model had CFI values >0.90. The RMSEA indicated how closely the model fit approximated an acceptable model, with values <0.10 representing good model fit. Total body fat was modeled as a single indicator of adiposity as a dependent variable influenced by REE. Initial analyses indicated that overall model fit would be substantially improved by including this measure as opposed to BMI percentile or percent body fat. REE was modeled as a determinant of total adiposity. Because REE is highly dependent upon lean mass, lean mass was controlled for in modeling REE. Reported calcium intake was modeled as a determinant of both adiposity and REE [16,34]. The hypothesized causal paths in the determination of total body fat and REE were estimated by linear regression coefficients (shown using single-headed arrows).

The base model (combined analysis; Figure 1) was adjusted for sex, pubertal status, height, SES, race/ethnicity, total energy intake, and genetic admixture. Race/ethnicity was dummy-coded with European Americans being the reference group (European Americans=0, African Americans=1, Hispanic Americans=2). Sex was

coded: males=0 and females=1. Since the measured value for each of the three genetic admixture components adds to one, only European and African admixture (as the two admixtures with the widest variation among our sample) were included as covariates to avoid overspecification of the statistical models. Specifically, the models tested: (1) if calcium intake significantly affected REE (2) if calcium intake significantly affected total body fat, (3) if REE had a significant effect on total body fat, and (4) if the relationship of calcium intake and total body fat was mediated by REE indirectly. We further analyzed these relationships without the variable REE included in the model to assess the degree of mediation.

RESULTS

Descriptive Statistics

Table 1. represents participant characteristics for the total sample and stratified by sex. Males were significantly older than females ($p<0.05$), had higher total lean mass and had higher REE ($p<0.01$), whereas females tended to have higher total fat mass ($p=0.0678$). However, there was no difference in BMI percentile between the sexes. Males had higher energy intake than females ($p<0.05$), but no difference in calcium intake.

Relationships between calcium intake, REE, and total body fat

Figure 1. illustrates the overall relationships between calcium intake, REE, and total body fat. All model fit indices indicated a good model fit ($\chi^2=15.13$, $p=0.13$, $df=10$, CFI=0.984, RMSEA=0.040). Specifically, the χ^2 which tested the hypothesis that the model implied variances and covariances were equal to those of the observed data was not rejected for our proposed model (Figure 1). Other fit indices were included to support that this was a well-fitting model. The CFI and the RSMEA were also indicative that this was a good fitting model with values above 0.95 and below 0.05, respectively.

The total amount of variation in REE and total body fat explained by the SEM base model was $R^2=0.455$ and $R^2=0.401$, respectively (both $p<0.0001$). There was a direct association between calcium intake and REE ($p<0.01$), but the observed relationship between calcium intake and total body fat was not statistically significant. REE had a direct effect on total body fat ($p<0.0001$). Furthermore, there was a significant indirect effect of calcium intake on total body fat, suggesting that REE mediated the influence of calcium intake on total body fat ($p<0.01$). In the model excluding REE, the effect of cal-

cium intake on total body fat was larger and showed a trend (p=0.056), thus indicating mediation.

The multigroup model (Supplement: Figure 2), in which the sexes were stratified, yielded the following values of selected fit indices: χ^2=18.04, p=0.45, df=18, CFI=1.000, and RMSEA=0.004. These values were indicative of a good fitting model, and standardized effects were equal across groups. There was somewhat greater predictive power for the males than for the females, such that the proportions of explained variance for calcium intake on total body fat were 0.470 and 0.362, respectively. Consistent with the combined analysis, the total amount of variation in REE and total body fat explained by this model for males was R^2=0.415 and R^2=0.470, and for females was R^2=0.474 and R^2=0.362, respectively (all p<0.0001).

For males, calcium intake was directly associated with REE (p<0.01), there was a trend toward an association of calcium intake and total body fat (p=0.065), and REE was directly associated with total body fat (p<0.0001). There was an indirect effect of calcium intake on total body fat, which was mediated by REE (p<0.05). In a model excluding REE, the direct effect of calcium intake on total body fat was significant (p<0.05), further indicating mediation in males.

For females (Figure 3), the only significant association was between REE and total body fat (p<0.0001). Therefore, unlike in males, there is not sufficient evidence for REE mediating the effect of calcium intake on total body fat in females.

DISCUSSION

We investigated calcium intake, and its contribution to REE and total body fat in a multiethnic sample of children. Although there was no direct relationship of calcium intake and total body fat, there was a mediating effect of REE between the two variables, explaining an indirect positive relationship of calcium intake on total body fat. Stratification of the model by sex revealed that these relationships and mediation effects were present in the males and there was a trend towards an effect of calcium intake on total body fat with and without REE included in the model. In females, the only relationship identified was that of REE and total body fat. Our findings of a positive relationship between calcium intake and REE, as well as a positive association of REE with total body fat, provides a mechanism by which dietary intake may influence energy balance and body composition.

The relationships observed herein contribute insight into the inconsistencies reported by other studies investigating the relationships among dietary calcium, REE and body fat. Consistent with our results, a randomized, controlled crossover study of 9-10 year-old children reported that milk consumption induced greater REE and thermic effect of food after six days of supplementation relative to supplementation with a sugar-only beverage [10]. Conversely, in a study evaluating calcium intake and total energy expenditure no effects of 24h energy expenditure was observed in diet groups with varying levels of dietary calcium [5]. In an adult weight-loss trial, there was no difference in total energy expenditure among various groups consisting of low calcium intake, calcium supplementation, and high dairy [35]. Whereas our analysis indicated a positive indirect relationship between calcium intake and body fat, in an analysis of NHANES III data and in rando-

mized trials, an inverse association between calcium intake and relative risk of obesity (suggestive of lower body fat) among adults has been observed [36]. Although the inconsistencies existing in the literature indicate a need for greater understanding of the role of calcium intake on obesity-related phenotypes (particularly across age groups), our results support the notion that the relationship between calcium and obesity traits is mediated by other aspects of energy balance that deserve careful consideration in future studies. Further, as the illustrated inherent virtues of SEM, evaluation using more sophisticated approaches that resolve issues of colinearity should be employed.

Exploring differences based on utilization of the race/ethnicity categorical variable could be an additional influential factor in the inconsistencies reported across studies. A uniqueness of our study is that we were able to evaluate the genetic contribution (assessed by ancestral genetic admixture) to our dependent variables. In exploratory analyses, the overall model and the model representing stratification by sex were also run without the inclusion of genetic admixture (data not shown). A trend towards significance directly relating calcium intake with total body fat for the overall sample was identified in this model. This may suggest that ancestral genetic background contributes, at least in part, to the relationship. Thus, genetic admixture as a tool to scientifically assess the heterogeneity of human populations allows for a more accurate assessment of individual variability and clearer understanding of the relationships among calcium, REE, and body fat.

Our findings related to the contribution of genetic admixture were of particular interest. In the overall model, neither European nor African admixtures were significant contributors to body fat. However, when investigating the model by sex, African admix-

ture was a negative predictor of total body fat in females, whereas European admixture was a positive predictor of total body fat in males. These findings suggest a differential contribution of ancestral genetic background in boys and girls that deserves further exploration in studies evaluating the role of genetic admixture in measures of sex differences in body composition among children.

The disparate findings in the relationships among calcium intake, REE, and body fat between males and females indicate inherent differential underlying physiology between the sexes. Inclusion of pubertal status and lean mass in the models, two factors which have been identified as contributors to the sexual dimorphism in REE, did not account for such differences. As such, the involvement of factors in addition to those evaluated here is evident. A plausible determinant of differential mediation could be the difference in hormonal milieu among males and females; estrogen is known to drive fat deposition [37], whereas testosterone is known to drive lean mass [38]. However, measurements of hormones were not available for this study. The evaluation of hormonal differences in explaining the relationship between calcium, REE and body composition in boys and girls deserves further exploration.

Children may be an ideal model system in which to explore the relationships between REE, dietary intake and body composition due to active growth and development, particularly during the peripubertal period. REE is known to be relatively high in children compared with adults, likely due to differences in oxidative requirements of the tissues needed for growth and development [34]. Increased REE translates into increased energy requirement. Theoretically, metabolic alterations that minimize positive energy flux by creating a greater caloric need have the potential to result in less fat accumulation

over time. By this theory, the positive association found between dietary calcium and REE could have a positive impact on long-term weight maintenance. However, among children there were opposing findings. In addition, other metabolic factors associated with body composition and energy substrate utilization could be contributing. For example, regulation of intracellular calcium levels by parathyroid hormone (PTH), further regulated by the circulating active metabolite of vitamin D, has been found to be positively associated with changes in fat mass and fat oxidation [36]. The impact of calcium levels and vitamin D status on PTH may in turn mediate the systemic effects of these dietary nutrients, but potential relationships with energy metabolism have not been well examined. Thus investigation of independently- and/or interactively-acting contributing factors is warranted.

Strengths of our study are that we had a large cohort of racially/ethnically diverse subjects and had robust measures of body composition. We also employed SEM which allowed for the simultaneous evaluation of multiple regression equations. There were, however, limitations. The data expressed are statistically significant, yet explain less than 50% of the variance. It is likely that unconsidered factors also impact the relationships evaluated. For example, calcium/dairy intake level has been shown to possibly affect fat oxidation [5,35,39], a measure we were unable to attain. Further, since serum PTH and vitamin D status are both proposed to have a role in the mediation of calcium and REE, inclusion of these measures would have likely enhanced our understanding of potential mechanisms [39]. Additionally, although considered an acceptable and appropriate tool for describing mean intakes of a large group of subjects, dietary assessment via 24-hour recall has limitations, particularly in the assessment of micronutrients. However, it is the

most commonly used method for dietary surveys in the US [40]. The cross-sectional design of the study prevents the inference of long-term relationships; thus, longitudinal data would be necessary to investigate the effects of calcium intake over time on our dependent variables.

In conclusion, our findings among a multiethnic sample of peripubertal children showed REE to be associated with calcium intake and total body fat, and a mediator of calcium intake on total body fat. As such, calcium intake may play a role in body fat accumulation and energy balance through its effects on REE in children. Future investigations evaluating mechanisms in which calcium, and possibly other key nutrients, affects energy balance and body composition are warranted.

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TABLE I

Descriptive statistics of total sample according to sex

	Total Sample	Sex	
	Overall (n=315)	**M (*n*=167)**	**F (*n*=148)**
Age (yrs)	9.6 ± 1.6	9.8 ± 1.6[a]	9.3 ± 1.5[b]
Tanner stage	1.5 ± 0.7	1.4 ±0.6[b]	1.6 ± 0.8[a]
Height (cm)	139.5 ± 10.6	140.1 ± 10.6	138.9 ± 10.6
Weight (kg)	36.7 ± 9.5	37.1 ± 10.2	36.1 ± 8.7
BMI percentile	66.3 ± 26.1	66.5 ± 26.1	66.1 ± 26.3
Race/Ethnicity (%)			
EA	38.7	20.0	18.7
AA	34.0	18.7	15.2
HA	27.3	14.3	13.0
SES	38.7 ± 14.5	38.6 ± 14.0	38.8 ± 15.0
REE (kcals/day)	1,192.33 ± 234.8	1,240.48 ± 248.3[a]	1,139.6 ± 207.3[b]
Total lean mass (kg)	25.6 ± 5.3	26.6 ± 5.3[a]	24.4 ± 5.0[b]
Total fat mass (kg)	8.9 ± 5.7	8.3 ± 6.3[b]	9.5 ± 5.0[a]
Energy (kcal/d)	1,886.5 ± 469.6	1,943.3 ± 486.7[a]	1,823.6 ± 443.0[b]
Calcium Intake (mg/d)	859.0 ± 333.8	872.8 ± 329.9	843.6 ± 338.5
European American admixture (%)	54.6 ± 38.8	54.2 ± 40.1	54.9 ± 37.5
African American admixture (%)	31.3 ± 38.4	31.7 ± 39.5[a*]	30.9 ±37.4[b*]

[a, b]superscripts represent differences between groups, *represents trend for difference ($p<0.10$)

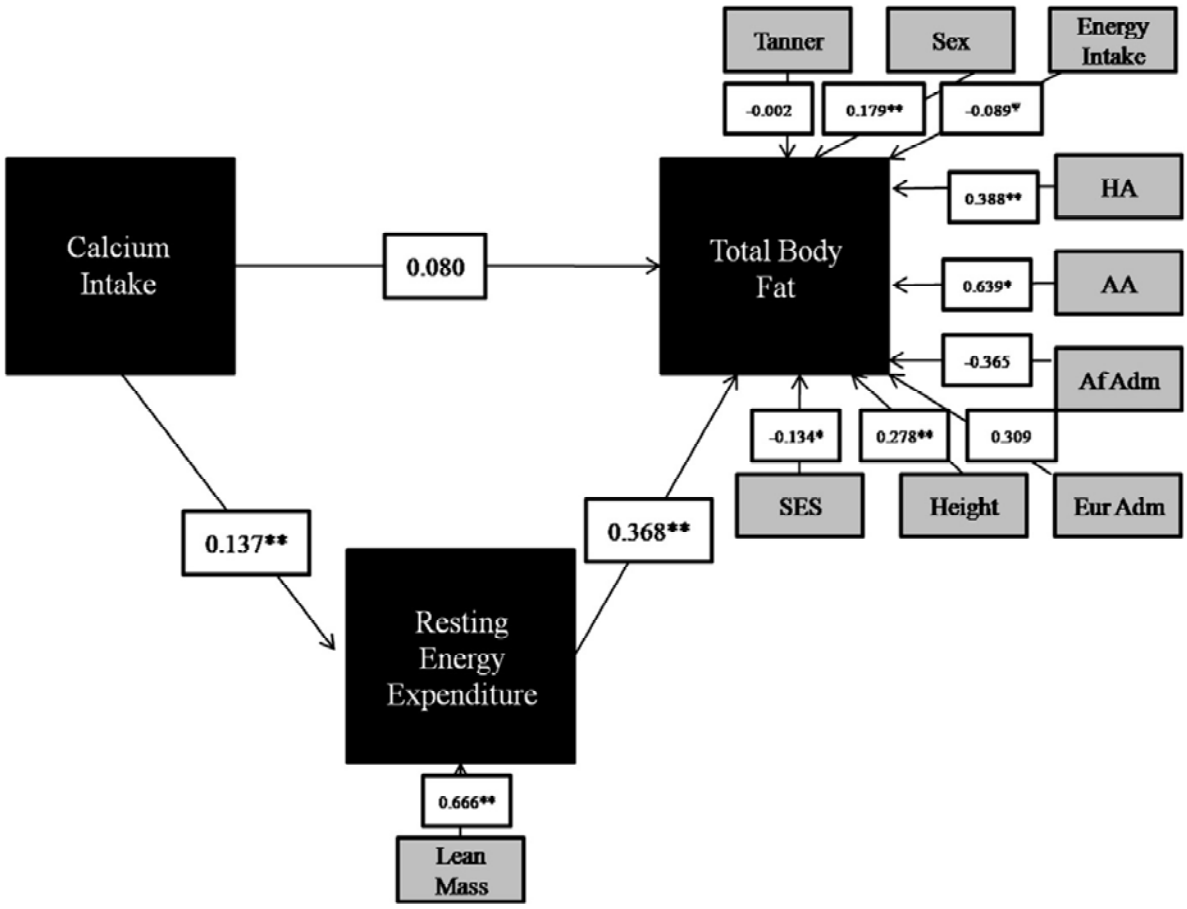

Figure 1. *Relationships between calcium intake, REE, and total body fat.*

The arrows represent the causal paths; specifically the arrowhead points to the presumed effect, and the line stems from the presumed cause.

HA = Hispanic American; AA = African American; Af Adm = African American Admixture; Eur Adm= European American Admixture; SES = Socioeconomic Status; Dark boxes = Dependent variables; Light boxes = Independent variables; White boxes = Parameter Estimate

(numbers represent β- coefficients); *<0.05, ** $\leq 0.01^{\Psi}<0.10$; $\chi^2=15.13$; p-value=0.127; df=10; CFI=0.984; RMSEA=0.040

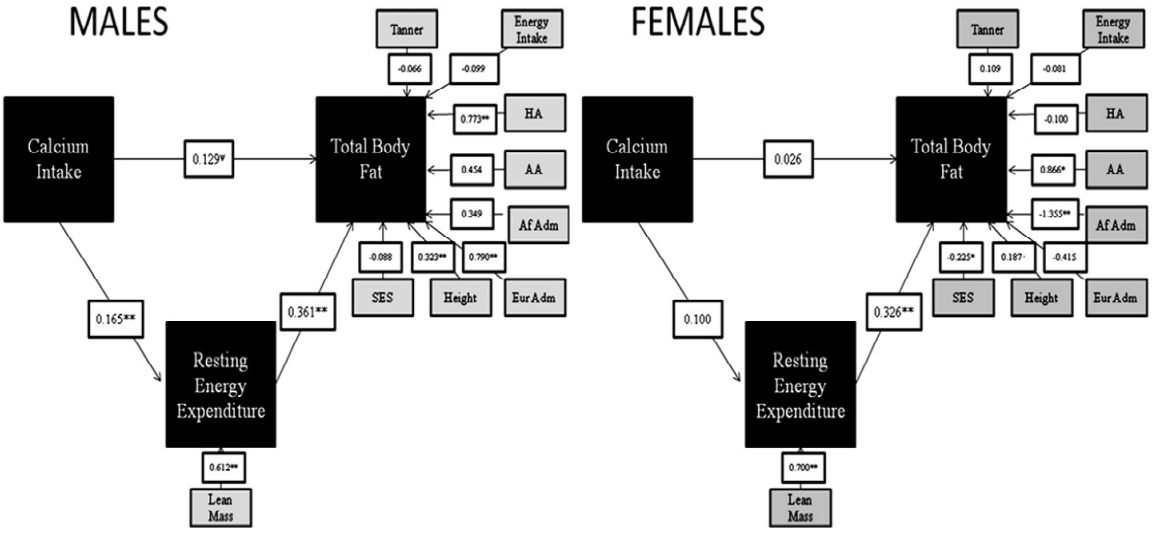

Figure 2. (Supplement) *Relationships between calcium intake, REE, and total body fat stratified by sex.* The arrows represent the causal paths; specifically the arrowhead points to the presumed effect, and the line stems from the presumed cause.

HA = Hispanic American; AA = African American; Af Adm = African American Admixture; Eur Adm= European American Admixture; SES = Socioeconomic Status; Dark boxes = Dependent variables; Light boxes = Independent variables; White boxes = Parameter Estimate

(numbers represent β- coefficients); *<0.05, ** ≤0.01$^{\Psi}$<0.10 ; χ^2=18.04; p-value=0.450; df=10; CFI=1.000; RMSEA=0.004

FACTORS ASSOCIATED WITH CALCIUM HOMEOSTASIS INFLUENCE RESTING ENERGY EXPENDITURE IN PERI-PUBERTAL GIRLS

by

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ABSTRACT

Objective: Regulation of calcium homeostasis is an emerging factor influencing resting energy expenditure (REE).

Methods: We investigated the association of dietary and hormonal factors involved in the regulation of calcium homeostasis with REE in peripubertal girls ages 7-12y (n=36). REE was assessed by indirect calorimetry, and the independent variables, body composition, dietary intake (calcium, vitamins D and K, phosphorus), and serum hormones (PTH, osteocalcin, 25OHD), were evaluated by DXA, 24h recall and serum assay, respectively.

Results: Evaluation of the relationship between calciotropic hormones with REE revealed a positive association between vitamin K and REE and an inverse association of PTH with REE ($p<0.05$). Multiple regression analysis using stratification by percent fat cut-offs revealed PTH and REE were positively related in those having normal adiposity ($p=0.03$) and inversely related in those with excess adiposity ($p=0.01$). The association of REE with vitamin K intake was evident in lean individuals ($p<0.001$), but was null in those with excess adiposity.

Conclusion: Overall, decreased calciotropic hormone levels along with increased related nutrient intakes were associated with greater REE, although these relationships differed according to adiposity. These findings stress the importance of achieving dietary adequacy essential for establishing a foundation for optimal body composition trajectory.

INTRODUCTION

Due to the substantial involvement in overall metabolism, particularly processes requiring significant energy allocation at rest, it is conceivable that calcium homeostasis plays a pivotal role in resting energy expenditure (REE). Beyond the typical function ascribed (maintenance of healthy teeth and bones), calcium is also an essential component of cellular physiology, where movement of the calcium ion into and out of the cytoplasm functions as a signal for vital cellular processes including (but not limited to) coagulation, electro-conduction of the heart, neurotransmitter release and muscle contraction. Thus, various energy- and cofactor-dependent mechanisms are in place in order to ensure calcium is kept within a tight range, and together comprise what is referred to as the "calcistat" network. To our knowledge, no study has investigated the extent to which the cascade of events involved in calcium homeostasis contributes to REE.

Undoubtedly dietary intake is a key player in calcium homeostasis. Adequate intake and absorption of key nutrients identified as having a role in calcium regulation serve to optimize energy coordination pathways. Although source-dependent, approximately 23-37% of dietary calcium is absorbed (Gueguen & Pointillart, 2000), and adequate intake and absorption allows for normalization of extra- and intra-cellular calcium levels. Insufficient availability of nutrients involved in the calcistat network leads to perturbation of obligatory function mediating the interplay between body composition compartments (e.g., bone mineral accretion, adipogenesis). For example, the vitamin D receptor in response to 25OHD activation, leads to increased adipocyte lipid storage (Zemel, 2009; Gilbert-Diamond *et al.*, 2010). Further, dietary phosphorus through calcistatic interactions also plays an influential role in the relationship of calcium homeostasis and REE action both at the intestine and in circulation. An excess of phosphorus in the intes-

tine has the capacity to bind calcium through salt formation, impeding absorptive capacity (Holick, 2003a; Holick, 2003c; Kidd, 2010a). In addition, circulating phosphorus influences PTH and 25OHD as increasing levels stimulate PTH release and subsequently inhibits 25OHD activation (Holick, 2003a; Holick, 2003b; Kidd, 2010b). Additionally, beyond its well-recognized essentiality for activating blood coagulation proteins, vitamin K is also intricately involved in calcium homeostasis via the facilitation of calcium deposition into the bone matrix and inhibition of calcium accumulation in soft tissues (Atkins *et al.*, 2009a; Kidd, 2010c). Thus the importance of achieving adequacy in nutrients identified in the regulatory pathways of calcium homeostasis (i.e. calcium, vitamin D, phosphorus, vitamin K) is highlighted in order to regulate hormone signaling underlying calcium homeostasis.

In the context of the current pediatric obesity epidemic, the period surrounding reproductive maturation, marked by distinct changes fundamental to energy balance, or more specifically REE, may be critical. Calcium intake, as well as potentially other key dietary nutrients, especially among girls (during this stage of development) is commonly inadequate (Bailey *et al.*, 2010), potentially altering physiological processes underlying REE and increasing risk of adverse outcomes related to body composition (i.e. lower bone mass and potentially greater fat mass) (Casazza *et al.*, 2008). To this end, the objective of the study was to evaluate relationships of body composition, as well as dietary and hormonal components relevant to calcium homeostasis, and potential contribution to REE in adolescent girls.

METHODS

Participants

Measures on 36 girls recruited from the Birmingham, Alabama area as a part of a larger cross-sectional (Casazza *et al.*, 2010), aged seven-12 years (pubertal stage ≤3 Tanner stage three), were used for current analyses. Girls were healthy and not on medications known to affect body composition (i.e. anti-hyperactivity, anti-asthmatic, steroidal drugs). Parents and children provided consent/assent, respectively, after reviewing the protocol with study personnel. The protocol was approved by the Institutional Review Board for human participants at the University of Alabama at Birmingham (UAB). All measurements were performed between 2005 and 2008.

Protocol

Participation required two visits within thirty days of one another. On the first visit, pubertal stage, anthropometric assessment and body composition were measured, and a 24-hour dietary recall was obtained. On the second visit, participants were admitted to the General Clinical Research Center for an overnight stay (ensuring ~10-hour fast) and a second 24-hour dietary recall was obtained. Upon completion of the overnight fast, REE assessment and fasting venipuncture were performed.

Outcome Variable

REE

REE was measured via computerized, open-circuit, indirect calorimetry system with a ventilated canopy (Delta Trac II; Sensor Medics, Yorba Linda, CA). The coefficient of variation (c.v.) for REE using repeated measures has been determined at <4% (Hunter *et al.*, 2008). One-minute average intervals of oxygen uptake (VO_2) and carbon

dioxide production (CO_2) were measured continuously for 30 minutes, in which the last 20 were used to calculate energy expenditure.

Independent Variables

Body Composition Assessment

Body composition compartments (fat, lean, bone mass) contribute uniquely to REE (Muller *et al.*, 2009; Hanks *et al.*, 2010b); thus, whole body composition was assessed by dual energy x-ray absorptiometry (DXA; GE Lunar Radiation corp., Madison, WI) with pediatric software (version 1.5e). Measurements assessed by this instrument differs by 4% or less (huffman dm *et al.*, 2005).

Serum Assays

PTH, 25OHD and OC were obtained from fasting sera drawn and assayed in the UAB Core Laboratory. Serum PTH was assessed by a two-site immunoradiometric assay, 25OHD with liquid chromatography/ tandem mass spectrometry technique and total OC using radioimmunoassay (Gundberg *et al.*, 1998). The intra-assay c.v.'s for the analysis of PTH, 25OHD and OC were 7.76, 4.83, and 5.36, and the mean inter-assay c.v.'s were 2.07, 4.94, and 5.76%, respectively.

Dietary Assessment

Total energy (kcal/d), calcium (mg/d), vitamin D (mcg/d), vitamin K (mcg/d) and phosphorus (mg/d) intake were assessed using the average of the two 24-hour dietary recalls, conducted using the "multiple pass" method, providing cup and bowl sizes to help estimate portion sizes. Each recall was performed in the presence of at least one parent. A registered dietitian coded and entered the data into Nutrition Data System for Research

version 2006 (Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN).

Confounding variables

Pubertal Status

Pubertal progression is associated with characteristic influences on body composition and energy metabolism. The one-to-five staging of Marshall and Tanner, based on examination of both breast and pubic hair development, was used for pubertal stage assessment, with one composite number representing the higher of the two assigned values (Marshall & Tanner, 1968).

Anthropometric Measures

Anthropometric measures were obtained by the same registered dietitian. Height (Heightronic 235; Measurement Concepts, Snoqualmie, WA) and weight (Scale-tronix 6702W; Scale-tronix, Carol Stream IL) were obtained in minimal clothing without shoes. BMI percentile was calculated using age-specific growth charts (2009).

Genetic Admixture Analysis

Inherent inter-population differences in calcium homeostatic mechanisms (transport, absorption, excretion) body composition and REE may confound data interpretation. Accordingly, genetic admixture was included as a genomic control variable representing population-based differences for statistical analysis (Casazza *et al.*, 2009a). Molecular techniques for the allelic identification and methodology for genetic admixture application have been described elsewhere (Parra *et al.*, 1998). Briefly, a panel of 140 ancestry informative markers was used to estimate the genetic admixture proportion of each subject, using maximum likelihood estimation based on an algorithm described by Hanis et

al (Hanis *et al.*, 1986). European genetic admixture, the most widely distributed component across racial/ethnic groups in this sample was used in analyses.

Physical Activity (PA)

After REE, PA represents the next greatest contributor to energy expenditure for most, also exerting an effect on REE (McKay *et al.*, 2010). The MTI Actigraph accelerometer (Actigraph GT1M – Standard Model 198-0100-02, ActiGraph LLC, Pensacola, FL, and accompanying software) was used to measure PA level and pattern for seven days prior to admission to the GCRC. Epoch length was set at one minute and data was expressed as counts per minute. Children were instructed to wear the monitor at the waist above the right hip, allowing removal for only sleeping, bathing and swimming. Actigraph monitors have previously demonstrated a high degree of inter-instrument reliability (Casazza *et al.*, 2009b). Daily and total counts per minute were summed and averaged.

Statistical Approach

Mean descriptive values for the overall sample and adiposity level (using body fat percent cut-off of 30, the standard value for females at which it is considered to be in excess (Williams *et al.*, 1992; Casazza *et al.*, 2010) were analyzed and differences were investigated using ANOVA. Preliminary analysis investigated the independent contribution of body composition compartments to REE was investigated using multiple linear regression. Bone mineral content was identified as the greatest contributor to REE ($p<0.008$), supporting the potential involvement of calcium in obligatory physiological function (via bone mineral accretion pathways). Next, the contribution of calciotropic hormones (PTH and 25OHD), OC and relevant dietary factors (calcium, vitamin D, vitamin K and phosphorus) to REE was evaluated, while accounting for relevant covariates

(described below). The sample was stratified by body fat percent and regression models were reanalyzed. Models investigating the contribution of nutrient intake on REE were additionally stratified by adequacy according to DRI recommendations, using 1300mg for calcium, 5 mcg for vitamin D, 60 mcg for vitamin K and 1250 mg for phosphorus (1994a). Inclusion of total energy intake as a control variable served to account for the correlation between micronutrient and overall caloric intake (*26*). It was determined that a minimum of 27 participants was necessary for an analysis providing 80% power with a corresponding effect size=0.25 at p=0.05. All models were adjusted for European admixture, pubertal stage, fat and lean mass. The nominal variable pubertal stage was orthogonally coded for regression analysis. Contribution of PA to REE was investigated, but was not identified as significant so was excluded from final models.

RESULTS

Sample characteristics are displayed in Tables 1 and 2. Evaluation of the contribution of calciotropic hormone analysis to REE revealed an inverse association of REE with PTH ($p<0.05$) and a trend for an inverse relationship of REE with OC ($p<0.10$). Investigation of associations between calcistatic dietary variables and REE demonstrated a positive relationship between vitamin K intake and REE ($p<0.0001$).

Although there were no differences in either mean dietary variables associated with calcium homeostasis or calciotropic hormone concentration by adiposity level (Table 2), multiple regression analysis using stratification by percent fat cut-offs revealed PTH and REE were positively related in those having normal adiposity ($p=0.03$) and inversely related in those with excess adiposity ($p=0.01$) (Table 3). The positive association of REE with vitamin K intake remained in lean individuals ($p<0.001$), but was no longer significant in those with excess adiposity. A trend towards a positive association between phosphorus and REE was observed only in those with excess adiposity ($p<0.10$).

Subsequently, stratification of the overall sample by dietary adequacy revealed a positive association with REE and vitamin K irrespective of dietary adequacy ($p<0.001$, adequate; $p<0.05$, inadequate). ANOVA analysis (Figure 1) revealed marginal associations between REE and calcium in those meeting or exceeding recommendations ($p<0.10$) and vitamin K ($p<0.10$) intake compared to those who did not.

DISCUSSION

Evidence is accumulating that homeostatic mechanisms related to calcium balance (i.e. hormone signaling) are an integral part of metabolic pathways influencing fuel utilization and resource partitioning (e.g. fat vs. bone), and ultimately energy required for metabolic processes at rest. Bone, as the body's calcium (and overall mineral) reservoir, requires substantial energy allotment vital for optimal physiologic function (Buchowski *et al.*, 2001; Eriksen, 2010; Kim *et al.*, 2010d). Accordingly, a variety of calciotropic dietary and hormonal factors centering on skeletal function were evaluated to assess potential underlying relationships with REE. Among these, vitamin K and PTH emerged as significant, and each of these relationships was influenced by level of adiposity. It is plausible that adequate intake and absorption of dietary nutrients involved in calcium maintenance may optimize mechanistic pathways involved in REE.

The relationship between vitamin K and REE is not surprising given the role of vitamin K in a number of physiologic processes including bone remodeling. A consistent line of evidence in human and animal studies clearly demonstrates that vitamin K influences bone health (Kalkwarf *et al.*, 2004; Gigante *et al.*, 2008b; Dougherty *et al.*, 2010). Acting as a coenzyme, vitamin K mediates the conversion of glutamate to gamma-carboxyglutamate which is essential to facilitate calcium incorporation into hydroxyapatite crystals, thus mineralizing bone (Atkins *et al.*, 2009). Particularly salient during the period surrounding the linear growth spurt (peri-puberty), vitamin K activity involves an increase in both bone deposition and resorption to ensure structural integrity (Yamauchi *et al.*, 2010a). Taken together, it may be inferred that adequate vitamin K status is bene-

ficial for optimal bone metabolism, and to this end modifies REE (Zemel, 2004; Fukumoto & Martin, 2009; Hanks *et al.*, 2010).

It is well-established that serum calcium levels are inversely associated with PTH (O'Toole, 2011), a hormone which may influence energy requirements directly and indirectly through body composition alterations. Consistent with existing literature, PTH was inversely associated with REE ($p<0.05$) in this cohort. Serum PTH increases in circulation secondary to dietary insufficiency of calcium and related nutrients, serving to regulate calcium serostatus (O'Toole, 2011). Continuously elevated PTH inhibits the bone modeling process, thereby down-regulating REE and increasing adipogenic pathways (Schmitt *et al.*, 2005a). Although causal inference cannot be established based on this cross-sectional study, it is plausible that PTH signaling influences REE via mechanisms associated with bone mineralization (i.e. modeling/remodeling) and adipose tissue accrual.

Adiposity may alter the contribution of dietary adequacy to resource allocation between tissue compartments, highlighting the importance of body composition in terms of both fat and bone in the metabolic regulation of calcium homeostasis. Interestingly, after stratification by percent fat cut-offs, relationships between vitamin K and PTH with REE varied. Such stratification indicated a positive relationship of dietary vitamin K and REE (as with the overall sample) in lean individuals, yet null in those with excess adiposity. In subjects with adiposity level beyond that which is considered normal, the attenuation of this relationship suggests the mechanism by which vitamin K influences energy expenditure may be perturbed upon excess adipose tissue. The magnitude of the relationship between PTH and REE in those with excess adiposity is particularly highlighted as

significance and direction remained parallel in the total sample despite difference among lean subjects. This may suggest potential altered bone turnover in overweight children. Indeed, adiposity has been reported to interfere with bone metabolism in children via secondary low bone turnover and reduced skeletal utilization of calcium (Viljakainen *et al.*, 2010b). The contribution of adiposity to the relationships found between both vitamin K and PTH with REE support the possibility of a possible detriment to bone accrual in early pubertal girls (Viljakainen *et al.*, 2010a); however, cumulative effects on body composition compartmentalization warrant further study.

Although statistical significance was not reached for each dietary nutrient's (i.e. vitamin D and phosphorus) association with REE, it is important to note an observation, warranting further investigation. Phosphorus metabolism in many respects parallels that of calcium (Bergwitz & Juppner, 2010). As calcium is liberated from bone, so too is phosphorus. In addition, PTH-stimulated enhancement of intestinal absorption (also involving vitamin D activation) of calcium extends to that of phosphorus. Evidently, alterations of PTH levels become concordant with phosphorus metabolism, thereby playing a role in calcium serostatus and resultant energy resource disposal (Schmitt *et al.*, 2005b; Saji *et al.*, 2010; Bergwitz & Juppner, 2010; Hori *et al.*, 2011).

In the context of our observed relationship between vitamin K and REE, the observed trend for an association of the hormone OC with REE ($p<0.10$) may also be noteworthy. Although physiologic implications of the bone marker OC are not fully understood, circulating levels are to an extent indicative of energy utilization (i.e. osteogenesis vs. adipogenesis) (Muruganandan *et al.*, 2009; Kim *et al.*, 2010c). Disconcordant with adult studies (Lee *et al.*, 2007; Kim *et al.*, 2010b), we observed an inverse relationship

between OC and REE. The discrepancy is not entirely inexplicable, but may rely on complexity of the hormone itself, as action relies on its bioactivity. In its vitamin K-required carboxylated form, OC confers calcium-binding properties of bone (Gigante *et al.*, 2008a). In its circulating undercarboxylated form, OC has been shown to be positively related to energy expenditure (Kim *et al.*, 2010a). Whereas total OC, as assessed in this sample, is dependent on bone turnover, the ratio of carboxylated to undercarboxylated OC, an unavailable measure, is dependent on vitamin K intake. The ability to assess the contribution of undercarboxylated OC (or ratio of both fractions) likely would have influenced the findings as novel studies suggest that it is this form that stimulates bone remodeling (Ferron *et al.*, 2010; Yamauchi *et al.*, 2010b). Future investigation regarding the relationship of OC with energy metabolism is warranted, particularly in those undergoing skeletal growth.

Major strengths of this study were use of objectively assessed data (in addition to self-reported intake) and robust measurement of body composition. Because of the interrelationships among the variables, the relatively small sample size may have precluded the detection of significant relationships. Finally, the cross-sectional nature of the study limits ability to infer causation; thus findings serve as observational data for future expansion.

Beyond commonly regarded pathways, dynamic parameters involved in calcium homeostasis influence energy balance through multiple mechanisms involving REE. Increased intake of calcium-related vitamin K nutrients and decreased circulating calciotropic hormone PTH were associated with higher REE. The relationships also differed according to levels of adiposity and nutrient intake, highlighting the importance of re-

source partitioning and dietary nutrient adequacy. Further, the associations were independent of ancestral genetic background and physical activity, thereby suggesting a substantial influence of diet on REE regulatory mechanisms. These findings stress the importance of achieving dietary adequacy essential for establishing optimal body composition trajectories, particularly throughout developing years. It is likely that assessment of these relationships studying a larger cohort longitudinally would strengthen the findings of our data.

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Table 1. Characteristic variables of overall sample (n=36)

Age (yr)	9.3 ± 0.3
Pubertal stage[2]	1.72 ± 0.15
European admixture	0.50 ± 0.06
Height (cm)	140.2 ± 1.7
Weight (kg)	36.0 ± 1.2
BMI percentile	63.6 ± 4.8
Total % Fat	25.2 ± 1.4
PA (min/d)	173.3 ± 17.1
REE (kcal/d)	1,152.8 ± 33.2
BMC (g)	1,260.6 ± 45.0

[1]On a scale of 1-5, based on the criteria of Marshall and Tanner (Marshall & Tanner, 1968)
PA=physical activity, REE=resting energy expenditure, BMC=bone mineral content

Table 2. Characteristic variables related to the calciotropic network and resting energy expenditure (REE) in the overall sample (n=36) and stratified by adiposity*

	Total sample	Normal adiposity* (n=25)	Excess adiposity* (n=11)
REE	1,153 ± 33	1,135 ± 42	1,194± 54
PTH (pg/ml)	47.2 ± 3.0	49.7 ± 3.8	41.6 ± 4.6
25OHD (ng/ml)	26.4 ± 1.1	25.9 ± 1.5	27.3 ± 1.6
OC (ng/ml)	12.0 ± 0.7	12.0 ± 1.0	11.9 ± 0.6
Calcium (mg/d)	831.7 ± 44.2	820.0 ± 56.4	858.5 ± 69.8
Vitamin D (mcg/d)	4.9 ± 0.6	4.9 ± 0.8	4.9 ± 0.6
Vitamin K (mcg/d)	64.0 ± 13.7	61.4 ± 17.1	70.0 ± 23.7
Phosphorus (mg/d)	1,099.1 ± 48.5	1066.5 ± 59.3	1173.2 ± 83.3

*lean <30% fat, obese ≥30% fat (Williams *et al.*, 1992; Casazza *et al.*, 2010)

PTH=parathyroid hormone, 25OHD=25-hydroxy vitamin D, OC=osteocalcin

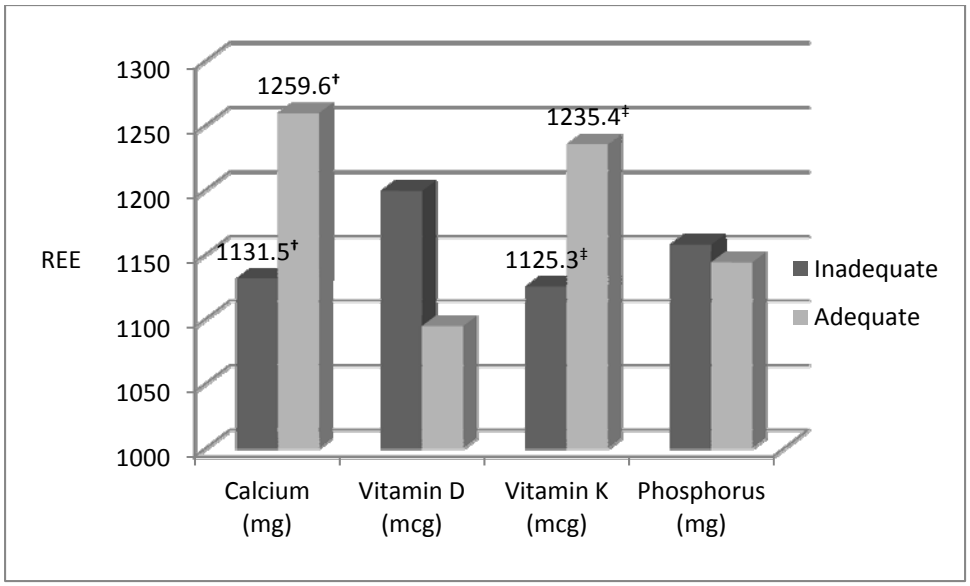
Table 3. Association between subjective and objective measures of calcium homeostasis and resting energy expenditure (REE) by linear regression analysis*

Subjective Measures	*p-value*
Calcium	0.28
Vitamin D	0.47
Vitamin K	<0.01
Phosphorous	0.15
Objective Measures	
PTH	0.05
25OHD	0.28
OC	*0.07*

* Adjusted for European admixture, pubertal stage, fat and lean mass; models evaluating subjective measures also controlled for overall energy intake.

Bolded values represents significance, $p<0.05$; *Italicized* values represent marginal significance, $0.10<p>0.05$.

PTH=parathyroid hormone, 25OHD=25-hydroxy vitamin D, OC=osteocalcin



REE=Resting Energy Expenditure

Figure 1. Mean† resting energy expenditure by dietary nutrient intake adequacy (determined by Dietary Reference Intakes (1994a). Dark gray bars represent nutrient intake meeting DRI, light gray bars represent intake lower than DRI. REE=Resting Energy Expenditure;†p=0.09, ‡p=0.08

†Adjusted for European admixture, pubertal stage, fat and lean mass, and overall energy intake.

VITAMIN D AND CALCIUM-SENSING RECEPTOR POLYMORPHISMS DIFFERENTIALLY ASSOCIATE WITH RESTING ENERGY EXPENDITURE IN PERIPUBERTAL CHILDREN

by

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ABSTRACT

Components of the calciotropic network impact variation in resting energy expenditure (REE). Genetic variants in the vitamin D receptor (VDR) and calcium-sensing receptor (CASR) may contribute to differences in REE through their role in calcium regulation.

A sample of 273 European- (EA; ;n=116), African- (AA; n=94), and Hispanic-American (HA; n=84) children (52% male), 7-12 years of age from whom DNA was available was used to evaluate contributions of VDR and CASR to REE, as measured via indirect calorimetry. SNPs for VDR (rs11568820) and CASR (rs1801725) were genotyped using the Illumina Golden Gate assay. Body composition was measured by DXA, and dietary measures were obtained from two averaged 24-hour recalls. Multiple linear regression models were used to determine the association between SNPs and REE using non-additive models. Group-wise stratification analysis was also performed such that the model including VDR was evaluated by ethnicity and adiposity, and the model including CASR was evaluated by sex and median calcium intake.

Presence of the 'A' allele of VDR was positively associated with REE in AA ($p=0.02$) and among children categorized as having normal body fat. For CASR, there was a significant association of the 'A' allele presence and greater REE in females ($p=0.01$); however, in males this relationship was inverse and was only marginally significant ($p=0.06$). Presence of the 'A' allele was associated with greater REE in those with high levels of calcium intake.

These findings support the notion that components of the calciotropic network influence REE through genetic variation at early stages of the lifespan, and that such relationships might be mediated by adiposity, sex and ethnicity. Future studies are warranted

to replicate these findings in order to understand the significant involvement of calcium-regulation genes on REE during influential periods in body composition trajectory.

INTRODUCTION

Though numerous researchers have sought to understand the etiology of pediatric obesity, causes explaining the ever-present proportion of affected children remain to be elucidated. A disruption in the balance between energy intake relative to expenditure is known to be fundamental in the accrual of fat mass; however, energy balance appears to be multi-factorial and highly variable among individuals (*1-3*). An underappreciated key factor encompassing simultaneous physiologic, behavioral and genetic influences on body composition patterning is resting energy expenditure (REE). As the largest constituent of overall energy output, REE exerts effects on body composition through obligatory metabolic pathways, serving as a dynamic host of regulatory processes. Studying the contribution of REE to pediatric body composition variation may provide significant insight towards understanding the etiology of pediatric obesity.

Dietary nutrients have been identified as having a role in energy balance. Scientific evidence supports a contribution of dietary calcium to REE that appears to be mediated by the pivotal role that serum calcium (Ca^{2+}) homeostasis plays in energy regulation (*4-9*). For calcium to be normalized in the body, a series of complex physiologic and metabolic processes occur that impact the storage and utilization of molecular, cellular and physiological resources. For example, when reduction in calcium concentration occurs, calcium-dependent processes (such as bone mineralization, lipolysis and protein synthesis) become compromised to normalize circulating levels. This maintenance of the calciotropic network thus impacts REE and, consequently, body composition patterning (*10*).

Limited exploration has been given to potential genetic mechanisms that may contribute to the calcium-REE relationship and its contribution to body composition.

Single nucleotide polymorphisms (SNPs) in the vitamin D (VDR) (*11;12*) and calcium-sensing (CASR) receptors (*13*) have been shown to regulate nutrient absorption, transport and excretion by altering the binding ability of specific metabolites. Activation of VDR enhances calcium absorptive capacity, and calcium binding to CASR regulates bone calcium resorption (Arai H 2001). It might be speculated that SNPs in VDR and CASR genetic regions could, in part, explain variability in calcium utilization (*14*). In this regard, the Cdx-2 rs11568820 polymorphism of VDR has been associated with alterations in transcriptional activity of the promoter, which may affect calcium absorption (*15*). Additionally, CASR variation at rs1801725 has been suggested to influence circulating calcium concentrations by modulating signal transduction, intracellular trafficking and cell surface receptor expression (*16*). It is possible that disturbances in calcium metabolism may be more pronounced in individuals with certain variants at the VDR and CASR loci, thereby impacting REE and ultimately body composition patterning.

In light of the above observations, it might be relevant to explore the extent to which genetic variants involved in the calciotropic network may play a significant role in REE and how such relationships may contribute to variation in body composition parameters. Such exploration becomes relevant when investigated at the peri-pubertal age, a period when the impact of modifiable factors most prominently contributes to long-term body composition patterning. The objective of this study was to evaluate if SNPs involved in the calciotropic network (VDR and CASR) are associated with REE, and how such involvement may be mediated by inherent (i.e., sex, adiposity, ethnicity) and/or dietary (i.e., energy, calcium, and/or vitamin D intake) factors.

METHODS

Participants. A sample (n=273; 145 male, 128 female) children, 7-12 years of age, were recruited to study the effects of genetic and environmental parameters on racial differences in metabolic outcomes. The participants were early pubertal (Tanner stage ≤3), healthy, and not on medications known to affect body composition. Parents and children provided consent/assent, respectively, after reviewing the protocol by study personnel. The protocol was approved by the Institutional Review Board for human participants at the University of Alabama at Birmingham (UAB). All measurements were performed between 2005 and 2009.

Protocol. Participants required two visits that were no more than thirty days apart. On the first visit, pubertal stage, anthropometrics and body composition were measured, and a 24-hour dietary recall was obtained. On the second visit, participants were admitted to the General Clinical Research Center for an overnight stay (ensuring ~10-hour fast) and a second 24-hour dietary recall was obtained. Upon completion of the overnight fast, indirect calorimetry was performed and serum was obtained for metabolite analyses.

Indirect Calorimetry. REE was measured via computerized, open-circuit, indirect calorimetry system with a ventilated canopy (Delta Trac II; Sensor Medics, Yorba Linda, CA). The coefficient of variation for REE using repeated measures has been set at <4% (*13*). One-minute average intervals of O_2 uptake and CO_2 production were measured continuously for 30 minutes, in which the last 20 minutes were used to calculate energy expenditure.

Genotyping. DNA was obtained from the study participants, and genotyping of VDR and CASR SNPs at rs11568820 and rs1801725, respectively, was performed at the UAB Hef-

lin Genotyping Core using the Illumina Golden Gate assay on the BeadXpress system (Illumina, Inc.). Briefly, the GoldenGate assay involves biotin-labeling of genomic DNA followed by capture of the labeled DNA onto streptavidin-coated sepharose beads. An artificial nucleotide-based molecule that contains universal priming sequences on either end and is complimentary to the target DNA sequence of interest is then created, amplified and hybridized to holographically-labeled silica bars that form arrays with up to 30-fold redundancy of each target to be interrogated. Once the array has been visualized with the BeadXpress reader, wavelength and intensity values of the fluorescence are used to determine genotype. A custom LIMS is used to track both samples and laboratory throughput. Allele detection and genotype calling were performed using the GenomeStudio software v3 (Illumina, Inc.).

Genotyping of ancestry informative markers for the measurement of genetic admixture was performed at Prevention Genetics (Marshfield, WI) using the Chemicon Amplifluor SNPs Genotyping System coupled with ArrayTape technology as previously described (*17*). A panel of 142 ancestry informative markers (AIMs) was used to estimate the genetic admixture proportion of each subject. Information about the AIMs along with previously parental population frequencies have been recently provided as supplemental materials by Klimentidis et al (*18*). Molecular techniques and methodology for marker genotyping have been described elsewhere (*19*). Genotypic information was translated into estimates of African, European, and Native American admixture for each subject using maximum likelihood estimation based on the algorithm described by Hanis et al. (*20*).

Anthropometric Measures. Anthropometric measures were obtained by the same registered dietitian. Height (Heightronic 235; Measurement Concepts, Snoqualmie, WA) and weight (Scale-tronix 6702W; Scale-tronix, Carol Stream IL) were measured in minimal clothing without shoes. BMI percentile was calculated using age- and sex-specific growth charts (*21*).

Body Composition. Body composition (i.e., bone mineral content, BMC; lean mass; and total and percent fat mass) was measured by dual-energy x-ray absorptiometry (DXA) using a GE Lunar Prodigy densitometer (GE Lunar Radiation Corp., Madison, Wisconsin) with pediatric software (version 1.5e). Subjects were scanned in light clothing, lying flat on their back with arms at their sides. Because excess fat has been associated with an imbalance of the calciotropic network, categorization according to adiposity level was performed such that females and males with > 30 and 25% fat mass, respectively, were characterized as having an excess adiposity level, and females and males with ≤30 and ≤25% fat, respectively, were characterized as having a normal adiposity level (*22;23*).

Diet. Dietary measures were obtained from two averaged 24-hour recalls using the "multiple pass" method, in which cup and bowl sizes were provided to help estimate portion sizes. Each recall was performed in the presence of at least one parent. A registered dietitian coded and entered the data into Nutrition Data System for Research version 2006 (nutrition Coordinating Center, University of Minnesota, Minneapolis, Minnesota). Total energy (kcal/d), calcium, and vitamin D intake were generated as variables from the analyses.

Pubertal Status. The Tanner stages have been demonstrated as reliable indicators of pubertal development. Assessment of pubertal stage was by direct observation by a pedia-

trician, the 'gold standard' for differentiating among the five stages of maturity (*24;25*). The staging based on the criteria of Marshall and Tanner (*26;27*) is according to both breast and pubic hair development in girls and genitalia and pubic hair development in boys. One composite number was assigned for Tanner staging, representing the higher of the two values defined by breast development and/or pubic hair (*28*).

Physical Activity by Accelerometer. The MTI Actigraph accelerometer (Actigraph GT1M – Standard Model 198-0100-02, ActiGraph LLC, Pensacola, FL and accompanying software) was used to measure physical activity levels and patterns for seven days prior to participant's inpatient visit at the GCRC. Epoch length was set at one minute and data expressed as counts per minute (counts min^{-1}). Children were instructed to wear the monitor on an elastic belt at the waist above the right hip, removing only for sleeping, bathing and swimming. Actigraph monitors have previously demonstrated a high degree of inter-instrument reliability (*29*). Daily and total counts per minute were summed and averaged.

Insulin/Glucose Dynamics. Insulin/glucose homeostasis is essential for maintaining energy balance. In addition, vitamin D has recently emerged as a potential mediator in this relationship (*30-34*). Measures of fasting insulin and glucose, along with insulin sensitivity, were obtained via intravenous glucose tolerance testing. Following the overnight fast, a topical anesthetic (Emla cream, AstraZeneca, Wilmington, DE) was applied to the antecubital space of both arms, and flexible intravenous catheters were placed in both arms. Subsequently, an intravenous glucose tolerance test was performed as previously described (*35*). The acute insulin response to glucose, an approximation of first-phase insulin secretion, was calculated as the incremental area under the curve for insulin dur-

ing the first 10 minutes after glucose injection using trapezoidal methodology (*36*). Values for fasting insulin and glucose were obtained from the average of the two baseline values, and were entered into the MINMOD computer program for determination of insulin sensitivity as described elsewhere (*37*). Fasting samples of glucose and insulin were analyzed using a SIRRUS analyzer (Standio Laboratory, Boeme, Texas). All analyses were performed in the Core Laboratory Nutrition Obesity Research Center at UAB.

Statistical Analyses. ANOVA was used to assess sex- and ethnic-specific differences in descriptive statistics. Lewontin's *D*' and r^2 were used to evaluate Hardy Weinberg Equilibrium (HWE) for each SNP (rs11568820 and rs1801725) for the overall sample and by ethnicity, in which a p value of <0.05 indicated deviation in goodness of fit. Hardy-Weinberg Equilibrium (HWE) for VDR was not apparent in the overall sample, yet was among ethnic groups, thus analysis was run according to ethnicity. To account for the genetic heterogeneity of the sample, to reduce Type I errors in the association analysis, and to control for the effects of population stratification, estimates of genetic admixture were added as covariates to the statistical models. Allele and genotype frequency for the overall sample and between groups (i.e. sex, ethnicity) were performed using the χ^2 test.

To test associations between each SNP and REE variation, a genotypic model was tested where values of 0, 1 and 2 were used as dummy codes to represent homozygous for the major allele, heterozygous, and homozygous for the minor allele, respectively. To evaluate the potential contributions of presence/absence of alleles, allelic models were evaluated where a dummy code of 0 was used to code for individuals homozygous for one allele, and 1 to code for individuals carrying at least one copy of the other allele. For all regression models, studentized residuals were evaluated for normality and logarithmic

transformations of the dependent variable was performed when necessary. In accordance with the assumptions of regression, the observations for which the residuals of the association models were above and below three standard deviations were removed from the analyses.

Step-wise multiple linear regression analysis was employed to guide inclusion of covariates in the association models, in which sex, fat mass index (total fat divided by height-squared), total lean mass, BMC, dietary variables (overall energy, calcium and vitamin D intake), and physical activity were investigated, with either VDR or CASR SNPs as the independent variable, and with entrance and stay criteria set at $p=0.15$ and <0.010, respectively. Insulin dynamics (insulin sensitivity, and fasting insulin and glucose), which has been reported to influence vitamin D metabolism (*38*) as well as energy utilization (*39*), was included in step-wise regression with VDR as the independent variable. Based on the stepwise exploratory analysis, the variables sex, fat mass index, total lean mass, fasting insulin and dietary calcium were considered as covariates for the association with VDR and sex, fat mass index, total lean mass, and dietary calcium were considered as covariates for CASR. In addition, to control for population stratification (*40-42*), genetic admixture was included in all analyses, as was Tanner stage to account for variability of REE according to pubertal status (*43*). Interaction variables between the genetic variants (i.e., VDR by CASR) and between each genetic variant and adiposity level, sex and median calcium intake, respectively, were also included in separate models. Models testing interaction variables included each individual component of the interaction term, in addition to the interaction variable. To explore potential differences in the associations due to ethnicity, calcium intake, sex and adiposity level, models were

evaluated according to these different categories. All analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). Significance level was set at $p \leq 0.05$; marginal significance was considered at $0.05 > p \leq 0.10$.

RESULTS

Preliminary analyses involving evaluation of additive and non-additive recessive-assumed models for VDR were not significant, thus only the non-additive dominant-assumed model was used for final analyses. For CASR, only one subject (EA female) was homozygous for the 'A' allele, therefore only the non-additive model was evaluated for genotype analysis. Overall and sex- and ethnic-specific sample characteristics are described in Table 1. Compared to girls, boys were older yet less reproductively mature, had a lower body fat percentage, lower total fat, greater lean mass, lower fasting insulin and greater resting energy expenditure, as well as marginally higher BMC. AA were reproductively the most mature (i.e., advanced Tanner staging), with the highest BMC and lean mass. HA were categorized as being in the highest BMI percentile. EA had the greatest amount of European admixture, followed by HA, who in turn had greater European admixture than AA. EA had the lowest levels of fasting insulin. In addition, boys had greater energy intake than girls, and HA had the greatest level of calcium intake, followed by EA and then AA.

The genotypic frequency of VDR for the entire group was 24.5%, 26.7%, and 48.7% for AA, AG, and GG genotypes, respectively (Table 2). VDR genotype was not in HWE in the total sample (thus the overall model was not analyzed); however, it was within ethnic groups. There were no ethnic or sex differences in genotype. The 'A' allele presence in the overall sample was 38.9%, and was greatest in AA (75.9%) compared to EA (22.9%) and HA (16.5), with no difference between males (35.6%) and females (40.3%).

The genotypic frequency of the CASR polymorphism for the entire group was 0.4%, 10.3%, and 89.4% for AA, AC, and CC genotypes, respectively (Table 2). CASR genotype was in HWE in the total sample (as well as within ethnic groups). EA had a significantly greater presence of the 'A' allele (14.4%) than AA (3.4%) and HA (5.8%), though there was no difference between males (9.3%) and females (8.6%).

Interactions

There was not a significant interaction between VDR and CASR in the overall model. Significant interactions were observed between VDR and adiposity (p=0.006), as well as between CASR and both sex and (p=0.0018) dietary calcium (p=0.0306). Thus, the VDR model (in addition to ethnic stratification in line with HWE results) was stratified according to adiposity for further analysis, and CASR was stratified by both sex and median calcium intake (838 mg; this model also controlled for overall energy intake).

REE and VDR (Table 3)

The model stratified by ethnicity indicated a positive association of 'A' allele presence and REE in AA, which was marginally significant in EA, and was not significant in HA. After stratification of the model by adiposity, this association was significant and was only in those characterized as having normal body fat.

REE and CASR (Table 4)

For CASR, there was no genotypic association with REE in the overall model. However, after stratifying by sex, there was a significant association genotype and REE in presence of the 'A' allele was associated with greater REE in females; however, in males this relationship was inverse and was only marginally significant. Stratification based on median calcium intake indicated that in those with high calcium intake the pres-

ence of the ‘A’ allele of CASR was associated with a greater REE, which was not significant in those with low calcium intake. Further stratification by both sex and median calcium intake indicated differential relationships. In males with low calcium intake, the presence of the ‘A’ allele was associated with lower REE, whereas in females with high calcium intake, the presence of the ‘A’ allele was associated with greater REE.

DISCUSSION

The calciotropic network influences a host of metabolic processes, many of which likely alter REE (*44*). Because the capacity for maintaining, sensing and absorbing calcium is at least in part under genetic control (*45*), investigation of potential influential genetic variants may provide some insight into metabolic consequences in terms of energy requirements. To date, associations of major fundamental genetic variants involved in calcium handling have not been investigated in terms of REE. Because this was an important consideration in children undergoing pubertal development, a formative period in which effects on metabolic health and body composition may be greatly impacted, we evaluated the association of VDR Cdx-2 and CASR A986S variants with REE. We observed VDR minor 'A' allele presence (A/A or A/G) was associated with greater REE in AA, and particularly among normal weight females. In addition, an association of the CASR minor 'A' allele presence (A/A or A/C) with REE varied based on sex and calcium intake. These findings suggest some degree of inherent capacity for calcium utilization, potentially by modulation of REE.

The observed positive association between the VDR Cdx-2 polymorphism and REE supports the hypothesis that through alteration in the affinity for the transcription factor-binding site, and subsequent VDR binding strength and transcription, the 'A' allele influences REE. In theory, circulating calcium concentrations increase due to greater intestinal binding capacity, thus providing greater availability of Ca^{2+} for energy-dependent processes (e.g. bone mineralization) and up-regulation of REE (*46-48*). In our study, greater REE among carriers of the 'A' allele supports the findings of greater intestinal calcium absorptive capacity. Based on the capacity of adipose tissue to sequester circu-

lating vitamin D (*49*), a nutrient integral for intestinal calcium absorption, the observation of the association of the 'A' allele of VDR with REE in only individuals characterized as having normal adiposity is not surprising. Indeed, an inverse relationship between vitamin D status and adiposity has been frequently reported (*50-52*), and excess fat has been associated with an imbalance of the calciotropic network, in which bone calcium resorption may be increased with excess adiposity (*53*). Thus excess fat accrual may impair the potential for greater calcium-absorptive capacity by the 'A' allele of VDR genotype. In addition, this association was significant in AA only, with a marginal significance in EA. This may be due to the fact that the highest level of mean fat mass was among HA, paralleling the observed absence of association among those with excess fat mass. Calcium intake was also the highest among HA, followed by EA, with AA having the least mean intake, potentially resulting in an overriding effect of calcium intake on associations between VDR and REE. In addition, racial/ethnic differences in calcium handling have been extensively noted in the literature, with AA being in general being less responsive to effects of PTH elevation in response to low circulating calcium, with greater calcium retention in comparison to EA and HA (*54-56*).

An association between CASR A986S polymorphism and REE was apparent, demonstrating sex-specificity in terms of directionality (albeit not significant in the overall sample). In females, REE was greater among those with the 'A' allele. In males, the relationship was inverse. Although this was contrary to our hypothesis, it is not completely unexpected. Females, closer to completing longitudinal bone growth, were mostly in their peak growth velocity, as well as that of bone calcium accrual. Although the exact period when peak bone mass is achieved isn't entirely agreed upon, it is general

agreed that the maximal accrual of bone mineral density is acquired in the years surrounding puberty (*57*). It is commonly noted in the literature that females undergoing puberty gain a greater proportion of fat mass relative to males in preparation for reproductive capacity (*22;58*), thus it is not surprising that consequences related to adipose accrual would be most apparent in females. Further supporting this notion is the observation of a positive association of the CASR 'A' allele in females with upper levels of calcium intake, while this relationship was inverse in males with low calcium intake. This may also translate into a heightened significance of dietary adequacy in young males.

The existence of a synergistic interaction between VDR and CASR is a strong possibility since the VDR gene is upregulated by CASR. It has been reported that CASR activation increases its own expression as well as that of VDR, the latter of which serves to enhance vitamin D action, which further increases CASR expression and action, potentiating the cycle. However, our analyses failed to detect an interaction (data not shown). There were, however, significant interactions of VDR with adiposity and of CASR with sex and calcium intake, highlighting the associations reported herein.

Elucidation of factors contributing to calcium homeostasis and calciotropic network is important for optimal body composition during this critical period of growth and development. This is the first study to our knowledge investigating the association of genetic variants involved in calcium regulation with REE, particularly in growing children. Although most investigations regarding calciotropic influence on REE are centered on absolute calcium intake (*59-66*), the body's response to diet has a significant impact on REE. The robust methodology used to measure the physiologic and genetic variables of interest, along with the specific peri-pubertal age group in which they were measured,

serve as strengths of this study. However, limitations of the study are acknowledged. Given the exploratory nature of this study, due mainly to a relatively modest sample size and consideration of only two polymorphisms, the necessity to perform corrections for multiple testing as performed for large-scale studies (e.g. Bonferoni corrections) was precluded. The level of significance which would have been indicated for these analyses would be 0.005 and we did not have the sample size to attain significance at this level. The Bonferoni approach has been identified as a stringent approach (*67*) that in samples like ours could increase the Type II error rate, which will counteract its purpose to reduce Type I error rate. Thus, findings may serve as a basis for future related investigations. Although both acute and chronic consequences of calcium status underlie the hypothesized contributing mechanisms through which genetic influence regulates REE, it is difficult to establish a true cause-effect relationship; nevertheless, our cross-sectional data provides valuable insight. Future studies of longitudinal design will be useful in further understanding relationships described herein.

In conclusion, these findings suggest that the VDR Cdx-2 and CASR A986S variants are associated with REE in peri-pubertal children, and the mechanisms underlying these associations are likely variably driven. Association with REE is likely adiposity- and ethnic-specific for VDR genotype, whereas sex and calcium intake seem to influence the association of REE and CASR genotype. Metabolically active processes associated with reproductive onset may impact genes involved in calcium regulation, reflected by differential findings of associations with REE. Future studies are warranted to replicate these findings in larger populations regarding influence of genes involved in calcium regulation on REE during this influential period in body composition trajectory.

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Table 1. Sample characteristics (overall and by sex and ethnicity)

	Overall (n=294)	Males (n=154)	Females (n=140)	EA (n=116)	AA (n=94)	HA (n=84)
Age (yr)	9.6 ± 0.1	9.8 ± 0.1[a]	9.3 ± 0.0[b]	9.6 ± 0.2	9.6 ± 0.2	9.4 ± 0.2
Pubertal stage	1.49 ± 0.04	1.38 ± 0.05[a]	1.62 ± 0.07[b]	1.35 ± 0.06[a]	1.76 ± 0.09[b]	1.40 ± 0.07[a]
Height (in)	54.9 ± 0.2	55.2 ± 0.3	54.6 ± 0.4	55.02 ± 0.39[ab]	55.57 ± 0.41[a]	54.06 ± 0.46[b]
Weight (kg)	36.8 ± 0.6	37.3 ± 0.8	36.1 ± 0.7	35.35 ± 0.81	37.69 ± 1.04	37.67 ± 1.10
BMI percentile	66.8 ± 1.5	66.8 ± 2.1	66.8 ± 2.2	66.6 ± 2.5[a]	64.6 ± 2.9[a]	77.9 ± 2.1[b]
Total % Fat	23.5 ± 0.6	21.1 ± 0.8[a]	26.2 ± 0.7[b]	22.3 ± 0.8[a]	20.8 ± 1.0[a]	28.0 ± 0.9[b]
Fat mass (kg)	9.0 ± .3	8.4 ± 0.5[a]	9.6 ± 0.4[b]	8.13 ± 4.7[a]	8.38 ± 6.7[b]	10.84 ± 6.4[a]
BMC (g)	1287.4 ± 18.9	1319.3 ± 25.7‡	1252.0 ± 27.6‡	1230.0 ± 25.9[a]	1395.8 ± 38.0[b]	1248.6 ± 33.1[a]
Lean mass (kg)	25.7 ± 0.3	26.7 ± 0.4[a]	24.5 ± 0.4[b]	25.3 ± 0.5[a]	27.2 ± 0.6[b]	24.8 ± 0.6[a]
European admix-ture	0.54 ± 0.02	0.54 ± 0.03	0.53 ± 0.03	0.96 ± <0.01[a]	0.14 ± 0.01[b]	0.39 ± 0.02[c]
Fasting insulin	12.6 ± 0.4	11.7 ± 0.5[a]	13.6 ± 0.6[b]	10.7 ± 0.4[a]	12.9 ± 0.6[b]	14.7 ± 0.9[b]
REE (kcal/d)	1192.7 ± 13.7	1239.8 ± 19.8[a]	1140.9 ± 17.8[b]	1181.7 ± 22.3	1190.2 ± 21.4	1210.7 ± 27.9
Calcium intake (mg/d)†	860 ± 19	862 ± 24	855 ± 25	874 ± 26[a]	745 ± 28[b]	968 ± 30[c]
Energy intake (kcal/d)	1898 ± 27	1956 ± 37[a]	1835 ± 39[b]	1880 ± 43	1891 ± 47	1932 ± 50

[a,b,c]superscripts represent significant difference between groups ($p<0.05$), † controlled for overall energy intake, ‡$p=0.07$

EA=European American, AA=African American, HA=Hispanic American, BMC=bone mineral content, REE=resting energy expenditure

Table 2. Allele and genotype frequency of vitamin D receptor (VDR) and calcium-sensing receptor (CASR) polymorphisms in the total sample and according to ethnicity

	Overall (n=273)	EA (n=108)	AA (n=87)	HA (n=78)	Males (n=145)	Females (n=128)
Genotype frequency						
VDR						
A/A	67 (24.5%)	8 (7.4%)	50 (57.5%)	1 (1.3%)	26 (17.9%)	33 (25.8%)
A/G	73 (26.7%)	33 (30.6%)	32 (36.8%)	23 (29.5%)	51 (35.2%)	37 (28.9%)
G/G	133 (48.7%)	67 (62.0%)	5 (5.7%)	54 (69.2%)	68 (46.9%)	58 (45.3%)
CASR						
A/A	1 (0.4%)	1 (0.9%)	0 (0%)	0 (0%)	0 (0%)	1 (0.8%)
A/C	28 (10.3%)	29 (26.9%)	6 (6.9%)	9 (11.5%)	24 (16.6%)	20 (15.6%)
C/C	244 (89.4%)	78(72.2%)	81 (93.1%)	69 (88.5%)	121(83.4%)	107 (83.6%)
Allele frequency						
Gene						
VDR						
A	38.9%	22.9%[a]	75.9%[b]	16.5%[a]	35.6%	40.3%
CASR						
A	8.8%	14.4%[a]	3.4%[b]	5.8%[b]	9.3%	8.6%

EA=European Americans, AA=African Americans, HA=Hispanic Americans; [a,b]superscripts represents significant difference between groups; VDR=Vitamin D Receptor, CASR=Calcium Sensing Receptor

Table 3. Association between VDR recessive genotype and resting energy expenditure (REE)

Model[†]	Group	N	β	p-value
By Ethnicity	EA	97	-0.060	0.0679
	AA	77	-0.111	0.0425
	HA	78	-0.037	0.2836
By Adiposity[‡]	Normal	172	-0.039	0.0481
	Excess	76	0.029	0.5174

[†]controlled for sex, pubertal stage, European admixture, fasting insulin, fat mass index (fat in kg divided by height in m^2), lean mass, and calcium; [‡]normal: <25% for males and <30% for females

Table 4. Association between CASR recessive genotype and resting energy expenditure (REE)

Model[†]	Group	N	β	p-value
Overall		261	-0.011	0.6273
By Sex	Males	140	0.062	0.0736
	Females	123	-0.079	0.0142
By Median Calcium	Low	129	0.023	0.4310
	High	134	-0.076	0.0578
By Sex and Median Calcium[*]	Males/Low	62	0.086	0.0494
	Males/High	78	-0.020	0.7391
	Females/Low	67	-0.043	0.2761
	Females/High	56	-0.127	0.0172

[†]controlled for sex, pubertal stage, European admixture, fat mass index (fat in kg divided by height in m^2), lean mass, and calcium; [*]Low: <838mg, model also controlled for overall energy intake

GENERAL DISCUSSION

Despite the substantial contribution of REE to overall energy balance, underlying factors which alter energy utilization pathways at rest remain relatively unexplored, with investigations centered on growth and development even more limited. Body composition largely influences REE, as tissue compartments comprising absolute total body mass vary greatly regarding energy maintenance requirements. The sentinel events of puberty, that is maturation of the skeleton and reproductive system, provide the foundations of metabolic phenotypes and body composition trajectories. Accordingly, identification of influential factors which may serve to optimize development (i.e. maximize skeletal accrual, while limiting adiposity) is essential for long-term energy balance.

Calcium represents a factor intricately involved in energy utilization at the cellular, tissue and systemic levels. Multiple physiologic mechanisms largely involving hormone signaling respond to circulating serum calcium levels to ensure its homeostatic maintenance. Diet is the major contributor to serum calcium, with additional influence provided by inherent genetic variation. Together, these obligatory function mechanisms impact body tissue compartmentalization and ultimately REE. The overall objective of this work was to elucidate the interrelationships of dietary, physiologic and genetic calciotropic factors with REE.

We sought to identify the associations among dietary intake, REE and body fatness (Aim 1), the underlying physiologic basis of the relationship between calcium homeostasis and REE according to calciotropic hormones (Aim 2) and the integration of these two based on inherent calcium handling capacity as represented by selected genetic polymorphisms previously demonstrated to influence calcium absorption and metabolism (Aim 3).

Aim 1

REE is known to be relatively high in children compared with adults, likely due to differences in oxidative requirements of the tissues needed for growth and development (*70*). Increased REE translates into increased energy requirement. Theoretically, metabolic alterations that minimize positive energy flux by creating a greater caloric need have the potential to result in less fat accumulation over time. In this context, a positive association between dietary calcium and REE could have a positive impact on long-term weight maintenance. With this premise, it was hypothesized that dietary calcium would be positively associated with REE, and that both dietary calcium and REE would be inversely associated with total body fat. These findings reveal a positive association between calcium intake and REE. Whereas there was no direct correlation of calcium intake and total body fat, structural equation modeling revealed REE as an indirect mediator of the two variables. Stratification by sex revealed a sexual dimorphism in the association such that the associations observed in the overall sample were apparent only in males. Among females, the only significant relationship identified was a positive relationship between REE and total body fat.

Our findings were in part contrary to that hypothesized. The concurring positive association calcium intake and REE was expected, however that of REE with body fat, and mediation by REE of a *positive* association of calcium with body fat was not expected. Although we do not have direct evidence, it may be rationalized in that adiposity has been reported to parallel measures of bone (*15*). As bone is mineralized rapidly throughout growth, sufficient calcium intake allows for optimal development, which to an extent may be mirrored by adipose accrual. However, conflicting reports exist on the

relationship between bone and adiposity, as adiposity has been reported to uncouple the bone remodeling process, thereby inhibiting normal development of bone (*15*). In addition, hormones associated with energy substrate utilization which regulate intracellular calcium likely also contribute. Consistent with this, associations of both PTH and 25OHD with changes in fat mass and fat oxidation have been reported[(71)]. The impact of calcium levels and vitamin D status on PTH may in turn mediate the systemic effects of these dietary nutrients, but potential relationships with energy metabolism have not been well examined, warranting investigation of independently- and/or interactively-acting contributing physiologic factors (Aim 2).

The reasons for sex-specific relationship are not entirely clear, but may be mediated by differential hormonal milieu (albeit direct evidence is again, limited). Estrogen is known to drive fat deposition [(72)], whereas testosterone is known to drive lean mass [(73),] each physiologically elevated in females and males, respectively, during pubertal development. During pubertal growth, the bone-muscle interface predominates in males, whereas in females, the bone-fat interface predominates (*74*). It is also plausible that differences in diet composition may have contributed to disparate findings. Although males had a greater overall caloric intake and no difference in calcium intake compared to females, males have greater variability in calcium retention by intake possibly attributed to the lack of hormonal fluctuations associated with the menstrual cycle in girls (*31*). Though no absolute differences in calcium intake was apparent by sex, it is conceivable that calcium intake values could possibly have deviated to a different degree in relation to physiologic requirements. The disparate findings between males and females are likely indicative of inherent differential underlying physiology, however, the evaluation of

hormonal differences in explaining the relationship between calcium, REE and body composition in boys and girls deserves further exploration.

Aim 2

Alterations in hormonal regulatory mechanisms involved in the calciotropic network responding to nutrient availability plausibly exerts effects on body tissue partitioning (i.e, proportions of lean, fat and bone mass), and thus REE. As such, our next hypothesis was that the cumulative effect of dietary and circulating factors within the calciotropic network would be associated with REE. Specifically, greater lean and bone mass as well as calciotropic dietary nutrient intakes would be associated with higher REE, and hormones targeting calcium repletion would be associated with lower REE. The relationships among metabolism, body composition and dietary adequacy may be of even greater importance when considering level of body fatness given the purported antagonistic relationship between bone and fat cells, both derived from a common precursor (*75*). In line with our hypotheses, bone and lean mass were significantly and positively associated with REE, whereas there was no significant relationship between fat mass and REE. An independent positive association of dietary vitamin K, and inverse associations of PTH and OC (albeit marginal significance) with REE were observed. Stratification by percent fat cut-points (</≥30, the standard value for females at which it is considered to be in excess (*76;77*) (rendered differential relationships, where as a positive relationship of dietary vitamin K and REE was found in individuals with normal adiposity level only. In addition, a positive relationship between PTH and REE was found in those having normal adiposity level, which was inverse in those with excess adiposity.

Signaling of metabolic information among tissue compartments (i.e. adipose, muscle, bone) responsible for production, storage and utilization of fuel resources is fundamental for energy expenditure coordination. The inverse relationship of PTH with REE was in line with our hypotheses, as PTH is increased in the face of low serum calcium targeting bone resorption in effort to restore level in circulation. A positive relationship between OC with REE was expected based on data in adults (*78;79*), however these disparate findings may be explained by the complexity of the hormone itself, as carboxylation status renders its bioactivity. In its fully carboxylated form, OC confers calcium-binding capacity of bone; however, in its undercarboxylated form, OC has been shown to act as a negative regulator of fat mass, and positive regulator of energy expenditure. The unexplored contribution of OC to energy balance in children warrants consideration, as the growth process itself may impart significant effects on metabolic action. Vitamin K was the only nutrient independently associated with REE, indicative of its involvement in bone mineralization and energy utilizing pathways. Vitamin K is integral in skeletal calcium binding properties, as well as partitioning of resources (i.e., osteogenesis or adipogenesis). Though conflicting findings have been reported (*80*), vitamin K has been linked with bone turnover markers in adults and children in states of both health (*81*) and disease (*82*) through its relationship with OC, and in this manner may affect energy metabolism. In context, dietary nutrient adequacy of those involved in calcium homeostasis is of importance in terms of resource partitioning and normal physiologic functioning.

In subjects with adiposity level beyond that which is considered normal, the attenuation of the relationship of REE with vitamin K suggests the underlying mechanism

may be perturbed upon excess adipose tissue, potentially via uncoupling of normal bone processes (*83*). As vitamin K is required for bone mineralization, subsequent REE would plausibly be attenuated. During pubertal-related bone remodeling, PTH increases energy-dependent mineral apposition (*84*), which may also be altered according to adiposity level by the same mechanism(s) driving the relationship between vitamin K and REE. Regulatory factors exerting effects on calcium homeostasis are complex and interwoven, and associated metabolic cost appears to link with body composition and ultimate metabolic health.

Aim 3

As SNPs in the VDR (*85;86*) and CASR (*87*) region have been shown to regulate nutrient absorption, transport and excretion through reliance upon binding ability of specific metabolites to respective receptors our final hypothesis was that genetic polymorphisms at loci previously demonstrated to regulate calcium homeostasis would be associated with REE. As VDR was not in Hardy-Weinberg equilibrium among the entire sample, yet was within ethnic/racial groups, analyses were run accordingly. Indeed, the 'A' allele of VDR was associated with greater REE in AA, which was marginally significant in EA, and was not significant in HA. After stratification of the model by adiposity, this association was only in those characterized as having normal body fat. Similarly, the 'A' allele of CASR was associated with REE, however the association differed by sex and calcium intake, such that in females with higher calcium intake this relationship was positive, whereas in males the relationship was inverse.

These findings suggest an association of inherent capacity for calcium utilization with energy required to maintain resting metabolic function, and seem to be dependent to

an extent on level of adiposity and dietary calcium adequacy. The observed positive association between the 'A' allele of VDR Cdx-2 polymorphism and REE supports the hypothesis that through alteration in the affinity for the transcription factor-binding site, and subsequent VDR binding strength and transcription, this SNP may exert influence on REE. A greater REE in those with the 'A' allele supports the plausibility of greater intestinal calcium absorptive capacity with its presence. However, as the contribution differed by adiposity level, excess fat accrual may mask the transference of greater calcium-absorptive capacity. Whilst the reproductively more mature females (likely undergoing relatively rapid adipose accrual with concurrent bone mineralization), similarly aged males overall gain less adipose tissue and more steadily gain bone mineral content. The differential racial/ethnic associations may lie in differences in calcium handling extensively noted in the literature, with AA reported to be less responsive to effects of PTH elevation in response to low circulating calcium, with greater calcium retention in comparison to EA and HA (*34*). Additionally, the mean fat mass was greatest among HA, paralleling the observed absence of association among those with excess fat mass.

Like the relationship with VDR, it is plausible that puberty-related sexual dimorphism in body composition trajectory also contributed to the association between CASR and REE. As there were no adiposity-specific association in males, adiposity was a significant contributor to calcium handling among young females. It is commonly noted in the literature that females in the period surrounding puberty are gaining a greater proportion of fat mass relative to males in preparation for reproductive capacity (*2;3*), thus it is not surprising that consequences related to adipose accrual would be most apparent in females. The opposed findings amongst males based upon median calcium in-

take may translate into a heightened significance of dietary adequacy, and/or a difference in calcium handling capacity with closer proximity to puberty, in this group. As there were no adiposity-specific association in males, adiposity seems to be a driving contributor to calcium handling among young females, whereas absolute calcium intake may be of more relevance than adiposity in males.

These findings suggest that the VDR Cdx-2 and CASR A986S variants are associated with REE in peri-pubertal children, and the mechanisms underlying these associations are likely variably driven. Association with REE is likely adiposity- and ethnic-specific for VDR genotype, whereas sex and calcium intake seem to influence the association of REE and CASR genotype. Metabolically active processes associated with reproductive onset also likely have indications regarding genotypic influence on calcium regulation, and, are worthy of consideration when investigating the relationships with REE. Investigating the impact of specific genetic factors involved in the determination of REE in this cohort contributes to our understanding of physiologic consequences associated with calcium regulation throughout growth and development.

Summary

Energy balance is complex and interactive, with substantial influence provided by REE. This effort provides preliminary evidence that the calciotropic network plays a role in energy balance via effects on REE. It is no surprise that calcium, as the main mineral comprising the skeleton and as an important chemical messenger in a multiplicity of metabolic functions, has been hypothesized to influence REE, particularly at the tissue level. Adequacy of dietary micronutrients involved in the calciotropic network contributes to greater REE hypothetically via allowance for maximal obligatory functioning capacity in terms of bone mineralization, lean tissue maintenance and fat oxidation. Alterations in the calciotropic network affecting these processes set the stage for relatively lowered energetic demand, indirectly feeding back on energy homeostasis. It is common that calcium and related dietary nutrients are insufficient throughout what is potentially the most sensitive lifestage, peri-puberty. It is this stage that body composition trajectory is established. Indeed, the highly metabolically active tissue bone reaches its peak in terms of mass during pubertal growth. Fat mass accrual, particularly in girls, is also occurring at an accelerated rate during this stage. The interactions among calciotropic genes, hormones and dietary nutrients during growth affect a range of cellular, tissue and systemic factors underlying intrinsic metabolic demand, and appear to link with body composition.

Future Studies

Taken together, the cumulative findings from the three investigations (Figure 2) provide the backbone for future studies, emphasizing the importance of achieving dietary adequacy for optimal growth trajectories, particularly throughout sensitive windows of

development. Initial support for assertions regarding calciotropic influence on REE was provided by the elucidation of mediation by REE on the relationship between calcium intake and body fat through utilization of complex statistical modeling (Aim 1). Hypotheses generated from this aim served as a basis for continuing investigations, and represent the evolution of this dissertation work. The need for inclusion of other dietary and physiologic aspects of the calciotropic network was the impetus for the subsequent study (Aim 2), identifying relationships of REE with lean and bone mass with, as well with vitamin K and PTH. These additional findings of significant influential calciotropic factors added strength to the proposed hypotheses, and indicated potential effects of sexual dimorphism related to linear growth as well as a differential impact by race/ethnicity. This then led to the need to investigate inherent factors, which comprised the final study of this work (Aim 3). Investigation of a genetic contribution added an additional layer to the interwoven relationships presented, and initiated speculation of potential epigenetic effects. When piecing together these findings, a comprehensive approach is necessitated, taking into account contribution of

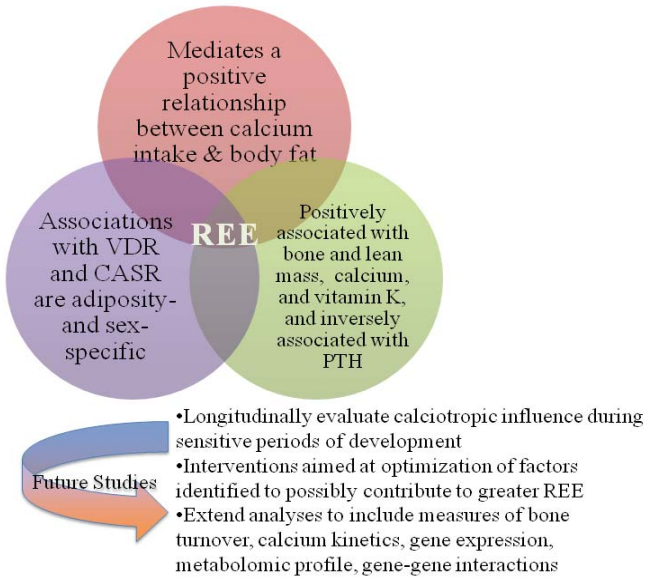

Figure 2. Future directions implied by specific aim-related findings

multiple factors including diet, genes, sex, ethnicity and adiposity level, representing multi-level influence.

Previous studies have suggested that there is a critical window for calcium utilization in growth-related processes; however, these studies were conducted in animals (*88*) or based on mathematical modeling using limited human data (*42;89*). To our knowledge this is the first study supporting a direct link of specific calciotropic micronutrients, hormones and SNPs with REE and body composition during peri-puberty, when the most impact is likely to be manifest. If the calciotropic network has a role in crucial aspects of development, specifically in terms of enhancing bone development and limiting fat mass accrual, then understanding physiologic consequences of regulatory factors is paramount during this stage, and may serve as a guide for future intervention studies. Though the findings of our data are based upon cross-sectional analyses and direct influence cannot be implied, in conjunction with previous investigations centering calcium regulation, a firm foundation for future studies is formed. Directive efforts implied by aim-related findings include the following: longitudinal evaluation of calciotropic influence during sensitive periods of development, interventions aimed at optimization of factors identified to possibly contribute to greater REE, and extension including measures of bone turnover, calcium kinetics, gene expression, metabolomic profile, as well as gene-gene interactions. By these targeted efforts, it may be possible to quantify the magnitude to which a) REE is altered, b) bone mass is accrued, and C) fat mass accrual is limited, based upon varying calciotropic-related dietary, physiologic and genetic factors. Further investigative efforts regarding influence of calcium regulation on REE during perhaps the most sensitive lifestage will be valuable for understanding the relation between the calci-

otropic network and current and future maximal energy utilizing capacity and body composition patterning.

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APPENDIX

IRB APPROVAL FORM

OMB No. 0990-0263
Approved for use through 1/31/2012

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule. Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [ ] CONTINUATION [X] EXEMPTION	[ ] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	
4. Title of Application or Activity Calciotropic Hormonal Influence on Energy Homeostasis (Cancer Preventioin & Control Training Program)		5. Name of Principal Investigator, Program Director, Fellow, or Other HANKS, LYNAE J

6. Assurance Status of this Project *(Respond to one of the following)*

[ ] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 09/29/2013 IRB Registration No. IRB00000196

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[X] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph 4.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[ ] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [ ] Full IRB Review on (date of IRB meeting) _____ or [ ] Expedited Review on (date) _____
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments	Title E110124002
Protocol subject to Annual continuing review.	Calciotropic Hormonal Influence on Energy Homeostasis (Cancer Preventioin & Control Training Program)

IRB Approval Issued: 4/18/11

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: smoore@uab.edu	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
14. Name of Official Sheila Moore, CIP	15. Title Director, IRB
16. Signature Sheila Moore, CIP	17. Date 4/18/11

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