A candidate-gene based approach for assessing genetic predisposition to childhood obesity

VASILIKI LAGOU

SUBMITTED FOR THE DEGREE OF
DOCTORATE OF PHILOSOPHY
IN THE FACULTY OF SCIENCE,
UNIVERSITY OF GLASGOW

PhD July 2007

Declaration

I hereby declare that this thesis has been composed by myself, that the work of which it is a record has been done by myself except where assistance has been acknowledged, that it has not been submitted in any previous application for a higher degree and that all sources of information have been specifically acknowledged by means of references.

Some of the results contained in this thesis have been submitted in a peer-reviewed journal as follows:

V. Lagou[1], Y. Manios[2], C.N. Moran[1], R.H. Wilson[1], M.E.S. Bailey[1], E. Grammatikaki[2], E. Oikonomou[2], E. Ioannou[2], G. Moschonis[2], Y.P. Pitsiladis[1] (2007) Developmental changes in adiposity in toddlers and preschoolers in the GENESIS study and associations with the *ACE I/D* polymorphism. *International Journal of Obesity*. 31(7): 1052-60.

Some of the results contained in this thesis have been presented in conferences as follows:

V. Lagou, Y. Manios, E. Grammatikaki, E. Oikonomou, E. Ioannou, G. Moschonis, G. Dedoussis, C. Vassilopoulos, M.E.S. Bailey, R.H. Wilson, C.N. Moran, Y.P. Pitsiladis. Impact of ACE polymorphisms on obesity-related phenotypes in Greek children aged 0-6 years. *European Congress of Obesity*. Athens, 2005.

V. Lagou, R.A. Scott, Y. Manios, T.L. Joshua Chen, C. Wang, E. Grammatikaki, E. Oikonomou, E. Ioannou, G. Moschonis, Y.P. Pitsiladis. Impact of peroxisome proliferator-activated receptor γ and δ on adiposity in toddlers and preschoolers in the GENESIS study. *17th Annual Meeting of European Childhood Obesity Group*. Athens, 2007.

Table of Contents

Acknowledgements

I would like to thank first and foremost my family for funding my PhD and for their endless love and patience during all these years of study, which have kept me away from them. I would also like to thank my husband, Felipe, for his constant love, encouragement, patience and the nice breaks to Brazil. Without them, I wouldn't be able to complete this long journey.

I would like to thank my supervisor, Dr. Yannis Pitsiladis, for his encouragement, for giving me the opportunity to carry out this very interesting project and for securing funding for the expensive lab work.

I thank Dr. R.H. Wilson and my assessor, Dr. M. Bailey, for the many stimulating discussions and their excellent scientific advice.

I thank Dr. C. Moran for his excellent technical assistance in the lab and for his catalytic help on the statistical analysis.

I thank Tun-Li for helping me with the genotyping during experiment 3.

I thank the members of CESAME and in particular Mrs. Heather Collins for their support and empathy.

I thank Dr. Y. Manios and his group from Harokopeio University of Athens for recruiting the subjects and for their help in the collection of DNA samples.

I would like to acknowledge the MRC/BBSRC associate programme in human nutrition research for partly funding this research. My final thanks go to all those subjects whose enthusiastic participation made this work possible.

Summary

The main aim of the present series of experiments was to assess the influence of selected candidate genes on several adiposity-related phenotypes in a large cohort of toddlers and preschoolers from Greece using both genotype- and haplotype-based approaches. Investigating the impact of genetic polymorphisms on adiposity in the young may reveal stronger associations than in older ages since the environment has had less time to take effect. Anthropometric measurements and buccal cell samples, from where genomic DNA was extracted, were obtained from 2374 children aged 1-6 years, all in public and private nurseries in Greece.

The aim of the first experiment (Chapter 3) was to assess developmental changes in adiposity in the present population of Greek toddlers and preschoolers. This was achieved by determining the prevalence of overweight and obesity in this population using specific BMI cut-off points based on UK and US national reference data. On average and irrespective of gender, 13.6% and 10.2% of children were found to be overweight and obese, respectively. These rates of obesity were comparable with those found in other national studies with high prevalence of overweight and obesity. When compared to the normative data from British children, Greek children were much taller and heavier and they showed excess BMI with advancing age. The present cohort of Greek children also showed gender- and age- related differences in several anthropometric indices, but similar patterns of growth as US preschool children with increased adiposity.

The aim of the second experiment (Chapter 4) was to assess the potential influences of *ACE* I/D polymorphism and its interaction with age and/or gender on adiposity-related phenotypes in the cohort of Greek toddlers and preschoolers. A significant main effect of the *ACE* I/D polymorphism on BMI and significant interactions between I/D genotype and age for the same phenotype were revealed. In boys and girls 1-2 years, the II genotype was

significantly associated, in a D-dominant fashion, with higher BMI. This association accounted for 6.4% of the variance and was not in accordance with previous studies in adolescents and adults. However, in the 4-6 years age group, subjects (mainly girls) with the DD rather than II genotype had higher BMI confirming the significant interaction between age and I/D genotype. The age, at which this alteration in the effect of *ACE* I/D polymorphism on adiposity was observed, coincided with the age at which BMI increased after its developmental nadir. These results suggest that the *ACE* I/D polymorphism associates with developmental changes in adiposity during early childhood in an age- and possibly gender-specific manner.

The aim of the third experiment (Chapter 5) was to investigate the potential influence of five genetic polymorphisms in *ADRB* genes on adiposity-related phenotypes in the Greek children and their potential interactions with age and/or gender. Significant main effects of *ADRB2* C16 on waist and hip circumferences and significant interactions between C16 genotypes and age for the same phenotypes were revealed. Significant interactions were also observed between C16 genotypes and gender. In boys and girls aged 4-6 years, the Gly16 allele was significantly associated in an additive fashion with higher waist circumference and this association explained 1.2% of the variance. As far as *ADRB2* C27 polymorphism is concerned, a significant main effect of C27 genotypes on hip circumference and significant interactions between these genotypes with age and gender for the same phenotype were observed. In boys, the Gln27 allele was significantly associated, in an additive fashion, with higher hip circumference at the age of 2-3 years that explained 6% of the variance. No significant main effects of *ADRB1* C49 and C389, as well as *ADRB3* C64 polymorphisms on adiposity-related phenotypes were found. Haplotype-based analysis did not reveal stronger associations compared to individual genotypes. *ADRB2* gene polymorphisms have subtle effects on adiposity in early childhood and these influences are manifested in a gender- and age-related manner.

The aim of the fourth and final experiment (Chapter 6) was to assess the influence of two polymorphisms in *PPARγ* gene on adiposity-related phenotypes in the Greek toddlers and preschoolers. Genetic analysis based on Pro12Ala genotypes revealed that the rare Ala12 and T1431 alleles had no significant main effect on adiposity-related phenotypes. Interactions with age and/or gender were either not significant. A synergistic effect of *PPARγ* Pro12Ala and *ADRB3* Arg64Trp polymorphisms, as well as a modulating eefect of BMI on the establishment of associations previously observed in adults was not confirmed in this study. Haplotype-based analysis including both *PPARγ* polymorphisms revealed no stronger associations of *PPARγ* diplotypes with adiposity-related indices compared to individual genotypes. Variation in the *PPARγ* seems not to contribute significantly to the high prevalence of early-onset obesity possibly due to differences in the dietary composition between children and adults.

The findings from the genetic analyses suggested that allelic variations in candidate genes simply predispose to the obesity phenotype. With well-conducted genetic studies and with thorough examination of the information with respect to genetic associations, progress in understanding and management of obesity can be foreseen.

List of Figures

List of Tables

Table of abbreviations

ACE	angiotensin I-converting enzyme 1
ACS	Acyl-CoA synthetase
ADRB	β-Adrenergic Receptor
ADBR1	β_1-Adrenergic Receptor
ADBR2	β_2-Adrenergic Receptor
ADRB3	β_3-Adrenergic Receptor
ANOVA	One-way Analysis Of Variance
αP2	Adipocyte fatty-acid binding protein
AT1	Angiotensin II Type 1 receptor
AT2	Angiotensin II Type 2 receptor
BMI	Body Mass Index
bp	base pair(s)
CAMP	cyclic-Adenosine Monophosphate
CI	Confidence Interval
D	Pairwise-disequilibrium coefficient
D'	Normalised estimate of linkage disequilibrium
DNA	Deoxyribonucleic Acid
EDTA	EthyleneDiamineTetraacetic Acid
ENPP1	EctoNucleotide Pyrophosphatase Phosphodiesterase
ERK	Extracellular signal-regulated kinase
GLM	General Linear Model
GPCR	G-protein coupled receptor
HSL	Hormone Sensitive Lipase
HWE	Hardy-Weinberg Equilibrium
IOTF	International Obesity Task Force
G_I	adenylyl cyclase inhibitory G protein
GENESIS	Growth, Exercise and Nutrition Epidemiological Study In preschoolers
G_S	adenylyl cyclase stimulatory G protein
Kg	kilogram(s)
LD	Linkage Disequilibrium
LPL	Lipoprotein Lipase

MAP	Mitogen Activated Protein
µl	microlitre(s)
µM	MicroMolar(s)
min	minute(s)
mRNA	messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
NHANES	National Health and Nutrition Examination Survey
P	Probability
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
pmol	picomole(s)
PPAR	Peroxisome Proliferator-activated Receptor
PPARγ	Peroxisome Proliferator-activated Receptor-γ
PPARγ$_1$	Peroxisome Proliferator-activated Receptor-γ$_1$
PPARγ$_2$	Peroxisome Proliferator-activated Receptor-γ$_2$
PPARγ$_3$	Peroxisome Proliferator-activated Receptor-γ$_3$
PPARγ$_4$	Peroxisome Proliferator-activated Receptor-γ$_4$
PPREs	Peroxisome Proliferator Response Elements
QTL	Quantitative Trait Locus (or Loci)
R	Correlation coefficient
r^2	Correlation of determination
RAS	Renin-Angiotensin System
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
rs	reference SNP identification number
RXR	Retinoid X Receptor
sec	second(s)
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SISA	Simple Interactive Statistical Analysis website
SNP	Single Nucleotide Polymorphism
U	Unit of enzymic activity
UTR	Untranslated Region

Chapter 1

General Introduction

1.1 Childhood obesity

Obesity can be defined as an excess accumulation of body fat that leads to increase risk of morbidity and/or premature mortality (Reilly, 2005). The health consequences of childhood obesity can be categorized into short- and long-term effects and include psychosocial ill health, asthma, increased cardiovascular risk factors such as hypertension, dyslipidaemia and insulin resistance, as well as persistence of obesity and cardiovascular risk profiles into adulthood (Dietz, 1998;Reilly, 2005). Previous reports indicate a dramatic increase in the prevalence of overweight and obesity in recent decades (Ebbeling et al., 2002). Rates have increased 2.3-fold to 3.3-fold in the USA from 1971 to 1994 and 2.0-fold to 2.8-fold in England from 1984 to 1994 (Ebbeling et al., 2002). Increasing obesity rates are not only observed in Europe (Lissau, 2004) and in the USA (Nicklas et al., 2001) but also in other countries such as China and Egypt (Ebbeling et al., 2002) and there is general agreement that this is due to a changing environment, which favours sedentary lifestyles and over-consumption of high energy-dense foods rather than changing genetic influences (Hill et al., 2003).

A consistent positive correlation between child and adult overweight and obesity has been seen with both parents of obese children being obese in almost 30% of the cases (Bouchard, 1997). Family and twin studies have shown heterogeneous estimates of heritability for BMI (Body Mass Index) and body fat distribution ranging between 10% to 80% (Bouchard and Perusse, 1988;Bouchard, 1995) and supported the notion of a strong genetic component in the development of obesity that involves the synergistic interaction between a number of genes important for adipogenesis and adipose tissue metabolism (Marti et al., 2004). Variation in obesity-related phenotypes cannot soley be attributable to genetic effects, but also environmental or lifestyle influences such as overfeeding (Koivisto Hursti, 1999) and lack of exercise (Meirhaeghe et al., 1999). Nevertheless, the alarming rates of obesity have occured in a constant pool of genes and thus, the influence of

environmental or behavioural changes on adiposity becomes progressively stronger (Prentice and Jebb, 1995). In Britain, for example, the consumption of a high fat diet has increased by 50% over a period spanning from 1940 to 1990, while the increase of physical inactivity, as indicated by proxy measures such as car ownership and hours of television viewing, seems to be the primary cause for the observed changes in the prevalence of obesity (Prentice and Jebb, 1995). However, these genetic and environmental interactions can exert a variable effect on adiposity with age as indicated in longitudinal studies (Maes et al., 1997) and this effect has been postulated to be due to different gene expression profiles at different ages, or to age-related variability in exposure to environmental influences (Maes et al., 1997). Moreover, genetic influences on phenotype may be stronger in young individuals, as the environment or behaviour has less time to take effect than at later ages (Maes et al., 1997) and therefore the study of young children becomes crucially important when attempting to quantify the extent of genetic predisposition to obesity.

The preschool years have been identified as a critical time for the development of adiposity later in life, since crucial behavioural and developmental processes occur during this period. In particular, habits related to food intake and physical activity become established and a decrease in BMI, preceding adiposity rebound, is observed (Dietz, 1997). Body composition undergoes marked changes during the first decade of life in absolute and relative proportions of water, lipid, protein and mineral mass; at birth, for example, approximately 80% of lean tissue is water, declining to approximately 75% at the end of the first decade (Fomon et al., 1982). Quantifying the main body components (i.e. body fat, lean mass, muscle mass, skeletal frame) is integral to the study of obesity in children (Pietrobelli et al., 2003) and studying quantitative traits that show high heritability and can be measured easily and accurately is particularly advantageous in large cohort studies (Newton-Cheh and Hirschhorn, 2005). BMI for age is a well-established diagnostic tool for obesity and its diagnostic accuracy depends on whether national BMI reference data or the international (IOFT) definitions of overweight and obesity in children are used (Reilly,

2006), Although it cannot distinguish between fat and lean mass (Wells and Fewtrell, 2006), several studies have shown good correlation between BMI and more direct fat measures obtained by methods that are highly precise and more accurate but have limited applicability to studying large populations (i.e. dual-energy X-ray absorptiometry and imaging techniques) (Pietrobelli and Tato, 2005). Skinfolds in the arm (biceps and triceps) and in the trunk (subscapular and suprailiac) are reliable indices of subcutaneous fat, while waist and hip circumferences are good predictors of abdominal fat (Pietrobelli and Tato, 2005;Wells and Fewtrell, 2006). Waist-to-hip ratio is a less accurate predictor of visceral fat and mortality compared to waist circumference in children (Fredriks et al., 2005), while equations for the prediction of total fat mass from skinfolds are inappropriate as they may be valid only in the population from which they were derived and they confound skinfold raw values with predictive error (Wells and Fewtrell, 2006).

1.2 Variation in obesity-related phenotypes

Obesity is a multifactorial trait and variation in an obesity-related phenotype, known as total phenotypic variance, is determined by both genetic and non-genetic factors (Bouchard, 1995). Total phenotypic variance can be described as the sum of genetic variance, environmental and lifestyle influences and gene-environment interactions (Bouchard and Perusse, 1988). Genetic variance results from the influence of the diverse genetic variants on adiposity (Marti et al., 2004;Rankinen et al., 2006)and can be further subdivided into additive, dominance and interaction variances generated by the additive, dominant and epistatic effects of alleles on the phenotype respectively, while environmental variance represents variation in diet habits or exercise levels. A gene-environment effect refers to a phenotype for which the response to an environmental influence is significantly influenced by the genotype (Moran et al., 2005). There are several types of variation in the coding and non-coding regions of the human genome, such as microsatellites (short tandemly repetitive DNA), short deletions and insertions, that

account for the inter-individual genetic differences and genomic variability has been shown to be population-specific with Africans showing greater gene diversity than Europeans or Asians indicative of the recent migration patterns and relationships between various populations (Stephens et al., 2001). SNPs are the most abundant (90%) and stable single-base changes in the human genome, occuring with an average frequency of approximately 1 every 1,000 nucleotides (nucleotide diversity index) (Brookes, 1999;Twyman and Primrose, 2003) and as such, they have facilitated the ability to map genes of complex diseases (Gray et al., 2000;Bhatti et al., 2006). Different alternatives (alleles) exist at these single base pair positions with the least frequent allele showing a frequency of 1% or more (Brookes, 1999). The frequency of SNPs in coding regions is fourfold lower compared to that in noncoding, with about 50% of them resulting in variation at the aminoacid level (non-synonymous changes) (Brookes, 1999;Twyman and Primrose, 2003). SNPs can affect the protein function if they occur in the coding or regulatory regions of the gene, but in many cases they show no functional relevance (Gray et al., 2000). For example, a single-nucleotide substitution (S127L) in the melanocortin-4 receptor gene has been found to alter the signalling properties of this G protein-coupled receptor resulting in severe early-onset obesity (Valli-Jaakola et al., 2004).

In human populations, functional and neutral variants can be associated by LD, which is the non-random association (co-segregation) of alleles at different loci (Goldstein, 2001) that arises because the variants share a joint population ancestry (Zondervan and Cardon, 2004). Genome-wide patterns of LD can vary significantly between populations as a result of natural selection and local variability in recombination rates, as well as genetic drift and population admixture (Cardon and Bell, 2001;Goldstein, 2001)and European populations generally show lower nucleotide diversity and greater LD than African populations (Ardlie et al., 2002). Considering *ACE (angiotensin I-converting enzyme 1)* gene as an example, a functional polymorphism has been identified in very strong LD with another marker in Caucasian populations (Soubrier et al., 2002). However, the same polymorphisms were not

found to be in tight LD in Nigerian families (Cox et al., 2002), suggesting that levels of LD in *ACE* gene differ in European and African populations. Furthermore, there is a great heterogeneity in the distribution of LD across the genome with GC-poor sequences, for example, showing stronger LD due to low recombination rates (Tiret et al., 2002) and genomic regions with consistent patterns of LD show reduced numbers of haplotypes; these are defined as groups of specific SNP alleles at multiple loci that tend to be co-inherited and are only rarely disrupted by recombination events (Weiss and Clark, 2002). Although there is a strong correlation between the level of LD and physical distance, physically close markers are not always in LD with each other (Ardlie et al., 2002;Tiret et al., 2002). Therefore, the selection of the appropriate SNPs based on the levels of LD is crucially important in the design of complex disease association studies, since genotyping of a few SNPs can be sufficient to represent most of the diversity in a genomic region (Goldstein, 2001)

1.3 Genetic analysis of childhood obesity

Currently, the two dominant strategies for identifying and locating genetic variants that contribute to obesity are linkage analyses and association studies (Cardon and Palmer, 2003). Most of the complex traits tend to aggregate in families, but rarely in a classic Mendelian fashion (Mayeux, 2005). Linkage analyses search for regions of the genome that co-segregate with the disease in many independent affected families or over many generations in an extended pedigree (Ardlie et al., 2002;Carlson et al., 2004)taking advantage of the strong correlation between markers in close proximity (Risch, 2000). Although linkage analysis has been, together with positional cloning, a powerful tool in identifying genetic loci for single-gene disorders, it may be less reliable for the study of complex diseases due to high false-positive rate (Risch, 2000) and less power in identifying susceptibility genes that have small effects (less than 5-10% of the variance) or weak genotype-phenotype correlation (Uhl et al., 1997;Risch, 2000). Given the fact that

the genetic element in complex traits is comprised of multiplex genetic variants each contributing a small effect (Marti et al., 2004), association studies can have more power in detecting genetic effects than linkage analysis, when the candidate gene has already been identified (Gray et al., 2000). Association studies can be broadly broken down into family-based (extended pedigrees, relative-pairs, parent-child trios, nuclear families) and population-based studies (case-control, cohort-based) (Cardon and Palmer, 2003). Family-based association studies are more suitable for studies of very rare conditions. Case-control studies compare exposure to risk genetic or environmental factors between affected subjects and unrelated healthy controls to find associations between risk factors and disease risk in relatively rare conditions (Clayton and McKeigue, 2001;Zondervan and Cardon, 2004), while cohort-based studies mainly attempt to associate polymorphic variants in candidate genes with influences on the phenotype of interest (Clayton and McKeigue, 2001)

To date, a limited number of population-based association studies have been performed on the influence of specific genetic variants on adiposity in young children, despite evidence suggesting that studying young cohorts can in some circumstances reveal genotype-phenotype associations more effectively than in adults, since confounding effects of the environment have had less time to take effect (Maes et al., 1997). Case-control studies in French children have shown that variation in the adiponectin encoding gene was associated with severe childhood obesity (Bouatia-Naji et al., 2006), while genetic variability in *ENPP1* (EctoNucleotide Pyrophosphatase Phosphodiesterase) and specifically, one three-allele risk haplotype was associated with chromosome 6q-linked childhood obesity (Meyre et al., 2005). Furthermore, a cross-sectional study on Scottish children has revealed that the *PPARγ* Pro12Ala polymorphism had smaller effects on BMI in younger ages compared to adult populations (Cecil et al., 2006). Other studies, however, have failed to reveal associations between genetic polymorphisms and increased adiposity in childhood. For example, a case-control study in a French population have shown that the Pro12Ala

polymorphism in *PPARγ* gene was not associated with childhood obesity (Ghoussaini et al., 2005), while variants in the gene encoding for uncoupling protein-1 were not found to contribute to juvenile-onset obesity in subjects of Danish ancestry (Urhammer et al., 1997). Similarly, the Bogalusa Heart cohort-based study, which has assessed the longitudinal effects of an *ADRB2* (β_2-Adrenergic Receptor) genetic variant on adiposity from childhood to adulthood, has detected no genetic effects on obesity in childhood (Ellsworth et al., 2002), while the PPARγ Pro12Ala variant was found not to contribute significantly to early onset obesity in a German population (Hamann et al., 1999).

According to the aforementioned examples, population-based association studies are widely applied for characterizing genetic determinants contributing to obesity in young children; however, in many cases they may suffer from several limitations that lead to inconsistency of the association data. During the case-control approach, although cases and controls are readily available and can efficiently be genotyped, several confounders such as gender, age and ethnicity differences (population stratification) between cases and controls can lead to spurious associations (Risch, 2000). Focusing on homogenous and randomly mating populations can overcome confounding by ethnicity, although such populations may be more of a theoretical ideal than a reality (Risch, 2000;Cardon and Bell, 2001). Furthermore, association studies, widely used to tackle the genetics of complex diseases, are usually plagued by the lack of reproducibility due to poor study design, overinterpretation of the findings and incorrect assumptions regarding the underlying genetic architecture (Cardon and Bell, 2001). For example, the power of association studies to detect significant genetic effects diminishes significantly with decrease in LD (Risch, 2000), while in cases of low LD, the required increase in sample size is inversely proportional to the estimate of LD (Zondervan and Cardon, 2004). Moreover, sample size is considered to be particularly important in association studies for generating robust data (Cardon and Bell, 2001). Considering, for example, the Pro12Ala polymorphism in *PPARγ* gene, a large study of 3000 subjects was necessary to confirm the association of the Pro12

allele with increased risk for type II diabetes and demonstrate that this genetic effect was more modest than originally described (Altshuler et al., 2000). Therefore, taking into consideration the modest effects of genes on the development of obesity, the tendency to analyse subgroups of subjects to accommodate age- or gender-related differences and the assessment of the genetic influence on phenotypes of variants with uncertain LD from the functional variants, sample sizes of 1000 to 10000 individuals may be necessary for revealing genuine associations (Cardon and Bell, 2001).

1.4 Candidate genes for obesity

Candidate genes selection can be based on the physiological role of their protein products in pathways crucial for the development of the disease. However, this approach can be unsatisfactory due to current incomplete knowledge about functional variants (Suh and Vijg, 2005). Alternatively, findings from linkage analyses and mouse models can be useful for the implication of putative genes (Suh and Vijg, 2005). There have been more than 420 findings of positive associations between obesity phenotypes and genetic variation in over 127 candidate genes in children and adult populations (Rankinen et al., 2006). *ACE*, *ADRB* (β-Adrenergic Receptor) family and *PPARγ* were among the putative genes widely studied for influences in obesity-related phenotypes.

1.4.1 ACE gene

ACE is a key component of RAS and converts angiotensin I (vasoinactive) into a biologically active hormone, angiotensin II (vasoconstrictor) (Bernstein et al., 1989) (Figure 1.1). *ACE* also has the ability to hydrolyze numerous other peptide substrates (Hooper and Turner, 2003). In humans, angiotensin II effects are mediated predominantly through two specific receptors, namely AT1 (Angiotensin II Type 1 receptor) (vasoconstrictor responses) and AT2 (Angiotensin II Type 2 receptor) (vasodilator

responses), which are G-protein coupled receptors in the plasma membrane (Crisan and Carr, 2000;Danser, 2003). Tissue RAS systems with all the necessary components for angiotensin II synthesis, have been also identified in a number of peripheral tissues including the adipose, the cardiac and the skeletal muscle tissue (Engeli et al., 1999), suggesting a paracrine action of the RAS (Lavoie and Sigmund, 2003). In adipose tissue, angiotensin II may play an important role in growth and differentiation, blood flow and lipolysis; however the exact effects of this hormone on adipose tissue are not yet established (Goossens et al., 2003). Furthermore, no effect of angiotensin II stimulation or *ACE* inhibition on lipolysis was observed in human adipocytes, suggesting that the RAS may serve more as regulator of the regional blood flow to adipose tissue rather than lipolytic regulator (Townsend, 2001).

The *ACE* gene is highly polymorphic, with over 100 polymorphisms listed in SNPs database (www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusID=1636). Historically, work is focused on *ACE* I/D (insertion/deletion) polymorphism, which involves the presence (insertion, allele 'I') or absence (deletion, allele 'D') of a 287bp (base pairs) *Alu* repeat sequence in intron 16 (Rigat et al., 1990). This polymorphism accounts for almost half of the variance in serum *ACE* activity, with homozygotes for the D allele showing higher plasma *ACE* levels than individuals with the ID (intermediate *ACE* levels) or II (lower *ACE* levels) genotypes (Rigat et al., 1990). However, the association between *ACE* activity levels and the I/D polymorphism was found to be weaker in people of African origin (Cox et al., 2002). Therefore, the I/D polymorphism may not be the causal variant for the variation of *ACE* activity but may be in strong LD with the causal variant in Caucasians and not so strong LD with the causal variant in Africans. This notion is also supported by the identification of 17 variant sites in tight LD with the *ACE* I/D polymorphism in 11 individuals, (5 African-American and 6 European-American) and the inference of 13 haplotypes suggestive of allelic heterogeneity (Rieder et al., 1999).

Previous studies have examined the possible association between the *ACE* I/D polymorphism and obesity in adult and adolescent populations with somewhat contrasting findings. Associations between RAS and obesity were assessed in a group of 449 Jamaican individuals, but no consistent differences between *ACE* genotypes and BMI were found (Cooper et al., 1997). However, the *ACE* I/D polymorphism was associated with variation in *ACE* levels with obese individuals having significantly higher serum *ACE* and angiotensinogen levels. In a study of 155 obese Korean women, no significant association between the *ACE* I/D polymorphism and obesity was found (Um et al., 2003). Other studies have demonstrated significant associations between the *ACE* I/D polymorphism and increased adiposity. In particular, the DD genotype was significantly associated with overweight and abdominal adiposity in adult Italian men (Strazzullo et al., 2003) and with obesity and abdominal fat deposits in a Spanish population with coronary heart disease (Riera-Fortuny et al., 2005). More recent data on Greek adolescents have also shown that the DD genotype is associated with greater triceps and subscapular skinfolds than the other I/D genotypes, but only in females who are relatively inactive, suggesting a gender-specific effect of *ACE* I/D polymorphism on obesity-phenotypes, as well as an interaction between I/D genotypes and physical activity (Moran et al., 2005).

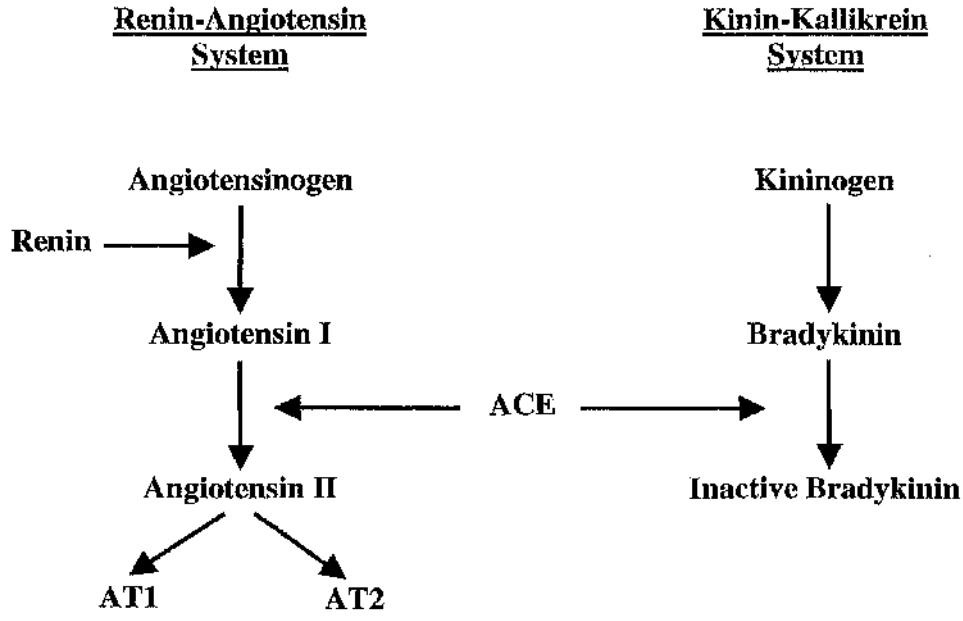


Figure 1.1 Role of *ACE* in the RAS and Kinin-Kallikrein systems (adapted from Crisan and Carr, 2000).

1.4.2 ADRB genes

Among the promising candidate genes for obesity are members of the *ADRB* family: *ADRB1* (β_1-Adrenergic Receptor), *ADRB2* and *ADRB3* (β_3-Adrenergic Receptor). *ADRBs* are G-protein coupled receptors in the plasma membrane and, typically of this superfamily, they have an extracellular N-terminus, seven transmembrane-spanning regions, three intracellular and three extracellular loops and an intracellular C-terminus (Green et al., 1993). *ADRB* are differentially expressed in several tissues throughout the body; *ADRB1* is expressed in cardiac muscle (inotropy and chronotropy), adipose tissue and kidney (renin secretion) (Mason et al., 1999), *ADRB2* in many cell types including adipocytes and *ADRB3* predominantly in adipose tissue (Small et al., 2003;Leineweber et al., 2004). In white and brown adipose tissue, *ADRBs* promote lipid mobilization and thermogenesis in a cascade of reactions (Collins et al., 2004). In particular, endogenous catecholamines (adrenaline and noradrenaline) stimulate the beta-adrenoceptors on the surface of

adipocytes, which signal to the interior of cell via the Gs (stimulatory) proteins that activate adenylyl cyclase. As a result, the levels of intracellular cAMP (cyclic-Adenosine Monophosphate) (a signaling molecule) are increased and subsequently PKA is activated. PKA phosphorylation of HSL (Hormone Sensitive Lipase) is followed by its activation and translocation to the lipid droplet and lipolysis stimulation (Figure 1.2) (Gonzalez Sanchez et al., 2003). *ADRB3* receptor can couple to both Gs (adenylyl cyclase stimulatory G protein) and Gi (adenylyl cyclase inhibitory G protein) (inhibitory) proteins leading to the simultaneous transduction of two independent signaling pathways, PKA and ERK/MAP (Extracellular signal-regulated kinase/ Mitogen Activated Protein) kinase respectively (Collins et al., 2001). This simultaneous activation may promote a more potent stimulation of lipolysis, since both pathways result in HLS phosphorylation in white adipose tissue (Soeder et al., 1999).

Single-nucleotide polymorphisms within the coding region of the *ADRB* genes can affect the expression and function of the gene/receptor and may contribute to the development of several disorders, such as hypertension, asthma, congestive heart failure or obesity (Small et al., 2003;Leineweber et al., 2004). In *ADRB1*, two nonsynonymous polymorphisms were identified, one in the extracellular N-terminus resulting in substitution of serine for glycine at codon 49 (Gly49Ser; C49) (Maqbool et al., 1999) and one between the seventh transmembrane-spanning domain and the intracellular tail of the receptor resulting in substitution of glycine for arginine at codon 389 (Arg389Gly; C389) (Maqbool et al., 1999;Mason et al., 1999). Due to their location, Gly49Ser (rs1801252) and Arg389Gly (rs1801253) polymorphisms can affect receptor trafficking or expression and Gs-coupling respectively. In particular, functional *in vitro* studies have shown that the Gly49 variant was susceptible to enhanced agonist-promoted down-regulation due to decreases in mRNA, while the Arg389 variant exhibited a greater agonist-promoted stimulation of adenylyl cyclase than Gly389 receptor (Leineweber et al., 2004). In *ADRB2*, a total of nineteen variable sites have been identified in the coding region (11 SNPs) and in the 5'

UTR (8 SNPs) upstream from the ATG start codon (Leineweber and Brodde, 2004). Among them, three non-synonymous polymorphisms have been identified in the coding region (Drysdale et al., 2000) and have been shown to have functional significance *in vitro* and *in vivo* (Small et al., 2003); work however, is usually focused on two of these SNPs in the extracellular region of the receptor, which result in substitution of arginine for glycine at codon 16 (Gly16Arg; C16, rs1042713) and glutamine for glutamic acid at codon 27 (Glu27Gln; C27, rs1042714). Functional studies *in vitro* have revealed that these N-terminus polymorphisms resulted in different properties of agonist-promoted down-regulation (Gly16 receptor displaying enhanced down-regulation and Glu27 receptor being resistant to such desensitization) and these differences accounted for by altered susceptibility to receptor degradation (Green et al., 1994). Moreover, the Gly16Glu27 and Gly16Gln27 receptors exhibited similar levels of down-regulation between them *in vitro* and both higher when compared to the Arg16Gln27 double mutant receptors (Green et al. 1994). One major SNP has been described in *ADRB3* (Clement et al., 1995;Walston et al., 1995;Widen et al., 1995) located either to the most distal residue within the first transmembrane spanning domain or the most proximal residue of the first intracellular loop resulting in a substitution of arginine for tryptophan at codon 64 (Trp64Arg; C64, rs4994). The functional significance of this polymorphism though has yet to be determined, since conflicting results regarding the Arg64 receptor's ability to promote cAMP accumulation (Leineweber et al., 2004). Numerous studies have examined the relationship between these *ADRB* polymorphisms and adiposity-related phenotypes. Despite the evidence from *in vitro* studies of Arg389Gly being a gain-of-function polymorphism, the Arg389 rather than the Gly389 allele has been associated with higher body weight and BMI, as a result of increased fat in Caucasian women (Dionne et al., 2002), whereas this polymorphism was found to have no effect on lipolysis regulation (Ryden et al., 2001). Furthermore, the *ADRB1* Gly49 allele, showing increased *in vitro* desensitization, was associated with greater long-term weight-gain and adult-onset overweight in women (Linne et al., 2005).

In *ADRB1*, genotyping of the Gly49Ser and Arg389Gly polymorphisms allows the inference of three haplotypes, namely Ser49Arg389, Ser49Gly389 and Gly49Arg389, which represent the majority of variation along the complete length of the gene. All four alleles in *ADRB2* gene have been previously associated with increased adiposity. For example, in Swedish women the Glu27, but not the Gly16 allele, was associated with increased body fat. In the same population, the Gly16Arg polymorphism was associated with altered *ADRB2* function, with Arg16 carriers showing a five-fold decrease in agonist sensitivity (Large et al., 1997). In contrast, men, but not women, homozygous for the Gln27 allele showed higher scores for obesity-related phenotypes and if they were also carriers of the Arg16 allele, the risk for obesity was increased (Meirhaeghe et al., 2000b). Twelve distinct haplotypes were identified in a population of 4 major ethnic groups (Drysdale et al., 2000). Genotyping of *ADRB2* C16 and C27 polymorphisms revealed the occurrence of three haplotypes, namely Arg16Gln27, Gly16Glu27 and Gly16Gln27, in these populations. As far as *ADRB3* Trp64Arg polymorphism is concerned, although the Arg64 variant has been initially associated with abdominal obesity and insulin resistance, as well as increased capacity to gain weight, lower age of onset of non-insulin-dependent diabetes mellitus and a lower metabolic rate (Clement et al., 1995;Walston et al., 1995;Widen et al., 1995), further population-specific and meta-analysis studies, albeit in obese and diabetic subjects, revealed conflicting results, with approximately one half of them reporting positive associations and another half failing to do so (Small et al., 2003;Leineweber et al., 2004).

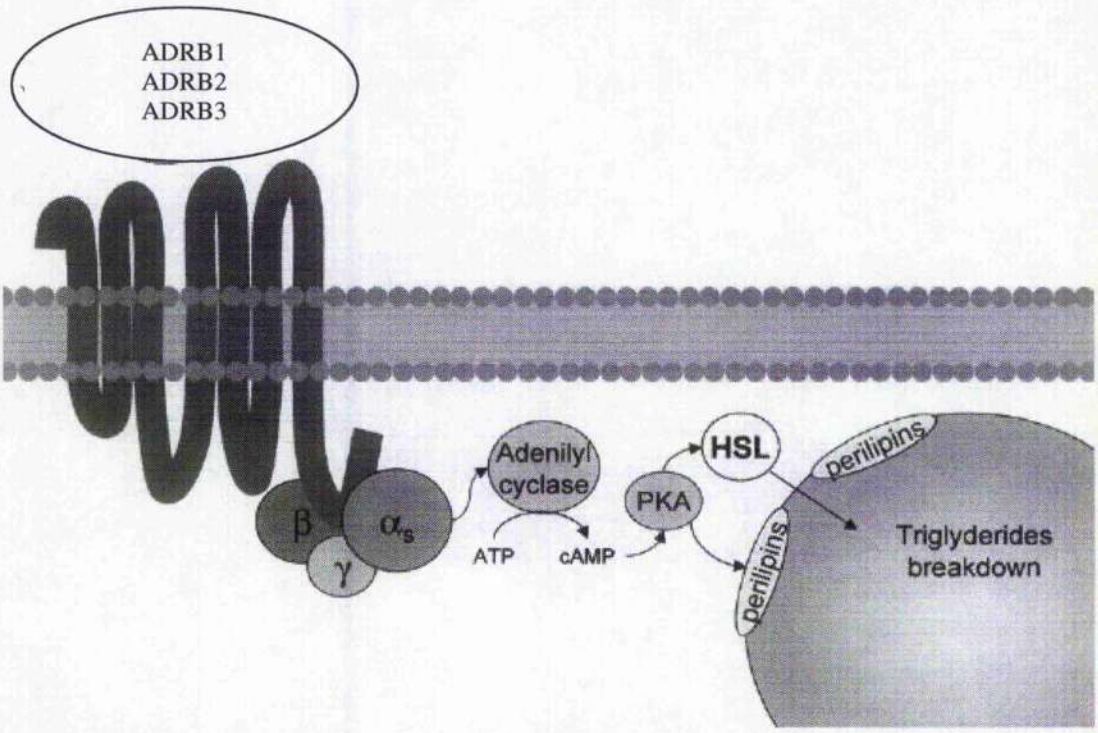


Figure 1.2 Signal transduction cascades of the *ADRB1*, *ADRB2* and *ADRB3* receptors for lipolysis regulation by catecholamines. The three *ADRBs* are coupled to the trimeric Gs, which consists of α, β and γ subunits, and they transmit an activation signal to adenylyl cyclase resulting in increased cAMP production. Then a cAMP-dependent protein kinase (PKA) is activated and leads to phosphorylation and activation of HSL and perilipin A in adipocytes (adapted from González-Yanes and Sánchez-Margalet, 2006).

1.4.3 PPARγ gene

PPARs constitute a subfamily of the nuclear hormone receptor gene superfamily and they have a key role in energy metabolism (Evans et al., 2004). *PPARγ* is a ligand-regulated transcriptional factor that controls transcription of target genes, such as aP2 (Adipocyte fatty-acid binding protein), LPL and ACS (Acyl-CoA synthetase), linked to lipid metabolism and energy balance by binding to PPREs located in the promoter or enhancer sites of these genes. *PPAR* heterodimerizes with the RXR to form a complex that binds to PPREs (Figure 1.3) (Martin et al., 1998;Gurnell, 2005). The activity of *PPARγ* is governed by the binding of small lipophilic ligands, mainly fatty acids derived from nutrition and

metabolism (Auwerx et al., 2003). Studies have shown that *PPARγ* is involved in several biological pathways, such as adipocyte differentiation and metabolism, as well as insulin sensitivity, type 2 diabetes, atherosclerosis, and cancer (Rosen and Spiegelman, 2001;Lehrke and Lazar, 2005). The importance of *PPARγ* in adipocyte differentiation is underlined by the finding that *PPARγ* can induce adipogenesis in fibroblasts (non-adipogenic cells), as well as myoblasts (Fajas et al., 1997). Moreover, targeted deletion of *PPARγ* in mice resulted in adipocyte hypocellularity (He et al., 2003), while heterozygous *PPARγ*-deficient mice have reduced adiposity (Barak et al., 1999;Rosen et al., 1999). In addition to that, antagonists for the *PPARγ* inhibited adipocyte differentiation (Wright et al., 2000). Similarly, humans with dominant-negative mutations in *PPARγ* showed partial lipodystrophy and severe insulin-resistance because of increased triglycerides and fatty acid deposition into skeletal muscle and liver (Savage et al., 2003). These findings make *PPARγ* a promising gene for susceptibility to obesity (Auwerx et al., 2003).

The *PPARγ* gene spans more than 100kb and contains 9 exons, which lead to 4 mRNA isoforms (*PPARγ1*, *PPARγ2*, *PPARγ3* and *PPARγ4*) by use of alternative promoters and differential splicing at the 5' end of the mRNA (Fajas et al., 1997). All four subtypes contain 6 common exons. *PPARγ1* contains also A_1 and A_2, *PPARγ2* exon B and *PPARγ3* only exon A_2. *PPARγ1*, *PPARγ3*, *PPARγ4* mRNAs give rise to the same protein (*PPARγ1*) encoding by exons 1-6, since exons A_1 and A_2 are untranslated. In *PPARγ2*, translation of exon B leads to the production of a protein (*PPARγ2*) with an additional 30 amino acids in the N terminus in humans (Fajas et al., 1997;Martin et al., 1998). The action of *PPARγ* is mediated by these two isoforms; *PPARγ2* is predominantly expressed in adipocytes, where it has a pivotal role in adipocyte differentiation and lipid accumulation (Tontonoz et al., 1994).

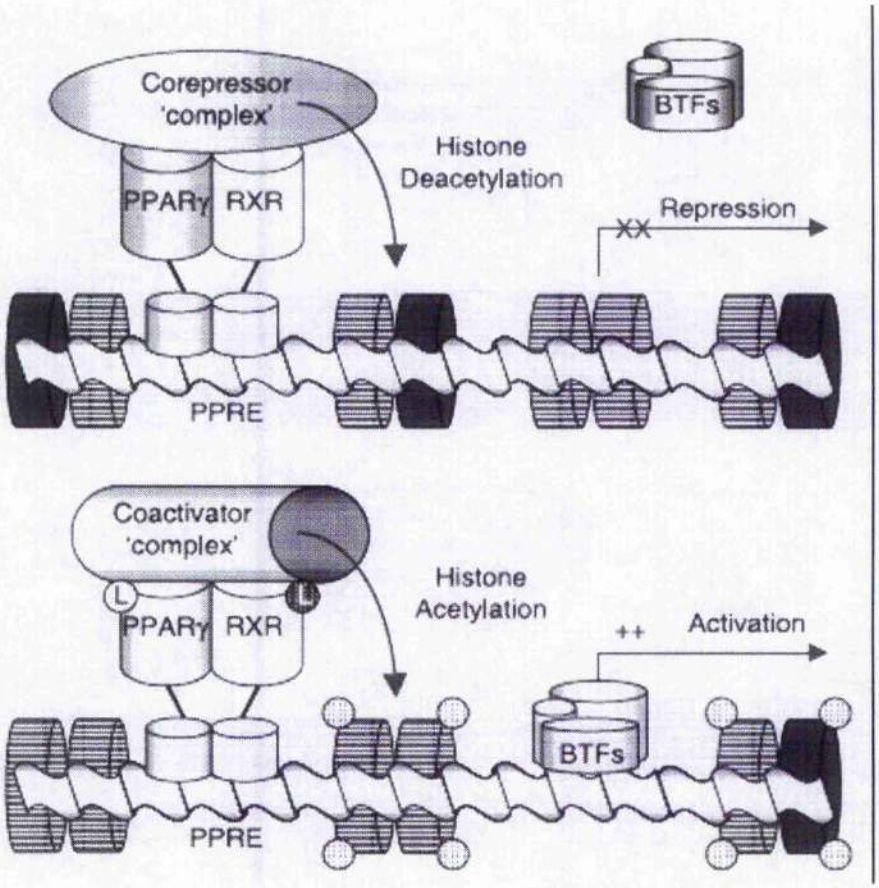


Figure 1.3 Function of *PPARγ*. In the absence of a ligand, *PPARγ* has the potential to actively silence genes, to which is bound, by recruiting transcriptional corepressor complexes. Binding of agonist ligands to *PPARγ* triggers a conformation change that results in release of corepressors and attracts transcriptional coactivator complexes, which promote gene transcription by altering the chromatin structure. These coactivators or corepressors exist in multiprotein complexes including histone-modifying enzymes. Acetylation of histone proteins is believed to relieve the tightly packed structure of the chromatin, allowing RNA polymerase II complex to bind and initiate transcription. RXR, PPRE, BTFs (Basal transcription factors) (adapted from Gurnell, 2005).

Several mutations and polymorphisms in *PPARγ* have been associated with adiposity (Meirhaeghe and Amouyel, 2004). A common structural polymorphism has been detected in the coding region of $PPAR\gamma_2$ gene consisting of a proline (Pro) to alanine (Ala) substitution (Yen et al., 1997) located at codon 12 (Pro12Ala). Functional *in vitro* studies have shown that Pro12Ala polymorphism induces a partial loss of function as a result of decreased DNA-binding affinity and reduced transcriptional activity (Deeb et al., 1998;Masugi et al., 2000). The Pro12Ala polymorphism has been extensively investigated for its association with obesity-related phenotypes in lean, obese and type 2 diabetic patients (Meirhaeghe and Amouyel, 2004). Inconsistent reports showed that the Ala12 allele is associated with a higher (Beamer et al., 1998;Valve et al., 1999;Meirhaeghe et al., 2000a) and a lower BMI (Deeb et al., 1998;Ek et al., 1999), whereas other studies found no association (Deeb et al., 1998;Mori et al., 1998;Hamann et al., 1999;Swarbrick et al., 2001). The effects of Pro12Ala polymorphism on adiposity have also been shown to be subject to modification by other genetic or environmental factors. For example, Hsueh et al. (2001) and Ochoa et al. (2004) have reported a synergistic contribution of *ADRB3* Trp64Arg and *PPARγ* Pro12Ala polymorphisms to obesity risk. Furthermore, variation in the dietary polyunsaturated-to-saturated-fat ratio was shown to influence BMI of Ala12 carriers, supporting the notion of gene-nutrient interactions at *PPARγ* locus (Luan et al., 2001). A second polymorphism has been detected in exon 6 at nucleotide 1431 of $PPAR\gamma_2$ resulting in a silent substitution from C to T (C1431T). Once again, conflicting results have been reported with the T1431 allele being associated with both higher (Valve et al., 1999;Doney et al., 2002) and lower BMI (Meirhaeghe et al., 1998;Knoblauch et al., 1999).

Research on the genetics of human obesity is continuing rapidly with the main goal being the identification of specific causative genes. According to the human Obesity Map (http://obesitygene.pbrc.edu), published each year, the number of candidate genes associated with human obesity rapidly increases with 113 candidate genes being reported in the 2004 update (Perusse et al., 2005) and 127 in the 2005 update (Rankinen et al.,

2006). These candidate genes, however, are not deterministic, since they are not sufficient individually to express the obese phenotype, but they only predispose individuals to particular traits. The current widely accepted hypothesis is that each susceptibility gene only has a modest effect on obesity-related phenotypes, while heterogeneity in complex phenotypes could suggest that rare genetic variants may also intervene in the predisposition to obesity (Marti et al., 2004). The large number of genes associated with obesity reflects the complexity of the disease and provides many additional challenges for scientists in identifying genetic variants implicated in the development of obesity. Although the candidate-gene approach has yielded intriguing insights into the genetics of human obesity, association studies often fail to provide convincing evidence of any involvement of a variant in the genetic predisposition to obesity. The reasons for the lack of replication of results in different populations are several, the most important being insufficient power to detect the modest genetic effects, unconsidered multiple testing effects, genotyping errors and publication bias (Clement, 2006). Other factors rendering the interpretation of genetic studies problematic include ethnic, gender or age differences that may influence the development of obesity (Clement, 2006). Designing the appropriate association studies using the correct analytical approach and appreciating the advantages and limitations of genetic methods as applied to complex diseases is crucial for defining genes involved in the development of obesity.

1.4.4 Hypotheses for the effects of each genotype tested on adiposity

1.4.4.1 ACE I/D polymorphism

Upon binding to AT1 receptor, angiotensin II has been shown to stimulate the production of prostacyclin *in vitro*. Prostacyclin, a local mediator of increased blood flow, regulates adipose tissue differentiation and increases the transcription rate of lipogenic enzymes

leading to elevating triglyceride content (Jones et al., 1997). As such, individuals with the DD genotype (higher ACE activity) and therefore higher plasma or tissue-specific angiotensin II will show higher adiposity (**Hypothesis 1**). Furthermore, ACE inhibitors and AT1 antagonists has been shown to lead to substantial weight loss and to reduce adipocyte size in rats fed with a fructose-rich diet. Nutritional status is known to regulate the expression of angiotensinogen with fasting resulting in reduced angiotensinogen levels and feeding in almost doubled. These effects are accompanied by effects in blood flow with reduced aniotensinogen expression leading to increased blood flow (less vasoconstriction) and *vice versa*. Based on these findings and on those from Cooper et al. (1997) study, which has found that angiotensinogen and ACE activity was higher in obese individuals, DD individuals would be expected to respond to feeding with higher adiposity compared to those homozygotes for the I allele. Finally, it has been demonstrated *in vivo* that reductions in locally produced angiotensin II through ACE inhibition were associated with parallel reductions in adipocyte leptin release and plasma leptin concentration under basal conditions (Cassis et al., 2004). However, elevations in systemic angiotensin II leads to sympathetic stimulation, which was shown to counterbalance the effects of locally produced angiotensin II leading to reduced leptin production (Cassis et al., 2004). Based on these findings, elevation in systemic angiotensin II (as expected in DD individuals) can increase sympathetic drive to adipose tissue, thereby decreasing leptin synthesis and release and resulting in higher adiposity.

1.4.4.2 Beta-adrenergic receptors

ADRBs has been found to promote lipolysis and thermogenesis in adipose tissue and mice lacking ADRBs were found to have a thermogenically inactive brown adipose tissue and were massively obese when fed with a diet high in fat and sucrose similarly to leptin-deficiency mice. SNPs affecting the expression or function of these receptors would thus have an effect on lipolysis or thermogenesis. Based on the findings from *in vitro* studies,

the ancestral *ADRB1*Gly49, *ADRB2*Gly16, *ADRB2*Glu27 alleles will be expected to be associated with higher adiposity due to a much faster densesitization in the presence of an agonist compared to their corresponding modern alleles, which signal for longer, while the ancestral form of *ADRB1*C389 (Arg389 receptor) will be expected to be associated with higher adiposity, as it signals at a higher level than its modern counterpart (Gly389) (Small et al., 2003) **(Hypothesis 2)**. Individuals with different alleles will respond differently to environmental stimuli, such as exercise and feeding **(Hypothesis 3)**. For example, a study in monozygotic twins have shown that subjects with the Gln27Gln genotype respond to 100 days of overfeeding and sedentary lifestyle with an increase in subcutaneous fat, whereas no *ADRB2* Gly16Arg genotype-specific responses were observed (Ukkola et al., 2001). In addition to that, inactivity was associated with higher BMI and waist and hip circumferences in Gln27Gln individuals than those bearing the Glu27 allele. In the same study, the inactive Gln27 carriers showed increased adiposity compared to active Gln27 carriers (Meirhaeghe et al., 1999), while in Greek adolescents the Gly16Gly genotype was associated with larger triceps in active compared to inactive subjects possibly due to Gly16 homozygotes responding less to the presence of catecholamines.

1.4.4.3 Peroxisome-proliferator activated receptor-γ

As far as *PPARγ* is concerned, it is known that it increases the expression of target genes, such as LPL and leads to higher production of fatty acids, which are directed into adipose tissue resulting in improved insulin-sensitivity in the muscle and increased triglyceride content in adipocytes due to increased fatty acid reesterification. Furthermore, synthetic ligands for *PPARγ*, such as TZDs, have been shown to increase appetite possibly due to the downregulation of leptin by *PPARγ* and treatment with these drugs has been associated with weight gain. A direct involvement of *PPARγ* in satiety or food intake though has yet to be confirmed. Although the Ala12 allele being associated with lower DNA binding and transcription activity would be expected to being associated with lower adiposity, it has

been postulated that this reduced activity may enhance the action of insulin leading to decreased lipolysis, increased accumulation of triglycerides in adipocytes and weight gain in the long term (**Hypothesis 4**). Furthermore, it has been shown that individuals with different Pro12Ala genotypes would respond differently to increased energy intake. For example, in a French-Canadian population, although Pro12 carriers had initially lower BMI, waist circumference and subcutaneous fat mass than Ala12 carriers, they respond to an increase in dietary fat with a gradual increase in BMI. This effect was not observed though in Ala12 carriers. In addition to that, the Ala12 allele was associated with lower BMI when subjects followed a diet enriched with unsaturated fatty acids, while it was associated with higher BMI when the ration of unsaturated to saturated fatty acids was lower (Luan et al., 2001).

1.5 Aims and objectives

In view of all the above, the main objectives of these experiments were:

a. Assess developmental changes in adiposity in a large cohort of Greek toddlers and preschoolers by:

   i. Estimating the prevalence of overweight and obesity by using specific BMI cut-off points based on national reference data and comparing these rates with findings from previous published reports.

   ii. Examining the evolution of various adiposity-related phenotypes with age and assessing differences in these growth curves between the two genders.

iii. Assessing gender and age-related differences in BMI, weight and height between the Greek population and normative data from British children of the same age obtained 2 decades ago.

iv. Assessing gender and age-related differences in other adiposity-related phenotypes between the Greek and similarly aged US children.

b. Assess whether variation within selected candidate genes can affect adiposity at different ages in a large cohort of Greek toddlers and preschoolers by:

i. Using a candidate gene approach: Candidate genes were *ACE*, *ADRB1*, *ADRB2*, *ADRB3* and *PPARγ* selected on the basis that they are involved in biological pathways crucial for the development of obesity.

ii. Using genotype- and haplotype-based approaches to better assess genetic variation effects on specific adiposity-related phenotypes in a large cohort of Greek toddles and preschoolers.

iii. Estimating the proportion of phenotypic variance explained by the genetic variants for significant genotype-phenotype associations and comparing this with the proportion of variance previously reported in adolescent and adult populations.

iv. Assessing the influence of genotype on the distribution of phenotypes for significant genotype-phenotype associations.

v. Discussing how significant findings could be explained biologically and relatively to the findings from *in vitro* and other cohort studies.

Chapter 2

General Methods

2.1 Subject population

The selected cohort comprised 2374 healthy children aged 1 to 6 years attending public and private nurseries, as well as day-care centers in five geographical districts of Greece: Athens (n=63), Aitoloakarnania (n=22), Thessaloniki (n=8), Halkidiki (n=12) and Helia (n=7). These counties are widely scattered across Greece and account for approximately 70% of the total Greek population (Manios, 2006). The sampling of the nurseries was random, multistage and stratified by the total population of children. The regions that took part in the study were classified as "large urban" (population >1,000000), "urban" (10,000-100,000), as well as "rural and small towns" (<10,000). The proportion of the total nursery population sampled was 55.5% (boys) and 56.8% (girls) in "large urban areas", 23.1% (boys) and 21.8% (girls) in "urban areas" and 21.4% (for both genders) in "rural areas and small towns". The prevalence of highly educated parents was higher in "large urban areas" compared to "urban areas" and "rural areas and small towns". Written informed consent to the participation of their children in the present study was obtained from all parents. The participation rate varied from 54% to 95%, with the highest rates being observed in rural areas and the lowest in urban areas (Manios, 2006). The study was approved by the Ethical Committee of Harokopio University of Athens and by all municipalities taking part in the study. There was no selection of subjects for inclusion on the basis of any phenotypic characteristic including health grounds, and the study is thus an unselected, cross-section of the general population in that age range.

2.2 Anthropometry

Subjects from each nursery school underwent a physical examination by two trained members of the research group in a classroom provided by the school. The protocols and equipment used for all measurements were the same for all regions. The physical examination included basic body composition measurements (weight, height, waist, hip

and mid-upper arm circumferences), as well as measurement of body fat at four sites (biceps, triceps, subscapular and suprailiac). Weight was measured to the nearest 10 g using a Seca digital scale (Seca Alpha, Model 770, Hamburg, Germany). Subjects were weighed in their underwear, without shoes. Recumbent length was measured to the nearest 0.1 cm in all subjects younger than 2 years without shoes using a portable measuring wooden board with a stationary headboard, a vertical moving foot piece and a horizontal back piece with a measuring tape on it (manufactured for the purposes of this study). Standing height was also measured to the nearest 0.1 cm in all subjects older than 2 years using a commercial stadiometer (Leicester Height Measure, Invicta Plastics Ltd., Oadby, UK). Weight and height were converted to BMI using Quetelet's Index (weight (kg) / height^2 (m^2)) (Garrow and Webster, 1985). Waist, hip and arm circumferences were measured to the nearest 0.1 cm using a non-elastic tape (Hoechstmass, Germany). Waist circumference was measured at the end of a gentle expiration after placing the measuring tape in a horizontal plane around the trunk, midway between the lower rib margin and the iliac crest. Hip circumference was measured at the point yielding the maximum circumference over the buttocks. Right arm circumference was measured at the midpoint of the upper arm, half the distance between the acromion and the olecranon. Biceps and triceps skinfolds were measured with the right arm hanging relaxed, while the skinfold was picked up about 1cm below the midpoint mark over the biceps and triceps muscle. Subscapular skinfold was measured with the shoulders relaxed. After identifying the inferior angle of the scapula, the skinfold was picked 1cm below the subscapular mark. Suprailiac skinfold was measured just above the iliac crest, along the axis of the anterior line. All skinfolds were measured on the right side of the body to the nearest 0.1 mm using a Lange skinfold caliper (Cambridge Scientific industries, Inc. Cambridge, Maryland, USA) and two repeated measurements were taken. Total skinfolds were calculated as the sum of the skinfolds at the four sites. The right side of the body was chosen for

anthropometry, as differences between left and right sides seem not to be biologically significant in prepubertal children (Moreno et al., 2002).

2.3 Genotyping, phenotyping and haplotype inference

2.3.1 DNA extraction

Buccal cell samples were obtained noninvasively by cytology brush (Medical Packaging Corporation, Camarillo, CA, USA) from 2102 subjects (1095 boys and 1007 girls) according to the manufacturer's recommendations and stored in eppendorf tubes containing cell lysis solution (0.1 M EDTA, 1% SDS, 0.1 M Tris HCl, pH 7.6). Genomic DNA was extracted using Nucleospin® Tissue columns for 373 samples and 96-Tissue purification kit (ABgene, Epsom, Surrey, UK) for the rest. Prior to mass extraction of genomic DNA from all samples, four different extraction methods were tested in 2 control samples. These methods were CST Genomic DNA purification kit (Invitrogen, Paisley, UK), Qiagen Blood DNA extraction kit (Qiagen GmbH, Hilden, Germany), Nucleospin Blood and Nucleospin tissue (Macherey-Nagel GmbH & Co. KG, Duren, Germany). The choice of the best DNA extraction method was based on the cost, as well as the assessment of the quality and the quantity of the extracted DNA, which was performed after separation of the DNA on 1.5% agarose gel. After purification, extracted DNA was stored in individual eppendorf tubes and 96-well Thermofast rigid semi-skirted plates (ABgene, Epsom, Surrey, UK) at -20°C.

2.3.2 Genotyping reactions

All PCR reactions were carried out on ice in 96-well Thermofast rigid semi-skirted plates (ABgene, Epsom, Surrey, UK) in a final volume of 25 μl (for *ACE* I/D genotyping), or 20 μl (for *ADRB* and *PPARγ* genes) comprised of ReddyMix™ PCR Mastermix containing

1.5mM $MgCl_2$ (ABGene, Epsom, Surrey, UK), 10 pmol of primers (Sigma-Genosys, Haverhill, Cambridgeshire, UK) (MWG-Biotech, Ebersberg, Germany), dH_2O and genomic DNA (approx. 100 ng). All digestions were also carried out in 96-well Thermofast rigid semi-skirted plates (ABgene, Epsom, Surrey, UK). Repeating genotyping in a randomly selected sample of 60 subjects assessed the validity of the genotyping for each polymorphism. Furthermore, after genotyping, allele frequencies were calculated for the total population to assess whether the observed genotypes and inferred diplotypes were in HWE (Hardy-Weinberg Equilibrium), using appropriate chi-square tests.

2.3.2.1 *ACE* gene

Primer design was based on NCBI reference sequence AF118569. The *ACE* I/D polymorphism was detected by PCR using a three-primer system; a forward primer recognizing the deletion (D) sequence, a forward primer specific for the insertion (I) sequence and a common reverse primer (Table 2.1). PCR reactions were performed using 10pmol of each of the three primers (Sigma-Genosys, Haverhill, Cambridgeshire, UK), dH_2O and genomic DNA. Thermocycling consisted of 2.5 min at 94°C followed by 35 cycles of 94°C for 45 sec, 56.5°C for 45 sec and 72°C for 20 sec and was completed with 10 minutes at 72°C and kept at 4°C prior to further processing. The amplified alleles were analyzed on 2% agarose gel. The presence of the I- and D-allele resulted in 252 bp and 197 bp products respectively (Figure 2.1). The forward D primer also imperfectly recognised a second site only present in the I-allele, which could result in the production of a 485 bp band. However, this was non-essential for genotyping and inconsistently produced with poorer quality DNA samples.

Table 2.1 Sequences and length of primers used for genotyping of *ACE* I/D polymorphism. The melting temperature (Tm), the probable secondary structures and the GC content were determined by the Sigma-Genosys calculator. None of the primers form dimmers.

	Name of primer	Sequence 5' to 3'	Length	Tm	Secondary Structures	%GC content
ACE I/D	DCP1CMDF3	CTCTAGACCTGCTGCCTATTACAGTC	26	64.21	Weak	46.15
	DCP1CMIF2	CGGGATGGTCTCGATCTC	18	63.35	Very weak	61.11
	DCP1CMIDR2	CCCTCCCATGCCCATAAC	18	65.09	None	61.11

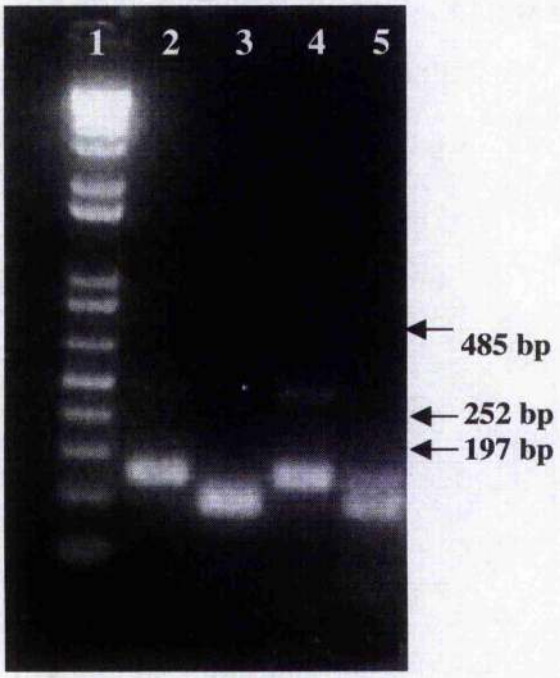


Figure 2.1 Genotyping of *ACE* I/D polymorphism. The figure shows an ethidium bromide stained 2% agarose gel of the *ACE* I/D-PCR amplified product. Lane 1: 1Kb plus DNA ladder (Promega, Southampton, UK). Lane 2: Individual homozygote for the I allele recognized by the presence of the 252 bp band and the absence of the 197 bp band. Lane 3: Individual homozygote for the D allele recognized by the presence of the 196 bp band and the absence of the 252 bp band. Lane 4: Individual homozygote for the I allele recognized by the presence of the 252 bp band and the absence of the 197 bp band. Lane 5: Individual heterozygote for the I/D polymorphism. The 485 bp band can also be seen in lanes 2 and 4.

2.3.2.2 *ADRB* genes

DNA amplification was carried out for five polymorphisms in *ADRB1* (Gly49Ser and Arg389Gly), *ADRB2* (Gly16Arg and Glu27Gln) and *ADRB3* (Trp64Arg) genes. Primer design was based on NCBI reference sequences NM_000684 (for *ADRB1*), AC011354 (for *ADRB2*) and X72861.1 (for *ADRB3*) (Table 2.2 and Table 2.3). Regions containing the *ADRB1*, *ADRB2* and *ADRB3* polymorphisms were amplified by PCR and genotypes were determined by RFLP analysis. For *ADRB1* Gly49Ser polymorphism amplification, the primers contained non-specific T-tails to facilitate their use in a multiplex reaction with the *ADRB3* Arg64Trp primers; however, due to poor DNA amplification by using this multiplex PCR, Gly49Ser and Arg64Trp polymorphisms were finally amplified separately. Thermocycling consisted of 5 min at 94°C followed by 35 cycles at 94°C, 60°C and 72°C each for 30 sec and was completed with 10 min at 72°C. For RFLP analysis, PCR products (5 µl) were then digested with *Eco0109*I RE (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK) overnight at 37°C in a final volume of 10 µl and separated on 2.5% agarose gel (Table 2.2). The Gly49 allele (G-allele) contained a unique restriction site for *Eco0109*I yielding two bands of 119bp and 117bp, whilst the Ser49 allele (A-allele) yielded a 236 bp band (Table 2.2, Figure 2.2a).

A 171 bp region, containing both the *ADRB2* Gly16Arg and Glu27Gln polymorphic loci was amplified using one set of primers (25 pmol each) (Sigma-Genosys, Haverhill, Cambridgeshire, UK) (Table 2.2 and Table 2.3). Thermocycling conditions were 5 min at 94°C followed by 35 cycles at 94°C, 56.5°C and 72°C each for 30 sec and was completed with 10 min at 72°C. The forward primer for the *ADRB2* gene polymorphisms (Bar2CM26NcoF2) contained a mismatched base that created a *Nco*I site in conjunction only with the Gly16 allele (Table 2.2).

Table 2.2 Sequences of the primers used for amplification of *ADRB1*, *ADRB2* and *ADRB3* gene polymorphisms, restriction enzyme used for the RFLP analysis and length of the resultant fragments.

SNPs	Primer sequence (pmol/reaction)	Size of PCR product (bp)	Restriction enzyme (Units/reaction)	Size of RFLP bands (bp)	Percentage of agarose gel
ADRB1 Gly49Ser	5'TTTTCGAGCCCGGTAACCTGTCG3' (25 pmol) 5'TTTTTTTTTTTTTTCGATGGCCACGATCACCAG3' (25 pmol)	236	*Eco0109*I (5 U)	236 (A-allele) 119, 117 (G-allele)	2.5%
ADRB1 Arg389Gly	5'AACGTGGTGAAGGCCTTCC3' (40 pmol) 5'ATCGTCGTCGTCGTCGTCC3' (10 pmol)	273	*Bst*NI (2.5 U)	273 (C-allele) 140, 133 (G-allele)	3.5%
ADRB2 Gly16Arg	5'CCTTCTTGCTGGCACCC**C**AT3' (25 pmol)	171	*Nco*I (2 U)	154, 17 (G-allele) 171 (A-allele)	3.5%
ADRB2 Glu27Gln	5'TCTGCAGACGCTCGAACTTG3' (25 pmol)		*Bbv*I (0.5 U)	171 (G-allele) 109, 62 (C-allele)	2.0%
ADRB3 Arg64Trp	5'CGCCCAATACCGCCAACAC3' (10 pmol) 5'CCACCAGGAGTCCCATCACC3' (10 pmol)	210	*Bst*NI (2.5 U)	158, 31, 15 (C-allele) 97, 61, 31, 15 (T-allele)	3.5%

***ADRB1* C49 primers contained non-specific T-tails to facilitate their use in multiplex PCR with the *ADRB1* Arg64Trp primers. However, this multiplex reaction was not finally carried out due to poor amplification. In the forward primer for Gly16Arg amplification, the base marked in bold is a mismatched base for creating a restriction site for *Nco* I RE (discussed in text).**

Table 2.3 Length and properties of primers used for genotyping of *ADRB1*, *ADRB2* and *ADRB3* polymorphisms. Melting temperature (Tm), probable secondary structures and GC content for each primer were determined by the Sigma-Genosys calculator. Bar1CM33F and Bar1CM250R primers contained non-specific T-tails to facilitate their use in multiplex PCR with the *ADRB3* Arg64Trp primers. However, these multiplexed reactions were not carried out here. The non-specific tails were not included in the calculation of Tm for these primers. None of the primers form dimmers.

SNP	Name of primer	Length	Tm	Secondary structures	% GC content
ADRB1 **Gly49Ser**	Bar1CM33F	23	73.3	Weak	56.5
	Bar1CM250R	33	77.2	Weak	36.4
ADRB1 **Arg389Gly**	Bar1CM1030F	19	66.3	Very weak	57.9
	Bar1CM1302R	19	68.8	None	63.2
ADRB2 **Gly16Arg**	Bar2CM26NcoF2	20	70.5	Weak	60.0
	Bar2CM196R	20	66.8	None	55.00
ADRB2 **Glu27Gln**	Bar3CM62F	19	70.5	None	63.2
ADRB3 **Arg64Trp**	Bar3CM271R	20	69.1	None	65.0

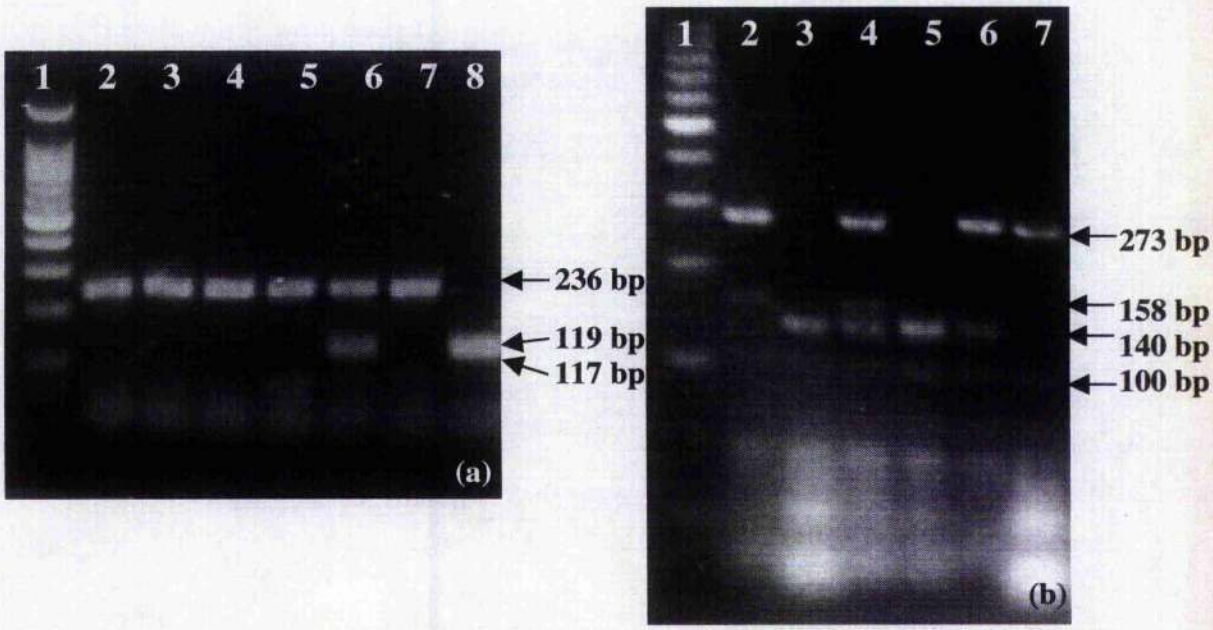


Figure 2.2 (a) Genotyping of rs1801252 (*ADRB1* C49) polymorphism. The figure shows an ethidium bromide stained 2.5% agarose gel of *Eco0109*I digested fragments of the *ADRB1* C49-PCR amplified product. Lane 1: 100 bp DNA ladder (Promega, Southampton, UK). Lane 2-5: Bands produced by homozygotes for the Ser49-allele (236 bp band). Lane 6: Bands produced by a heterozygote (Gly49Ser) individual. Lane 7: Bands produced by an individual homozygote for the Ser49-allele (236 bp band). Lane 8: Bands produced by an individual homozygote for the Gly49-allele (119/117 bp doublet).

(b) Genotyping of rs1801253 (*ADRB1* C389) and rs4994 (*ADRB3* C64) polymorphisms. The figure shows an ethidium bromide stained 3.5% agarose gel of *BstNI* digested fragments of the *ADRB1* C389/*ADRB3* C64-PCR amplified product. Lane 1: 100 bp DNA ladder (Promega, Southampton, UK). Lane 2: Bands produced by an individual homozygote for the Arg389-allele (273 bp band) and heterozygote for the Arg64Trp (158 bp and 100 bp bands). Lane 3: Bands produced by an individual homozygous for the Gly389-allele (140 bp band) and for the Trp64-allele (100 bp band). Lane 4: Bands produced by an individual heterozygous for both Arg389Gly and Arg64Trp polymorphisms. Lane 5: Bands produced by an individual homozygote for Gly389-allele and Trp64-allele. Lane 6: Bands produced by an individual heterozygote for the Arg389Gly polymorphism and homozygote for the Trp64-allele. Lane 7: Bands produced by an individual homozygote for Arg389-allele and Trp64-allele.

The amplified 171 bp PCR product was then used for two separate digestions. For Gly16Arg RFLP analysis, 5 µl of PCR product was digested with *Nco*I RE (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK) overnight at 37°C in a final volume of 10 µl and separated on 3.5% agarose gel. The Gly16 allele (G-allele) yielded two bands of 154 bp and 17 bp, whilst the Arg16 allele (A-alelle) remained uncut (Figure 2.3a). For Glu27Gln polymorphism, 5 µl of PCR product was digested with *Bbv*I RE (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK) overnight at 37°C in a final volume of 10 µl and separated on 2.0% agarose gel. The Gln27 allele (C-allele) contained a restriction site for *Bbv*I yielding two products of 109 bp and 62 bp, whilst the Glu27 (G-allele) allele remained uncut (Figure 2.3b).

A multiplexed PCR and digestion was carried out for the *ADRB1* Arg389Gly and *ADRB3* Arg64Trp polymorphisms. Thermocycling consisted of 5 min at 94°C followed by 35 cycles at 94°C, 60°C and 72°C each for 30 sec and was completed with 10 min at 72°C. Bar1CM1030F and Bar1CM1302R amplified a fragment of 273 bp, whilst Bar3CM62F and Bar3CM271R a product of 210 bp. A double digestion with *Bst*NI RE (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK) was carried out at 60°C overnight in a total volume of 20 µl (5 µl from each PCR product) (Table 2.2). The Gly389 allele (G-allele) contained a unique site for *Bst*NI giving two fragments of 140 bp and 133 bp, whilst the Arg389 allele (C-allele) remained uncut (Figure 2.2b). The Trp64 allele (T-allele) contained four *Bst*NI sites producing bands of 97, 61, 31, 15 and 6 bp, whilst the Arg64 allele (C-allele) contained three *Bst*NI sites producing bands of 158, 31, 15 and 6 bp (Figure 2.2b). Digested products were separated on 3.5% agarose gel. Due to poor amplification of Arg64Trp polymorphism for most of the samples, PCR for the *ADRB3* gene was repeated with each reaction containing only the primers specific for the *ADRB3* Arg64Trp region.

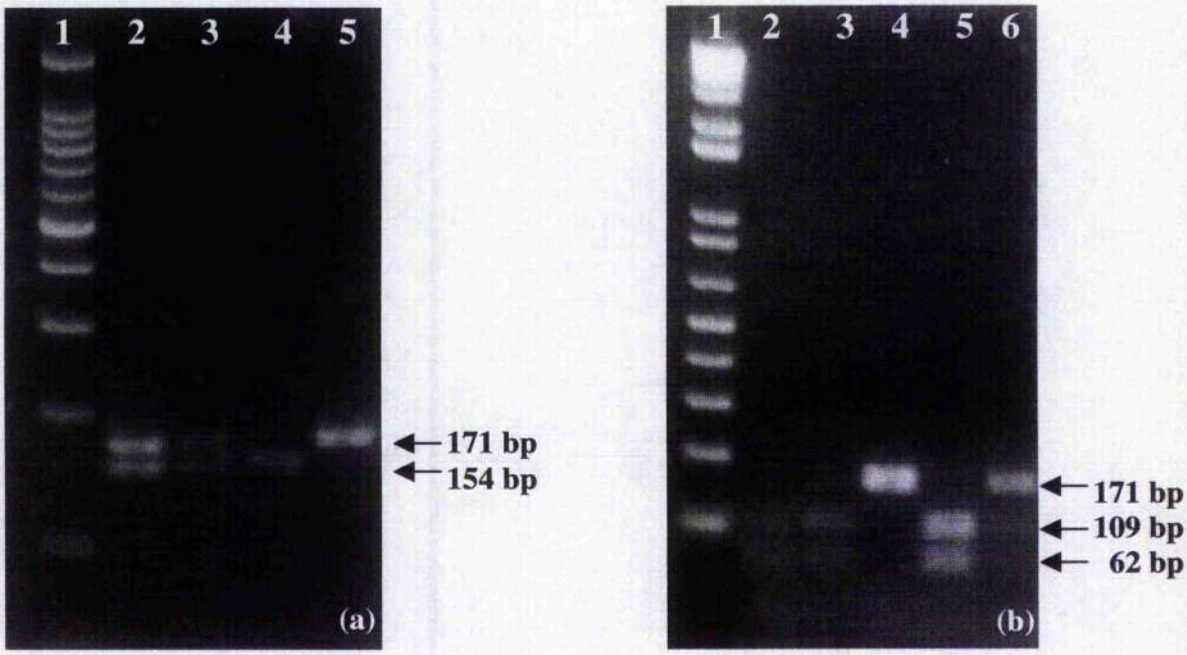


Figure 2.3 (a) Genotyping of rs1042713 (*ADRB2 C16*) polymorphism. The figure shows an ethidium bromide stained 3.5% agarose gel of *Nco*I digested fragments of the *ADRB2*-PCR amplified product. Lane 1: 1Kb plus DNA ladder (Promega, Southampton, UK). Lanes 2 and 3: Bands produced by heterozygotes (Gly16Arg) individuals. Lane 4: Band produced by an individual homozygote for the Gly16-allele (154 bp bands). Lane 5: Band produced by an individual homozygote for the Arg16-allele (171 bp).

(b) Genotyping of rs1042714 (*ADRB2* C27) polymorphism. The figure shows an ethidium bromide stained 2.0% agarose gel of *Bbv*I digested fragments of the *ADRB2*-PCR amplified product. Lane 1: 100 bp DNA ladder (Promega, Southampton, UK). Lanes 2 and 3: Bands produced by individuals homozygotes for the Gln27-allele (109 bp and 62 bp). Lane 4: Band produced by an individual homozygote for the Glu27-allele (171 bp). Lane 5: Bands produced by an individual homozygote for the Gln27-allele (109 bp and 62 bp). Lane 6: Bands produced by a heterozygote (Glu27Gln) individual.

2.3.2.3 *PPARγ* gene

DNA amplification was carried out for both *PPARγ* polymorphisms, Pro12Ala and C1431T. Primer design was based on NCBI reference sequence NM_015869.3 (Table 2.4). Genotypes for all three polymorphisms were determined by PCR amplification and RFLP analysis. PCR reactions were carried out using 10 pmol/µl of each primer (MWG-Biotech, Ebersberg, Germany). For Pro12Ala polymorphism, thermocycling conditions consisted of 5 min at 94°C followed by 35 cycles at 94°C for 45 sec, 58°C and 72°C each for 30 sec and was completed with 10 min at 72°C. The reverse primer used for Pro12Ala amplification, contained a mismatched base that created a *Bsl*I restriction site in conjunction only with the Pro12 allele (Table 2.4). For RFLP analysis of this polymorphism, PCR products (5 µl) were digested with 4 Units of *Bsl*I RE (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK) overnight at 55°C. The digested fragments were resolved on a 3.5% agarose gel. The Pro12 allele (C-allele) contained a unique restriction site for *Bsl*I yielding two products of 168 bp and 17 bp, while the Ala12 allele (G-allele) yielded an uncut 185 bp fragment (Figure 2.4a). For C1431T polymorphism, thermocycling conditions consisted of 5 min at 94°C followed by 35 cycles at 94°C for 30 sec, 57°C and 72°C each for 30 sec and was completed with 10 min at 72°C. For RFLP analysis, each PCR product (5 µl) was digested with 12 Units of *Pml*I RE (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK), 10x Nebuffer and 1% BSA in total volume of 10 µl at 37°C overnight to obtain optimal digestion. The digested fragments were separated on 3.0% agarose gel. The C-allele contained a unique restriction site for *Pml* I producing two fragments of 146 bp and 57 bp, while the T-allele yielded an uncut 203 bp fragment (Figure 2.4b). Due to partial digestion of some samples with *Pml*I RE, 3 U of *Nla*III RE were used instead when required. The T-allele contained a unique site for *Nla*III yielding two products of 147 bp and 56 bp, while the C-allele remained uncut (203 bp).

Table 2.4 Sequences and properties of the primers used for amplification of *PPARγ* Pro12Ala and C1431T gene polymorphisms, restriction enzyme used for the RFLP analysis and length of the resultant fragments. In PPARGVL190R, the base marked in bold is a mismatched base for creating a restriction site for *BslI* RE (discussed in text). Melting temperature (Tm) and %GC content were determined for each primer by the MWG calculator. None of the primers form dimmers.

		Name of primer	Sequence 5' to 3'	Tm	%GC content	PCR product (bp)	RE (units)	RFLP bands (bp)	% agarose gel
PPARγ	Pro12Ala	PPARGVL6F	TGTCTTGACTCATGGGTGTATTC	58.9	43.5	185	*Bsl*I (4U)	168, 17 (C-allele)	3.0
		PPARGVL190R	ATCAGTGAAGGAA**C**CGCTTT	55.3	45			185 (G-allele)	
	C1431T	PPARGVL1418F	TGAATGTGAAGCCCATTGAA	53.2	40	203	***Pml*I (12U)** *Nla*III (3U)	146, 57 **(C-allele)** 203 (C-allele)	3.5
		PPARGVL1621R	GAGCGGGTGAAGACTCATGT	59.4	55		***Pml*I (12 U)** *Nla*III (3U)	203 **(T-allele)** 147, 56 (T-allele)	

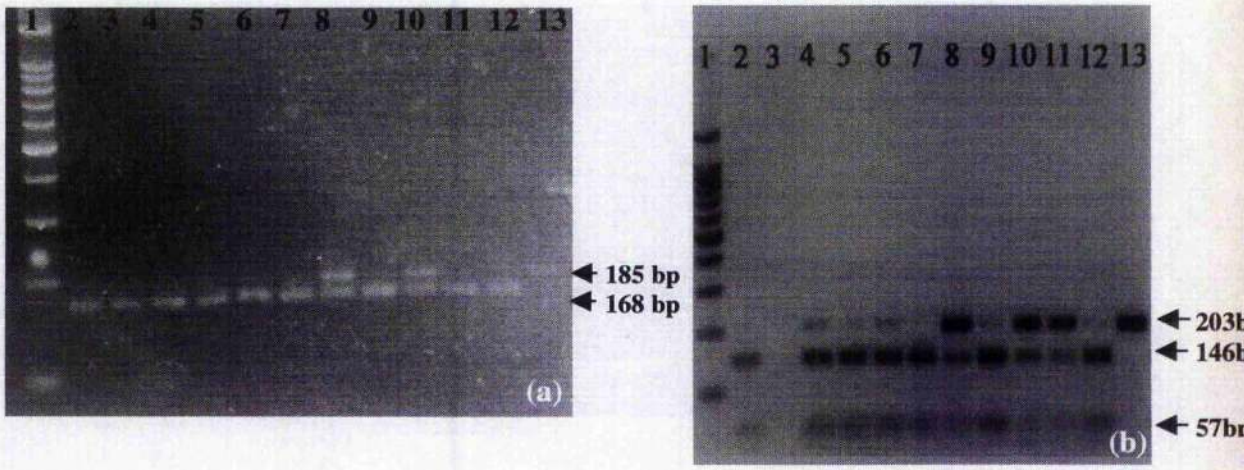


Figure 2.4 (a) Genotyping of rs1801282 (Pro12Ala) polymorphism. The figure shows an ethidium bromide stained 3.5% agarose gel of *BslI* digested fragments of the Pro12Ala-PCR amplified product. Lane 1: 100 bp DNA ladder (Promega, Southampton, UK). Lane 2-7, 9, 11, 12: Bands produced by individuals homozygotes for the Pro12-allele (168 bp and 17 bp bands). Lane 8, 10: Bands produced by an individual heterozygote for the Pro12Ala allele. Lane 13: Bands produced by an individual homozygote for the Ala12-allele (185 bp band).

(b) Genotyping of rs3856806 (*C1431T*) polymorphism. The figure shows an ethidium bromide stained 3% agarose gel of *PmlI* digested fragments of the C1431T-PCR amplified product. Lane 1: 100 bp DNA ladder (Promega, Southampton, UK). Lane 2, 4-7, 9, 12: Bands produced by individuals homozygotes for the C-allele (146 bp and 57 bp). Lane 3: Individual that the genotyping did not succeed. Lane 8, 10-11: Bands produced by heterozygotes individuals. Lane 13: Bands produced by an individual homozygote for the T-allele (203 bp).

2.4 Statistical analysis

Prior to any analysis, anthropometric data for all subjects were scrutinized for outliers, using descriptive statistics and normal probability plots. Outliers, as well as individuals with any missing phenotypic data, were identified and excluded from the dataset.

Allele frequencies were calculated for all polymorphisms and chi-square tests (χ^2) were performed to assess whether the observed genotype frequencies were in HWE. Haplotypes frequencies were calculated by using Arlequin software (Arlequin 3.01, 2006, CMPG, Switzerland). The basic *D* (Pairwise-disequilibrium coefficient) (defined as the difference between the probability of observing two marker alleles on the same haplotype and observing them independently), as well as the normalized estimate of LD, D' (Normalised estimate of linkage disequilibrium) (defined as D/Dmax), between the two sites were calculated (Zondervan and Cardon, 2004).

GLM (General Linear Model) analysis on the total population was carried out to assess whether the genetic effects on the total population were influenced by gender and/or age. GLM modelling allows predictor variables to be assessed as factors explaining dependent distributions simultaneously. In the present analysis, gender and age were assessed as possible confounding factors in the analysis. Initially, the models considered in the current analysis were the individual effects of gender, age and genotype, the three two-way interactions (gender * age, gender * genotype and age * genotype) and the three-way interaction (gender * age * genotype). However, due to the fact that models should have as few parameters as possible and should have simple types of relationship (the simplest may be a power relationship), models were pared down to the minimal adequate model, in which most terms were significant. Although all the phenotypes of interest in the present study are potentially related, as they are all descriptive of adiposity, different models best

explained the phenotypes, while unnecessary variables were removed (model simplification). If an interaction was found to be significant, then the main effects would be considered as being important, regardless of the level of significance of each variable, since the interaction includes the main effects of each variable. If significant interactions were found between genotype and/or gender, ANOVA (One-way Analysis Of Variance) analysis stratisfied by age and/or gender would be carried out. Apart from GLM analysis on the total population, an alternative approach involving gender and age subgrouping was also employed. This was based on the fact that previous studies, albeit in adolescents and adults, have indicated the presence of gender-specific effects of *ACE* I/D (O'Donnell et al., 1998), *ADRB* (Meirhaeghe et al., 2000b;Gonzalez Sanchez et al., 2003) and *PPARγ* (Beamer et al., 1998;Valve et al., 1999) gene polymorphisms on phenotype. Subjects from each gender were also assigned to single-year age groups: 13-24 months (1-2 years), 25-36 months (2-3 years), 37-48 months (3-4 years) and 49-71 months (4-6 years) to account for marked changes in body composition during the first decade of life (Fomon et al., 1982) and to control for secular trends in adiposity (Manios, 2006). To maintain adequate numbers of subjects for analysis within each age group, 4 to 5 years old and 5 to 6 years old were grouped together prior to any statistical analysis (Table 2.5). Isolated significant findings from this age and gender subgrouping analysis were compared to those from the main GLM analysis. Reflection of these isolated significant findings in the main analysis could confirm that the isolated findings are not Type I errors.

Phenotypic distributions for the total population or each gender or age group were tested separately for normality using the Ryan-Joiner test and datasets with non-normal distributions were transformed by Box-Cox transformation (Table 2.6). Mean values and 95% CI (Confidence Interval) for each phenotype were subsequently back-transformed for presentational purposes. The influence of the *ACE* (I/D), *ADRB* (*ADRB1* C49 and C389,

ADRB2 C16 and C27, *ADRB3* C64) and *Par* (Pro12Ala and C1431T) genotypes on adiposity-related variables was evaluated by one-way analysis of variance (ANOVA) using MINITAB 13.30 (Minitab Ltd., Coventry, UK). For *PPARγ* gene, because of the small number of individuals homozygous for the less common allele (i.e. Ala12 and T1431) at each locus, these were collapsed with the heterozygous and compared with the homozygous for the common alleles for all further analyses. Multiple testing was controlled by the use of adjusted P values for significance (the α value) within each gender and age group using the Dunn-Sidak method with correction for repeated tests and correlations between datasets as implemented on the SISA website (SISA website http://home.clara.net/sisa/bonferroni.html). For each age group, the mean correlation (r) between the adiposity-related phenotypes evaluated in these analyses was calculated as the square root of the mean of the squared partial correlations (coefficients of determination) between the assessed phenotypes.

Singificant associations were further assessed by correlation analyses to evaluate possible genetic models underlying the association (Moran et al., 2005). The proportion of phenotypic variance explained by the ANOVA (r_A), which is model-free, was compared to the proportion of variance explained under each model by correlation analysis (r_C^2) (MINITAB 13.30, Minitab Ltd., Coventry, UK). The ratio of these proportions (r_C^2/r_A) gives the proportion of the genetic variance at the tested locus explained under a specified model. For *ACE* I/D polymorphism, models tested were the allelic additive model and the two fully dominant models. Genotypes were expressed as dummy variables in the correlation analysis (0, 1, 2 for the additive model and 0, 0, 1 or 0, 1, 1 for the dominant genetic models depending on which allele was being tested for dominance). The additive model is the easiest to explain mechanistically, with the mean of the heterozygotes being exactly intermediate to the means of the two homozygotes groups. A dominant model

explains the data best when the heterozygote mean is close to one of the homozygote means. For *ADRB* polymorphisms, genotypes were also expressed as dummy variables in the correlation analysis (0, 1, 2 for the additive model and 0, 0, 1 or 0, 1, 1 for the dominant and recessive genetic models). The Gly49, Arg389 (*ADRB1*), Gly16, Glu27 (*ADRB2*) and Arg64 (*ADRB3*) alleles were identified as the ancestor variants, after comparison of human with other Great Ape sequences (NCBI database; http://www.ncbi.nih.gov/). A dominant model explains the data best when the heterozygote mean is close to the homozygote for the ancestor allele mean, while a recessive model explains the data best when the heterozygote mean is close to the homozygote for the modern allele (Ser49, Gly389 for *ADRB1*, Arg16, Gln27 for *ADRB2*and Trp64 for *ADRB3*) mean. No genetic models were tested for any of the *PPARγ* polymorphisms, since no homozygotes for the rare alleles were identified in some age groups. Odds ratios were calculated after dividing the population into 3 groups based on low (0-25%), pooled-middle (25-75%) and high (75-100%) quartiles to assess whether the influence of genotype on phenotype took place over the whole phenotype range or only at one extreme. Odds ratios were calculated as the likelihood of a given genotype being present in a particular quartile compared to its likelihood of being in the remaining quartiles. Significant associations were assessed by using 2x2 chi-square tests and 95% CIs.

For haplotype-based analysis in *PPARγ* gene, individuals with the Pro-C/Pro-T diplotype were combined with those homozygotes (Pro-T/Pro-T) for the Pro-T haplotype, as previously suggested (Doney et al., 2002). Furthermore, heterozygotes (Pro-C/Ala-C) and homozygotes (Ala-C/Ala-C) for the Ala-C haplotype were also collapsed into a single group, whereas double heterozygotes (Pro-C/Ala-T) were combined with heterozygotes (Pro-T/Ala-T, Ala-C/Ala-T) and homozygotes (Ala-T/Ala-T) for the Ala-T haplotype (Doney et al. 2002). Differences in the phenotypic means by these four diplotype groups

were assessed by one-way ANOVA using MINITAB 13.30 (Minitab Ltd., Coventry, UK) for each age group and gender. Significant associations ($P<0.05$) were further analyzed by performing two-tailed pairwise *t*-tests, which allowed comparison of Pro-T, Ala-C and Ala-T haplotypes with the common Pro-C haplotype.

To evaluate the improvement in the associations with the phenotypes produced by assessing diplotypes rather than *ADRB1* (C49, C389), *ADRB2* (C16, C27) or *PPARγ* (Pro12Ala, C1431T) genotypes, the sum of squares from the individual genotype ANOVA was subtracted from the sum of squares from the diplotype ANOVA (as were the degrees of freedom) and new F and P values were calculated.

Table 2.5 Number of boys and girls in each age group. To maintain adequate number of subjects for analysis, subjects from 49-60 months and 61-71 months age groups were assigned into a single group.

	Prior to Grouping		After Grouping	
Age (months)	**Boys**	**Girls**	**Boys**	**Girls**
13-24	91	90	91	90
25-36	249	202	249	202
37-48	437	384	437	384
49-60	285	298	318	331
61-71	33	33		
Total	1095	1007		

Table 2.6 The best estimate of lambda (λ value) for the transformation of each phenotype for each age group in both genders. The resulting transformation is Y^{λ}. A lambda value of 0 corresponds to the natural log transformation, while λ values of 2, 0.5, -0.5 and −1 correspond to squared, squared root, $1/\sqrt{Y}$ and 1/Y transformations respectively (Y is the raw data).

	Boys				Girls			
	1-2	**2-3**	**3-4**	**4-6**	**1-2**	**2-3**	**3-4**	**4-6**
BMI		0.000	-1.573	-1.461		-0.113	-1.461	-1.461
Arm circumference	2.022	-2.472	-1.573	-2.471	1.910	-0.675	-0.113	-1.235
Waist circumference	0.787	-1.236	-2.022	-2.022	-1.349	0.562	-0.337	-2.472
Hip circumference	2.697	-0.899	-1.686	-2.248	1.011	-2.134	-1.461	-1.686
Biceps skinfold	0.112	0.000	-0.224	-0.562	0.562	0.225	0.000	-0.113
Triceps skinfold	0.786	0.000	0.113	-0.113	0.787	0.112	0.000	-0.112
Subscapular skinfold	0.337	-0.450	-0.674	-0.562	-0.449	-0.899	-0.899	-0.899
Suprailiac skinfold	0.225	-0.337	-0.224	-0.225	0.449	0.113	0.000	-0.224

Chapter 3

Growth and adiposity in Greek children

3.1 Introduction

The prevalence of overweight and obesity has remarkably increased in childhood and adolescence over the past three decades (Dehghan et al., 2005). Around 22 million children under the age of five are obese worldwide (Miller et al., 2004), while a high prevalence of overweight and obesity has been reported in Greek children (Magkos et al., 2005) and adolescents (Mamalakis et al., 2000;Krassas et al., 2001). Increased adiposity in childhood is predictive for obesity in adulthood (Whitaker et al., 1997;Magarey et al., 2003) and accounts for other morbidities in young children, such as dyslipidemia, hypertension, insulin resistance, orthopaedic difficulties (Dietz, 1998). Published studies reported prevalence of overweight and obesity that vary between most European countries. However, it is not clear whether these discrepancies in the findings are due to differences in measurements or they represent true results. Furthermore, although a considerable number of reports have assessed the rates of overweight and obesity in school-aged children, adolescents and adults, there is lack of information regarding the corresponding prevalence in very young children.

The aim of this study was to assess the prevalence of overweight and obesity in a large representative population of toddlers and preschoolers from Greece by using specific BMI cut-off points based on national reference data and by comparing the rates of overweight and obesity in the Greek children with the findings of other published reports. Moreover, the evolution of various physiological- and adiposity-related indices with age and according to gender in the present cohort of Greek children was described. The final aim of this study was to compare specific physiological-related phenotypes from the current Greek population with normative data from British and US children.

3.2 Methods

3.2.1 Subjects and measurements

The number of subjects that took part in the study, as well as information on the assessed adiposity-related phenotypes and the protocols used for anthropometry, are given in Chapter 2 (General Methods). British normative data was obtained from the British 1990 growth reference published by the Child Growth Foundation (2 Mayfield Avenue, London W4 1PW). The British growth reference provides anthropometric data (BMI, weight, length or height and head circumference) from 17 distinct surveys representative of England, Scotland and Wales and includes 37,700 children aged 0 (23 weeks of gestation) to 23 years (Cole et al., 1995).

3.2.2 Data analysis

Although BMI cut-off points are well established in adult populations (25 kg/m^2 for overweight and 30 kg/m^2 for obesity), defining childhood overweight and obesity in childhood is a more difficult task, since BMI changes substantially with age and there are no internationally accepted definitions. A variety of reference-data sets for BMI in childhood exist, such as the British 1990 Growth reference (Child Growth Foundation, Chiswick, UK) (Cole et al., 1995), the US CDC 2000 charts (Kuczmarski et al., 2000) and the international BMI reference data by the IOTF (Cole et al., 2000). In the current study, the centile-based cut-off points adopted currently by Child Growth Foundation were used to analyse prevalence and obesity in the Greek population. Due to significant differences in the rates of overweight and obesity between the present population (based on the UK1990 reference data) and those reported by Manios, 2006 in a larger subset of the GENESIS cohort (based on CDC 2000 percentiles), the CDC 2000 growth charts were also used to

determine the prevalence of overweight and obesity in the present population. The Nutstat module of EpiInfo was used to determine the age- and sex-specific Z-scores for weight, height and BMI, according to the CDC 2000 growth charts. The UK1990 and CDC 2000 BMI-for-age growth charts were used for classifying the children as overweight (UK1990) or at risk of overweight (CDC 2000) (85^{th}-95^{th} percentile) and as obese (UK1990) or overweight (CDC 2000) ($\geq 95^{th}$ percentile). IOFT (International Obesity Task Force) definitions of obesity were not preferred in the current study, since they have low sensitivity, they are highly sex-specific in comparison to national definitions and they don't extend to children under the age of 2 years (Chinn, 2006;Reilly, 2006).

In the Greek population, mean and standard deviations for all anthropometric were estimated for each gender and age group. A randomisation test available by SMART (Statistics and Mathematics as Advanced Research Tools) website (www.bioss.ac.uk/smart) was used to assess the statistical significance of differences between the means of each phenotype in boys and girls; statistical significance was accepted at $p<0.05$. The phenotypic data was not transformed to achieve normality prior to the randomisation test, since this test makes no distribution assumptions about the data like, for example, *t*-tests. The descriptive statistics for all anthropometric parameters within age group and gender are shown in Table 3.2. The 75^{th}, 50^{th} and 25^{th} percentiles for all anthropometric measurements for each gender are shown in Figure 3.1. Growth curves were constructed by using MINITAB statistical software (MINITAB 13.30, MINITAB Ltd., Coventry, UK).

By using a linked software program provided by the Child Growth Foundation, BMI, weight and height were converted into age- and gender-specific standard deviation scores (SDS, Z-scores) of their distribution in the British 1990 reference data (Table 3.3 and 3.4). Significant differences between the values of each measurement and the reference

population were assessed by an independent two-tailed *t*-test taking into account the fact that for the UK 1990 population each SDS has a normal distribution mean of 0.0 and a standard deviation of 1.0.

Reference data for specific body composition indices are not available for Greek children. Age- and sex-specific data have been compiled for specific anthropometric indices other than BMI in the United States and United Kingdom. In the present study, anthropometric measurements, such as mid-upper-arm and waist circumferences, as well as triceps and subscapular skinfolds, from the Greek children were compared with reference data derived from the NHANES III (1988-1994) (Flegal et al., 2001) by using the Welch's approximate *t*-test for unequal variances. NHANES III is a stratified, multistage probability design of the noninstitutionalised US population aged ≥2 months conducted by the CDC National Centre for Health Statistics (HCHS) (Flegal et al., 2001). Data from this survey, along with others, have been used for the development of the US CDC 2000 growth charts (Kuczmarski et al., 2000).

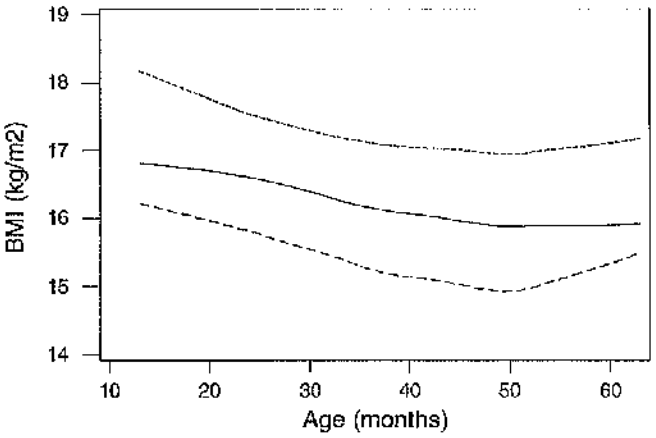
Table 3.1 Prevalence of overweight and obesity in Greek toddlers and preschoolers based on the UK 1990 and CDC 2000 growth reference percentiles.

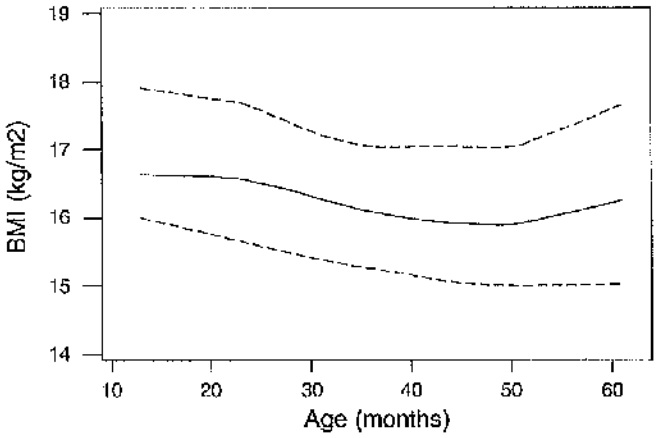
	N	Overweight (%) UK 1990	At risk of overweight (%) CDC 2000	Obese (%) UK 1990	Overweight (%) CDC 2000
Boys					
1-2 years	91	7.7%	-	5.5%	12.2%
2-3 years	249	10.8%	10.4%	4.0%	6.4%
3-4 years	437	11.2%	12.1%	8.5%	10.1%
4-5 years	318	12.6%	14.1%	11.9%	12.3%
Girls					
1-2 years	90	7.8%	-	10%	9%
2-3 years	202	12.4%	13.4%	5.9%	5.4%
3-4 years	384	10.9%	15.1%	9.6%	10.9%
4-5 years	331	10.3%	16.6%	13.6%	13.9%

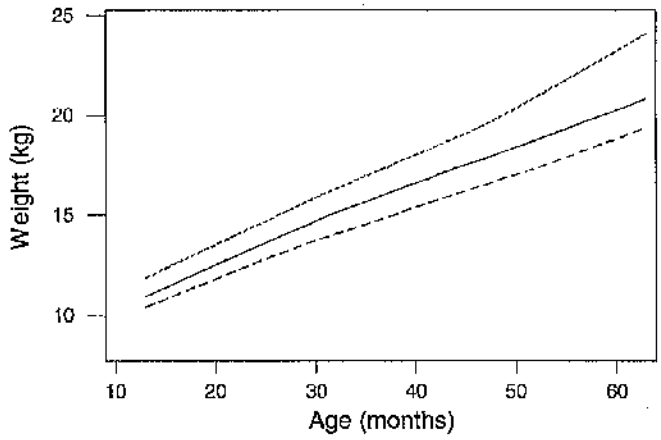
Overweight (UK1990) or at risk of overweight (CDC 2000) were defined as a BMI SDS >1.04, equivalent to the 85th percentile, while obesity (UK 1990) or overweight (CDC 2000) were defined as a BMI SDS ≥ 1.64, equivalent to the 95th percentile. N= the total number of subjects in each age group. Children up to 24 months were classified as overweight (≥95th percentile) using the CDC weight-for-length growth charts.

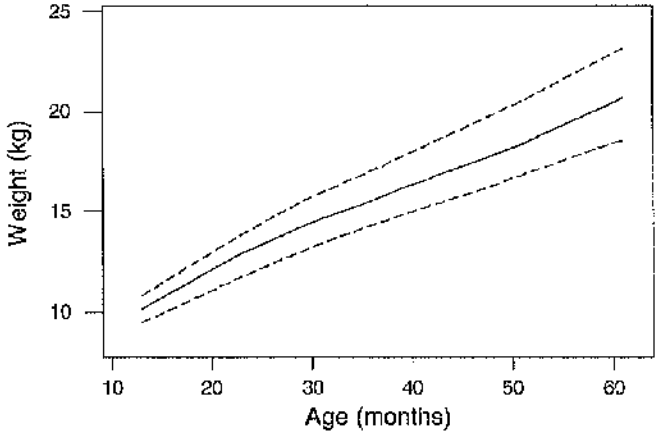
Table 3.2 Descriptive statistics of anthropometric measurements in each age group for boys and girls.

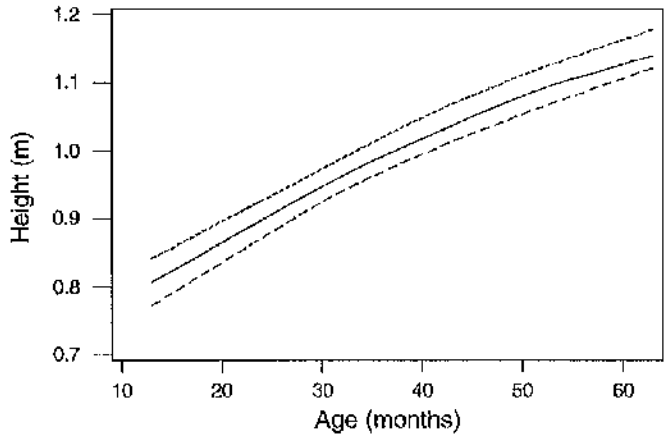
12-24 months Age group	Boys	Girls	P Value 1
Mean age (months)	19.9	19.2	
BMI (kgr/m^2)	16.90 ± 1.52	16.87 ± 1.53	0.894
Weight (kgr)	12.56 ± 1.46	11.78 ± 1.70	**0.001**
Height (m)	0.86 ± 0.05	0.83 ± 0.05	**<0.001**
Arm circumference (cm)	15.47 ± 1.09	15.32 ± 1.28	0.400
Waist circumference (cm)	46.72 ± 2.88	46.34 ± 3.36	0.420
Hip circumference (cm)	49.19 ± 3.09	49.12 ± 4.03	0.895
Biceps skinfold (mm)	5.72 ± 1.67	5.39 ± 1.62	0.172
Triceps skinfold (mm)	8.90 ± 2.49	8.80 ± 2.4	0.797
Subscapular skinfold (mm)	6.54 ± 1.67	6.53 ± 1.61	0.964
Suprailiac skinfold (mm)	5.24 ± 1.64	5.21 ± 1.95	0.929
25-36 months Age group	**Boys**	**Girls**	**P Value**
Mean age (months)	31.7	31.6	
BMI (kgr/m^2)	16.40 ± 1.37	16.29 ± 1.5	0.403
Weight (kgr)	15.19 ± 1.81	14.76 ± 1.98	**0.015**
Height (m)	0.96 ± 0.04	0.95 ± 0.04	**0.009**
Arm circumference (cm)	16.11 ± 1.19	16.13 ± 1.38	0.859
Waist circumference (cm)	49.61 ± 3.04	49.6 ± 3.54	0.888
Hip circumference (cm)	52.86 ± 3.3	53.17 ± 3.69	0.337
Biceps skinfold (mm)	5.79 ± 1.76	5.92 ± 1.87	0.462
Triceps skinfold (mm)	9.43 ± 2.32	9.90 ± 2.76	**0.049**
Subscapular skinfold (mm)	6.4 ± 1.82	6.98 ± 2.33	**0.002**
Suprailiac skinfold (mm)	5.35 ± 2.0	5.68 ± 2.26	**0.101**
37-48 months Age group	**Boys**	**Girls**	**P Value**
Mean age (months)	42.6	42.5	
BMI (kgr/m^2)	16.16 ± 1.61	16.19 ± 1.64	0.806
Weight (kgr)	17.29 ± 2.29	17.1 ± 2.55	0.177
Height (m)	1.03 ± 0.04	1.02 ± 0.05	**0.006**
Arm circumference (cm)	16.5 ± 1.45	16.55 ± 1.43	0.580
Waist circumference (cm)	51.6 ± 3.84	51.7 ± 4.25	0.816
Hip circumference (cm)	55.3 ± 4.06	56.1 ± 4.4	**0.007**
Biceps skinfold (mm)	5.55 ± 1.97	6.01 ± 2.0	**0.001**
Triceps skinfold (mm)	9.2 ± 2.7	9.9 ± 2.8	**<0.001**
Subscapular skinfold (mm)	6.3 ± 2.0	7.2 ± 2.3	**<0.001**
Suprailiac skinfold (mm)	5.2 ± 2.6	6.1 ± 2.7	**<0.001**
49-71 months Age group	**Boys**	**Girls**	**P Value**
Mean age (months)	54.5	54.3	
BMI (kgr/m^2)	16.11 ± 1.7	16.16 ± 1.74	0.741
Weight (kgr)	19.80 ± 2.93	19.38 ± 3.0	0.072
Height (m)	1.11 ± 0.05	1.09 ± 0.05	**0.001**
Arm circumference (cm)	16.95 ± 1.51	17.18 ± 1.65	0.061
Waist circumference (cm)	53.38 ± 4.26	53.69 ± 4.5	0.376
Hip circumference (cm)	58.1 ± 4.9	59.05 ± 4.9	**0.010**
Biceps skinfold (mm)	5.28 ± 1.86	6.4 ± 2.2	**<0.001**
Triceps skinfold (mm)	9.00 ± 2.5	10.6 ± 2.9	**<0.001**
Subscapular skinfold (mm)	6.00 ± 1.9	7.13 ± 2.6	**<0.001**
Suprailiac skinfold (mm)	4.7 ± 2.2	6.11 ± 3.4	**<0.001**

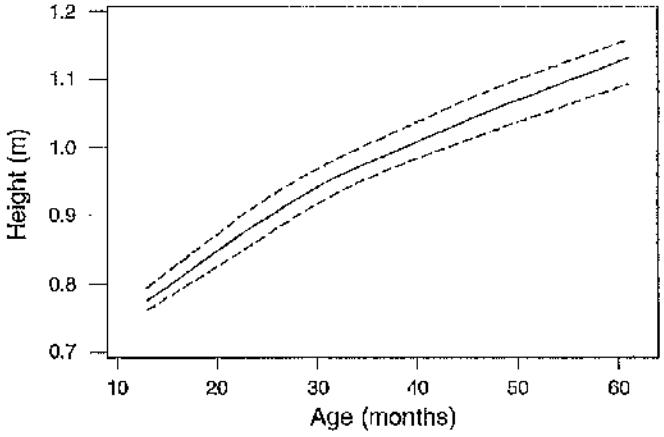

BMI (kg/m2)
19
18
17
16
15
14
10
20
30
40
50
60
Age (months)

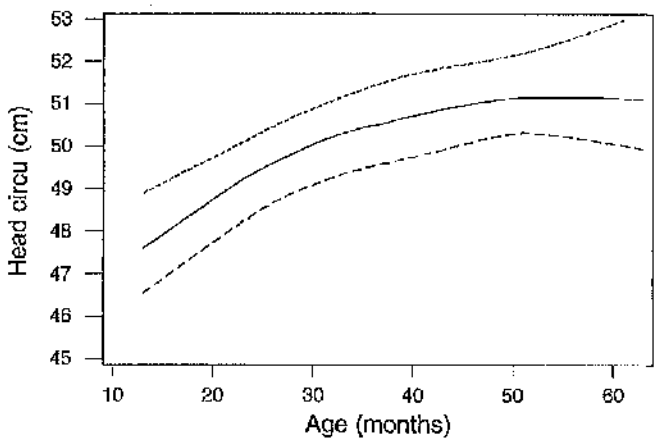

BMI (kg/m2)
19
18
17
16
15
14
10
20
30
40
50
60
Age (months)

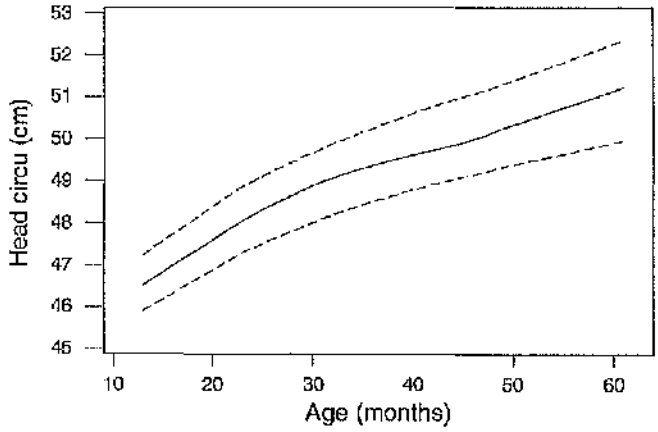

Weight (kg)
25
20
15
10
10
20
30
40
50
60
Age (months)

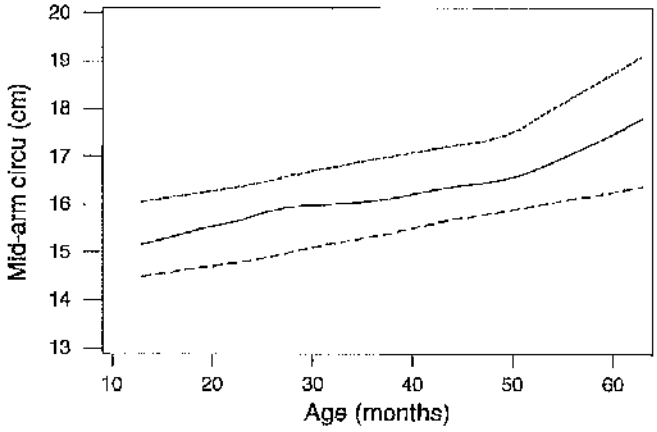

Weight (kg)
25
20
15
10
10
20
30
40
50
60
Age (months)

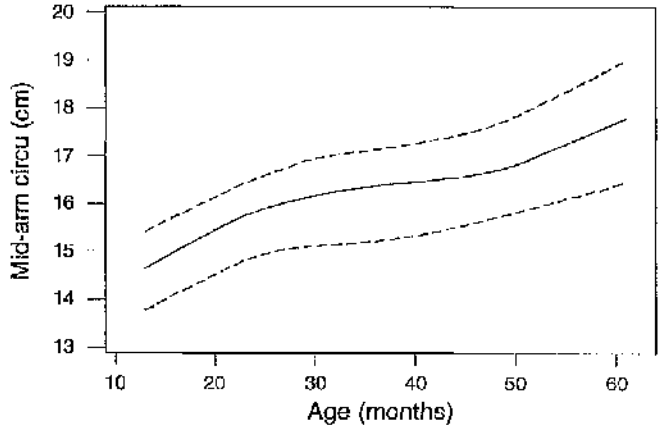

Height (m)
1.2
1.1
1.0
0.9
0.8
0.7
10
20
30
40
50
60
Age (months)

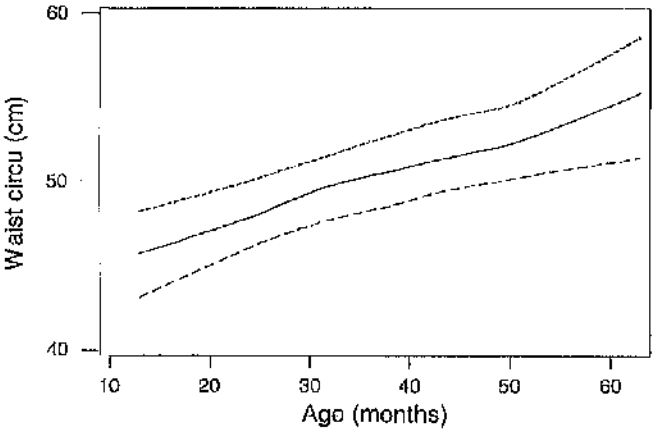

Height (m)
1.2
1.1
1.0
0.9
0.8
0.7
10
20
30
40
50
60
Age (months)

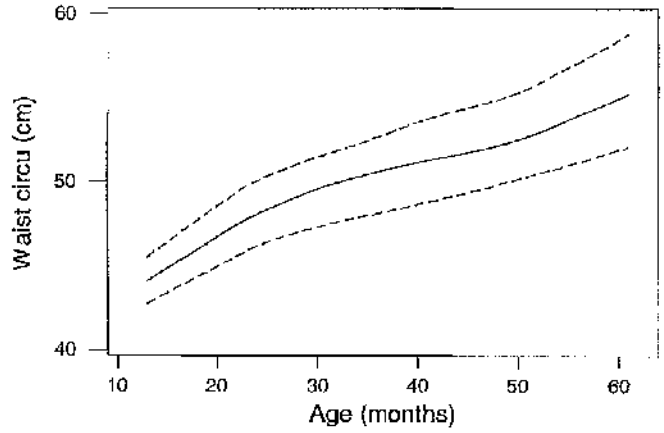

Head circu (cm)
53
52
51
50
49
48
47
46
45
10
20
30
40
50
60
Age (months)

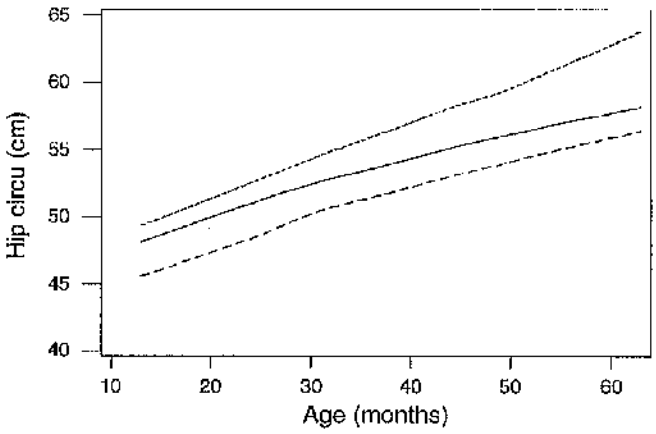

Head circu (cm)
53
52
51
50
49
48
47
46
45
10
20
30
40
50
60
Age (months)

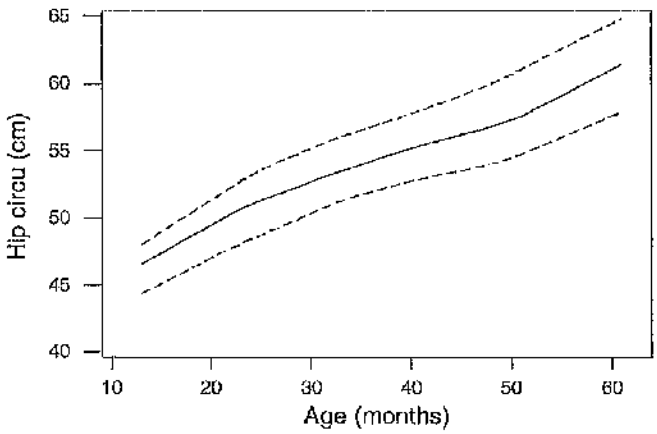

Mid-arm circu (cm)
20
19
18
17
16
15
14
13
10
20
30
40
50
60
Age (months)

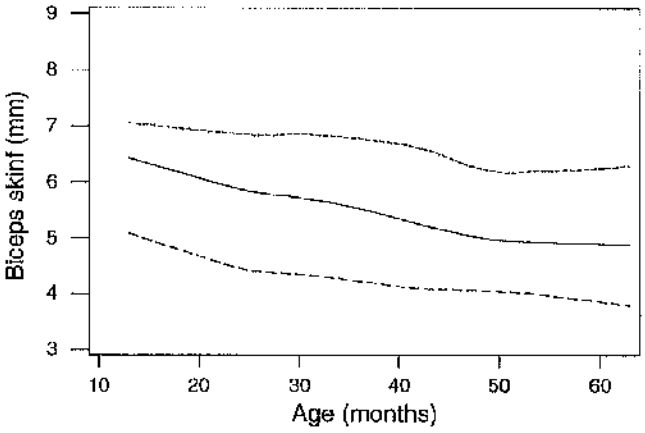

Mid-arm circu (cm)
20
19
18
17
16
15
14
13
10
20
30
40
50
60
Age (months)

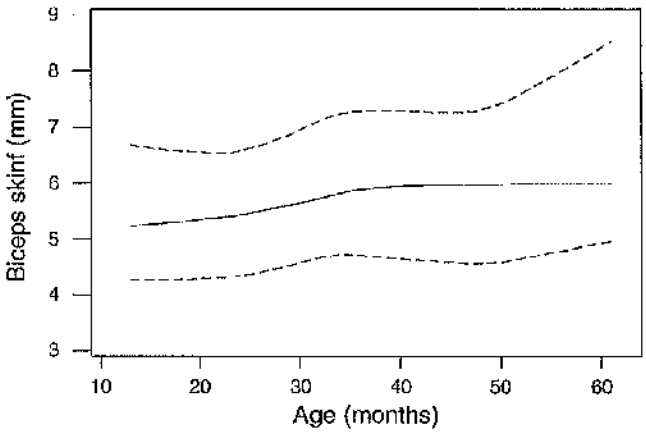

Waist circu (cm)
60
50
40
10
20
30
40
50
60
Age (months)

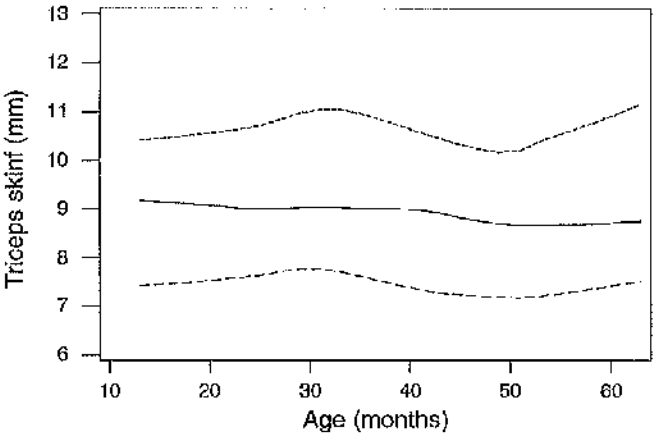

Waist circu (cm)
60
50
40
10
20
30
40
50
60
Age (months)

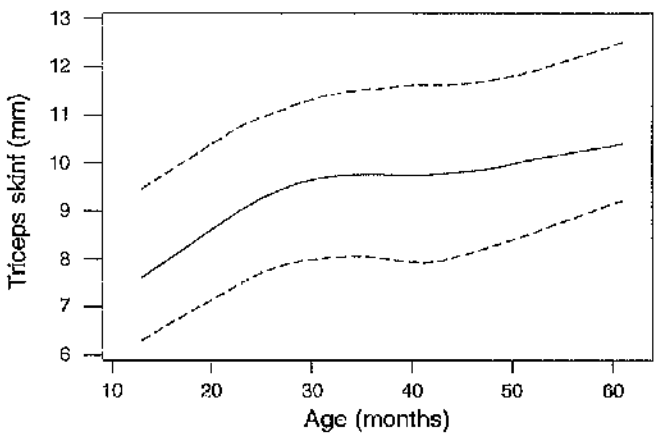

Hip circu (cm)
65
60
55
50
45
40
10
20
30
40
50
60
Age (months)

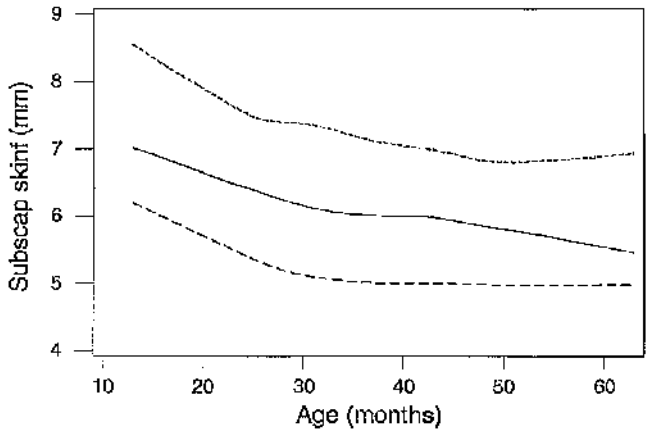

Hip circu (cm)
65
60
55
50
45
40
10
20
30
40
50
60
Age (months)

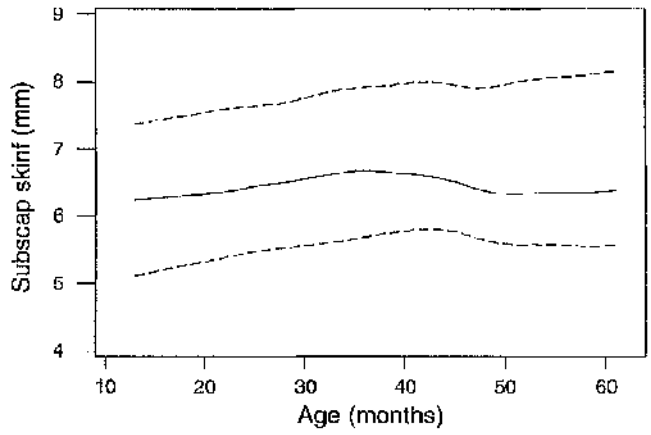

Biceps skinf (mm)
9
8
7
6
5
4
3
10
20
30
40
50
60
Age (months)

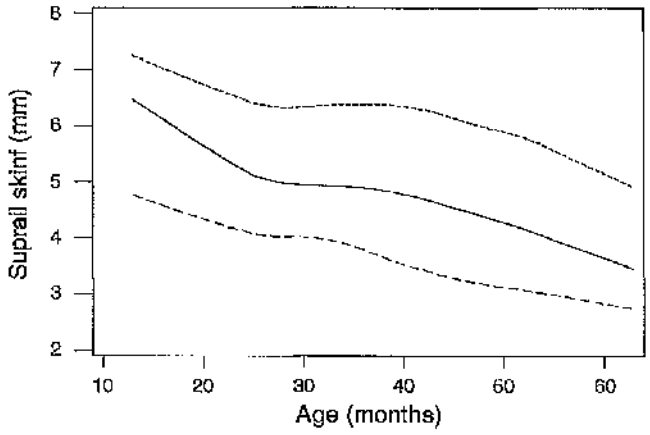

Biceps skinf (mm)
9
8
7
6
5
4
3
10
20
30
40
50
60
Age (months)

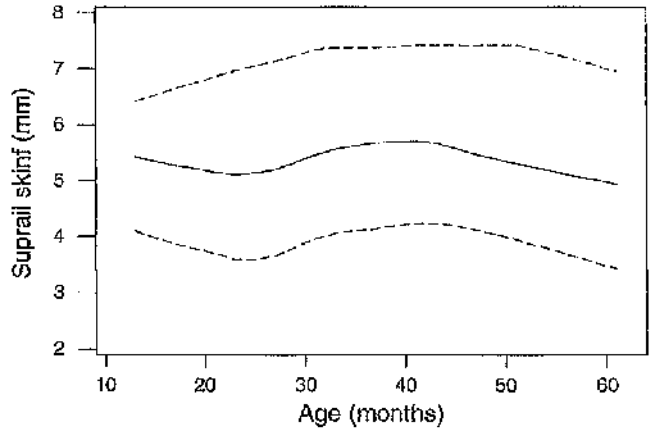

Triceps skinf (mm)
13
12
11
10
9
8
7
6
10
20
30
40
50
60
Age (months)

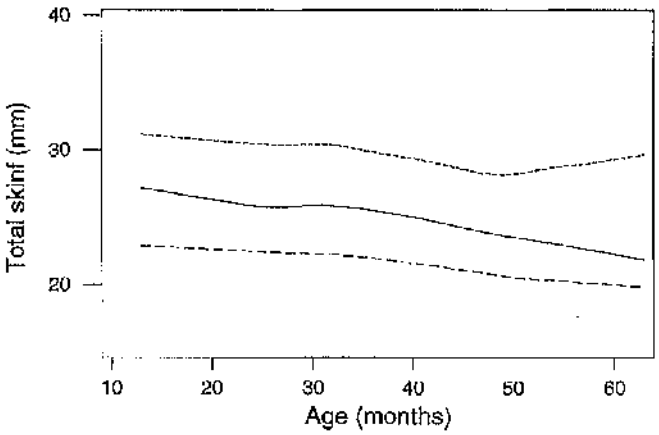

Triceps skinf (mm)
13
12
11
10
9
8
7
6
10
20
30
40
50
60
Age (months)











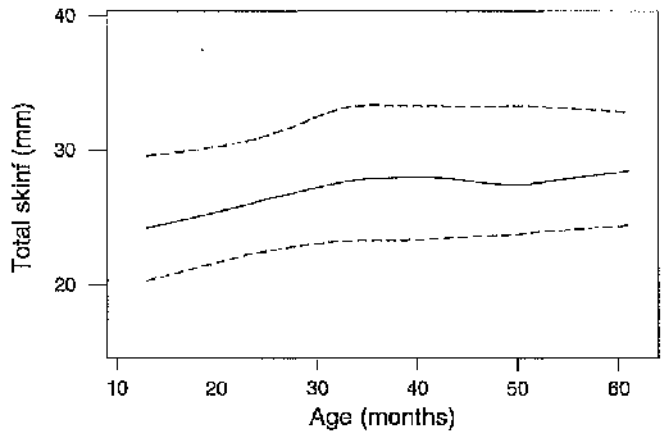


Figure 3.1 Percentiles for anthropometric indices in boys (left panel) and girls (right panel) aged 13-63 months. The 75th (top line), 50th (middle) and 25th (bottom line) distributions are shown in each graph.

3.3 Results

3.3.1 Prevalence and overweight of obesity

The prevalence of overweight and obesity in the present population showed an increasing tendency with age for both genders (from 5.5% to 11.9% in boys and from 10% to 13.6% in girls for obesity) (Table 3.1). The same tendency was observed when determining these rates based on the CDC 2000 growth charts (from 6.4% to 12.3% in boys and from 5.4% to 13.9% in girls for overweight). Although, there were no significant differences in BMI between boys and girls for any of the age groups (Table 3.2), obesity was more prevalent in girls than in boys in all age groups (for example, 13.6% of girls were obese at the age of 4-5 years compared to 11.9% of boys). A summary of the results for the prevalence of overweight and obesity based on both UK1990 and CDC 2000 reference data is given in Table 3.1.

3.3.2 Greek versus British growth reference data

The mean SDS and SD for weight and height from the Greek population were found to be significantly different from the reference population for age groups 2-3, 3-4 and 4-6 years and both genders (Table 3.3 and 3.4). In particular, Greek children of both genders were found to be heavier and taller than the British counterparts (Table 3.3 and 3.4). These differences were more prominent with increasing age in both genders. Although heavier and taller for all age groups, the Greek children differ significantly in SDS for BMI from the British ones only at 4-6 years of age. A summary of the mean SDS values and SD for BMI, weight and height and a graphical presentation of the differences between these values and the reference population for both genders are given in Tables 3.3, 3.4 and Figure 3.2 respectively.

Table 3.3 Mean SDS, 95% CIs, and SD for Greek boys produced from the 1990 British Growth Reference Charts.

Measurement	Age Group	Mean SDS	95% CI	SD	P value
BMI	1-2 years	-0.15	(-0.38 to 0.08)	1.12	0.205
	2-3 years	-0.004	(-0.14 to 0.13)	1.06	0.953
	3-4 years	0.07	(-0.04 to 0.18)	1.20	0.223
	4-5 years	0.23	(0.10 to 0.36)	1.21	**0.001**
Weight	1-2 years	0.56	(0.37 to 0.75)	0.91	**<0.001**
	2-3 years	0.73	(0.61 to 0.85)	0.95	**<0.001**
	3-4 years	0.71	(0.61 to 0.81)	1.05	**<0.001**
	4-5 years	0.83	(0.71 to 0.95)	1.06	**<0.001**
Height	1-2 years	1.03	(0.72 to 1.34)	1.51	**<0.001**
	2-3 years	1.09	(0.96 to 1.22)	1.01	**<0.001**
	3-4 years	0.99	(0.89 to 1.09)	1.04	**<0.001**
	4-5 years	1.02	(0.91 to 1.13)	0.99	**<0.001**

Table 3.4 Mean SDS, 95% CIs, and SD for Greek girls produced from the 1990 British Growth Reference Charts.

Measurement	Age Group	Mean SDS	95% CI	SD	P value
BMI	1-2 years	0.05	(-0.18 to 0.28)	1.13	0.676
	2-3 years	0.10	(-0.05 to 0.25)	1.09	0.194
	3-4 years	0.20	(0.09 to 0.31)	1.10	**<0.001**
	4-5 years	0.27	(0.15 to 0.39)	1.11	**<0.001**
Weight	1-2 years	0.52	(0.31 to 0.73)	1.00	**<0.001**
	2-3 years	0.76	(0.61 to 0.91)	1.09	**<0.001**
	3-4 years	0.78	(0.67 to 0.89)	1.07	**<0.001**
	4-5 years	0.74	(0.63 to 0.85)	1.03	**<0.001**
Height	1-2 years	0.70	(0.44 to 0.96)	1.26	**<0.001**
	2-3 years	1.04	(0.88 to 1.20)	1.14	**<0.001**
	3-4 years	1.06	(0.95 to 1.17)	1.09	**<0.001**
	4-5 years	0.94	(0.82 to 1.06)	1.10	**<0.001**

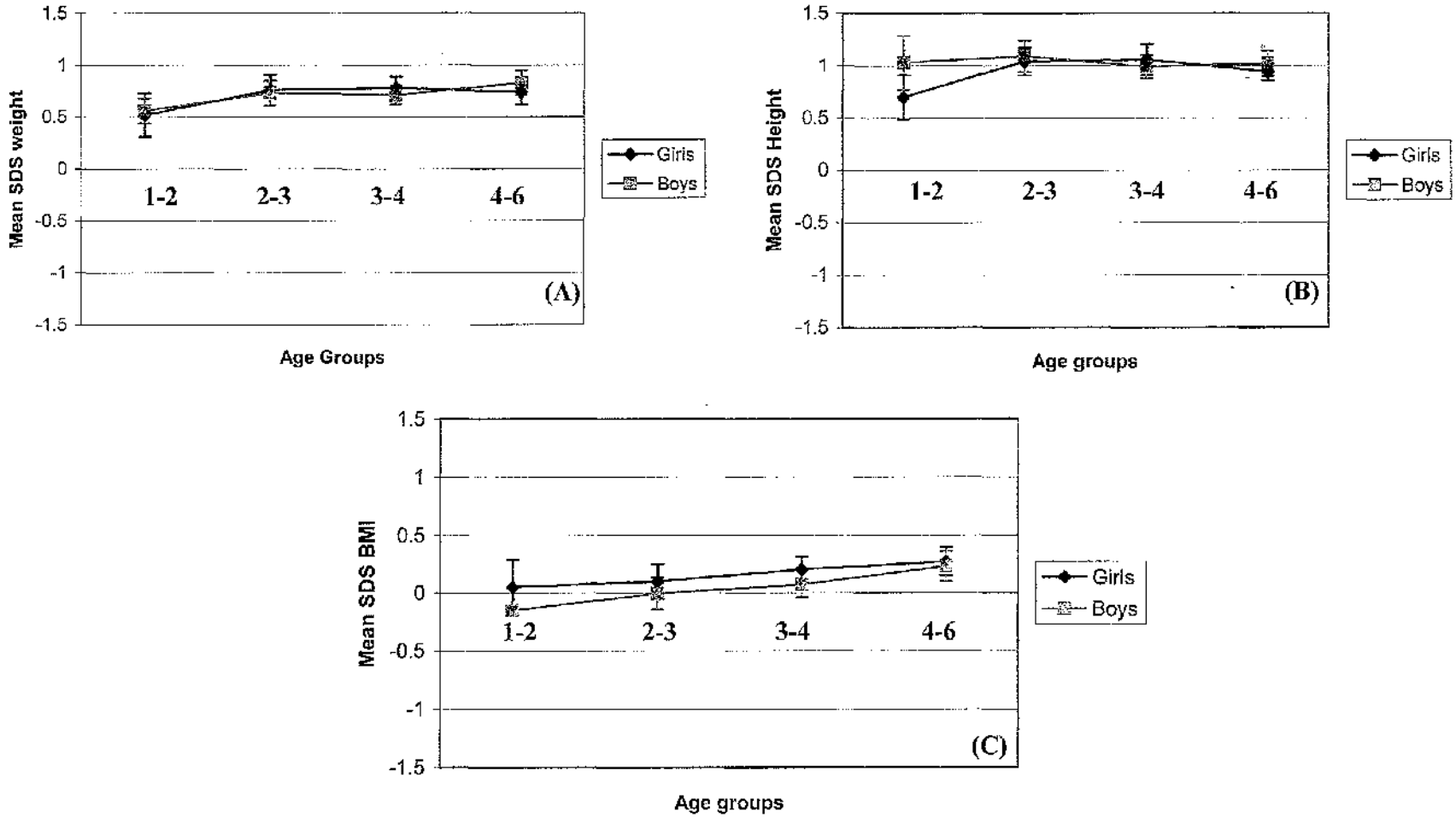


Figure 3.2 (A) Mean weight SD score and 95% CI relative to the revised UK 1990 reference data. (B) Mean height SD score and 95% CI relative to the revised UK 1990 reference data. (C) Mean BMI SD score and 95% CI relative to the revised UK 1990 reference data.

3.3.2 Greek versus US reference data

Comparison of anthropometric indices, such as arm and waist circumferences, as well as triceps and subscapular skinfolds between the present population and reference data from US children (NHANES III) revealed similar patterns of development for both populations (Figure 3.3). In both Greek and US boys (2-6 years), the waist and arm circumference increase with age. Greek boys showed significantly higher waist circumference measurements compared to their US counterparts at all ages; for example the mean waist circumference for Greek boys (3-4 years) was 52.95 cm versus 51.4 cm in the US boys ($P \leq 0.001$) (Figure 3.3). In contrast, US boys had significantly higher arm circumference compared to Greeks at ages 1-5 years (Figure 3.3). Although significant differences between the present cohort and the US population were observed in waist and arm circumferences, Greek boys did not differ significantly from the US boys in triceps and subscapular skinfolds at any ages (Figure 3.3), but showed similar patterns of adiposity development. In particular, triceps and subscapular skinfolds decrease with age in both Greek and US boys, with the former showing slightly higher (but not significantly) scores for these subphenotypes than the latter at most of the ages (Figure 3.3). However, the aforementioned decline in triceps skinfold was not obvious for the present population at the ages of 1-2 and 5-6 years, probably due to the smaller number of subjects at these age groups in the Greek (79 and 43 respectively) compared to the US population (644 and 492 respectively).

In both Greek and US girls, waist and arm circumference increased with advancing age similarly to those in boys (Figure 3.3). Although Greek girls were found to have significantly higher waist circumference than their US counterparts at all ages, the former showed significantly lower arm circumference measurements than the latter at ages 1-2, 3-4 and 4-5. Although significant differences between the Greek and the US girls have been

observed in waist and arm circumferences, they did not differ significantly in triceps and subscapular skinfolds at ages 2-5 (Figure 3.3). Both triceps and subscapular skinfolds increase with increasing age with Greek girls showing slightly (but not significantly) higher mean values at these ages (Figure 3.3). Significant differences in these skinfolds observed between Greek and US girls at the ages of 1-2 and 5-6 years may be again due to the smaller number of individuals in these groups in the Greek population (80 and 39 respectively) compared to those in the US (625 and 554 respectively).

3.4 Discussion

In the present study (2102 children), although the prevalence of overweight was found to be similar for both genders (10.6% for boys and 10.3% for girls), the rates of obesity were higher in girls compared to that in boys (9.7% and 7.5% respectively) based on the 1990 UK reference data (Table 3.1). These estimates were higher when based on the CDC 2000 growth charts for both genders (10.6% of boys and 15.03% of girls being overweight, while 10.25% and 9.8% being obese) although the 85^{th} and 95^{th} percentiles, which correspond to similar BMI values between different national reference data (Reilly, 2002) were used in both cases (Table 3.1). In a larger subset of the GENESIS (Growth, Exercise and Nutrition Epidemiological Study In preSchoolers) cohort (2374 Greek children), the prevalence of overweight and obesity according to the CDC 2000 growth charts were found to be even higher (Manios, 2006) than those estimated in the present population (17.4% and 17.2% of boys being overweight and obese, respectively), possibly due to the inclusion of additional children with high BMI in the analysis. These differences between estimates of the UK 1990 and the CDC definition could suggest that the former underestimates overweight and obesity in the Greek population. When compared to findings from the Health Survey for England (2004) that assessed overweight and obesity

rates in children aged 2-10 years (15% and 16% of boys, as well as 15% and 13% of girls were found to be overweight and obese respectively) based on the 1990 Growth reference dataset, Greek children showed lower prevalence of overweight and obesity in both genders (Figure 3.4). A higher prevalence of overweight and obesity, compared to those in the present population, was also observed in a representative US population of a similar age, where 27.3% and 15% of boys, as well as 25.2% and 12.6% of girls aged 2-5 years were found to be overweight and obese according to the CDC 2000 growth reference (Ogden et al., 2006) (Figure 3.5). When compared to the findings from other large and similarly aged populations from Europe, Australia, China and Mexico, Greek children of the present cohort show similar alarming rates of overweight and obesity based on the IOFT cut-off points (Figure 3.6). Previous studies in Greek children, although limited, have also reported high rates of overweight and obesity in specific geographical regions (Krassas et al., 2001;Magkos et al., 2005), rather than in a representative sample of the population. The lowest prevalence rates were observed in a nationwide study in Greek children aged 11-16 years (13.4% overweight and 3.7% obese, CDC 2000 charts), but these were based on self-reported data for weight and height, which can underestimate obesity (Karayiannis et al., 2003). Disparities in these estimates may be due to several potential factors, such as the employment of different BMI cut-off points, the representativeness of the population (national or regional), the sample size, the age and sex of the subjects, as well as differences in measurement methods and time periods of data collection (Lissau et al., 2004).

Body composition undergoes marked changes in absolute amounts and relative proportions of water, protein, lipid and mineral mass during the first years of life (Fomon et al., 1982). Growth of both fat mass index (FMI) and fat-free mass index (FFMI), which constitute the two main components of BMI, are influenced by age and gender; FFMI increases steadily

with advancing age, whilst FMI decreases rapidly during childhood after reaching a peak in infancy and starts increasing again towards adulthood. These changes in BMI during early childhood for both genders are illustrated well in Figure 3.1 where BMI was observed to increase (adiposity rebound) after its developmental nadir. Children from the current study experience an early adiposity rebound (as defined between the 48th to the 61st month of age), reflecting along with other studies (Dorosty et al., 2000) a secular trend towards earlier adiposity rebound possibly due to strong environmental influences. This increase in BMI after 48 months seems to be faster in girls than boys and especially in girls above the 75th percentile (Figure 3.1). However, it is not clear whether this rebound in BMI is attributed to FM or FFM, since BMI does not distinguish lean or fat masses. Furthermore, skinfold thickness, although being reliable indices of regional fat and being highly correlated with arm circumference, they give no information on lean mass (Wells and Fewtrell, 2006). In the present study, biceps skinfold decrease, while arm circumference increase in boys suggesting that BMI rebound may be attributed to changes in FFMI (muscle and/or bone mass) rather than FMI. In girls, in contrast, arm, waist and hip circumferences, as well as biceps, triceps and suprailiac skinfolds are rising simultaneously during this period indicating that this is a genuine increase in FMI rather than FFMI (Figure 3.1). As for BMI, the increase in these indices seems to be faster in girls above the 75th percentile.

In the present study, the mean values for anthropometric measurements, such as BMI, weight, height and waist circumference were similar to those reported in other national surveys in Europe (Haschke and van't Hof, 2000;McCarthy et al., 2005;Savva et al., 2005) indicating a similar pattern of growth in preschoolers. There is also increased evidence that modern children are getting fatter faster than those in the past (Reilly, 2005). However, such secular trends in adiposity during early childhood are not always recognizable by

changes solely in BMI, since quantitative changes in body composition (defined as the ratio between FFM and FM) do not always reflect a change in BMI (Hall and Cole, 2006).

Previous studies have shown that, although modern children had higher FMI than those in the past, these changes in body composition were not obvious by changes in BMI, since they were accompanied by a reduced FFMI. In the present population, children of both genders were found to be significantly taller and concomitantly heavier than their British counterparts (Tables 3.3 and 3.4, Figure 3.2). The observed increase in mean height in the present population could be an indicator of a secular trend for accelerated natural growth and improved nutritional status and could explain the absence of significant differences in the mean BMI between the Greek children in the present and the British children 2 decades ago, since BMI is independent of height (Freedman et al., 2001). Additionally, an early adiposity rebound observed in the Greek children could also be suggestive of a rapid growth (Freedman et al., 2001). An age-related trend towards higher BMI in the present population, when compared to the UK reference data, in association with the evolution of the growth curves (Figure 3.1) could further support the notion of a continuing secular trend to an increase in childhood fatness. Nevertheless, previous studies, in which the BMI of British toddlers (Stenhouse et al., 2004) and preschoolers (Reilly et al., 2006) were compared with the 1990 UK reference data, have shown that the excess BMI SDS was 0.26 and 0.39 respectively. These values are very similar to the ones in the present population and could be indicative of increased fatness in modern children. Additionally, when compared to the US population, in which the onset of childhood obesity epidemic occurred a decade before that in other populations (Goran, 2001), Greek children showed a similar development of other anthropometric indices, as illustrated in Figure 3.3.

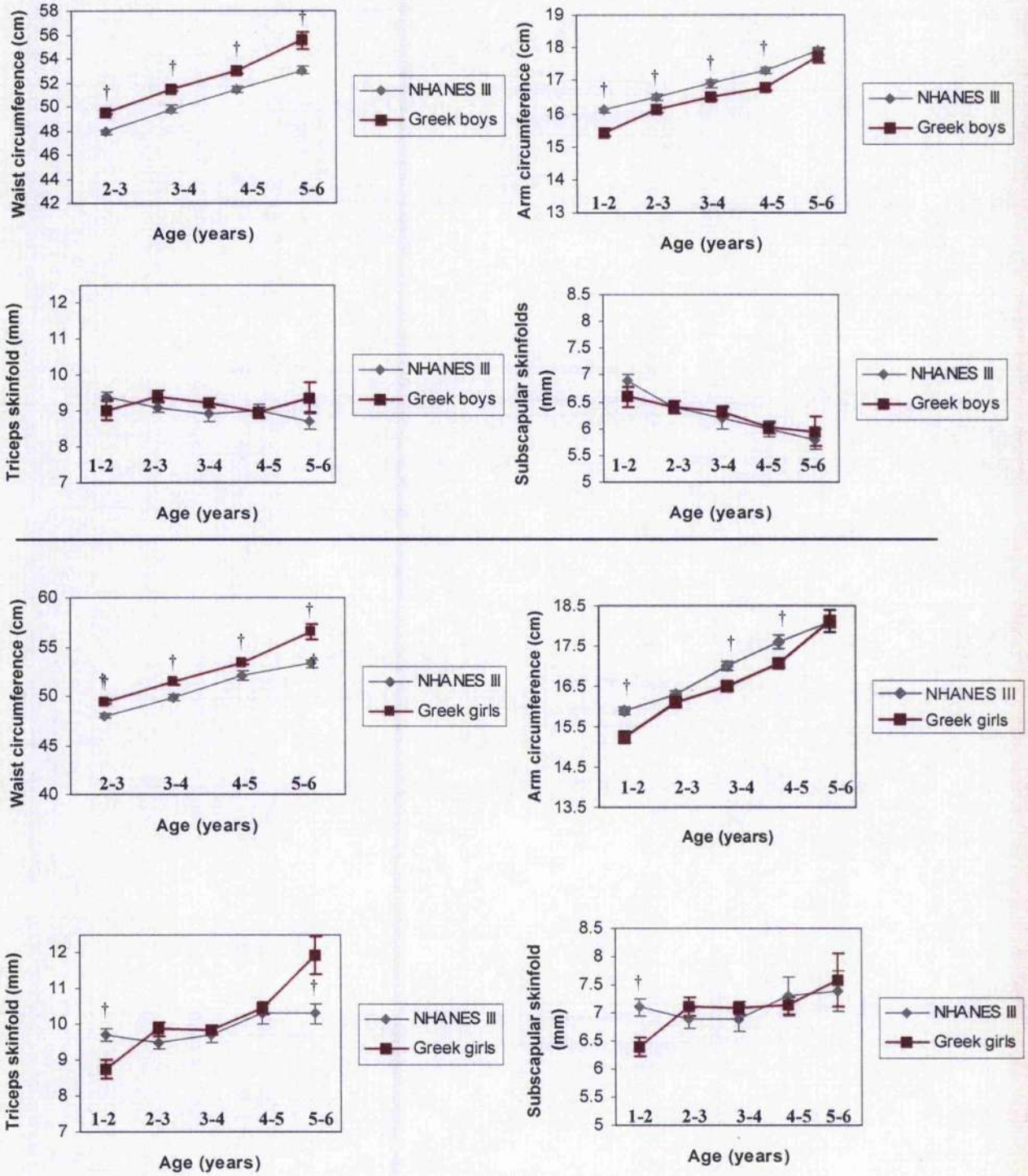


Figure 3.3 Comparison of mean values for waist and arm circumferences, triceps and sub scapular skinfolds between NHANES III (1988-1994) data and Greek children (present study) for boys (top panel) and girls (bottom panel) at different ages. 95% CIs for these phenotypes are also shown. (†) indicates statistically significant differences (P<0.05).

In conclusion, the present study, carried out on a large representative sample of toddlers and preschoolers from Greece, is the first cohort of toddlers and preschoolers that includes such a wide range of adiposity-related phenotypes. The present population had similar prevalence of obesity relative to other preschool populations. Compared to normative data from British children, Greek children were much taller and concomitantly heavier suggestive of improved nutritional status and accelerated growth and they showed a tendency for increasing BMI with advancing age. The excess BMI levels were similar to those found in modern preschoolers and most likely attributed to increased adiposity.

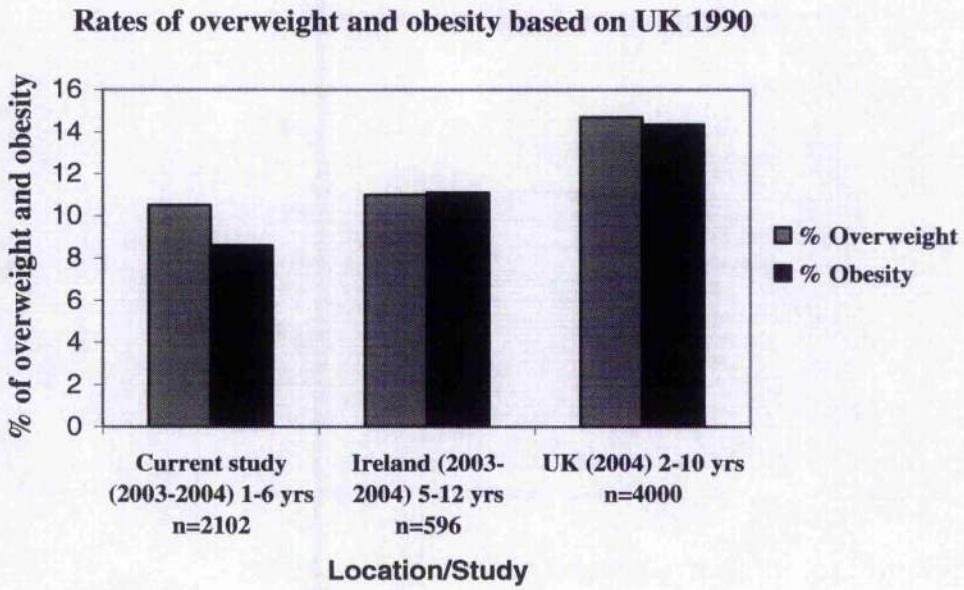


Figure 3.4 Prevalence of overweight and obesity based on the UK1990 reference data. Grey and black bars represent % of overweight and obesity respectively. The period during which each study was carried out, as well as the number and the age of subjects in each study are also given. Data were nationally representative for both Ireland ((O'Neill et al., 2007) and UK (Health Survey for England 2004).

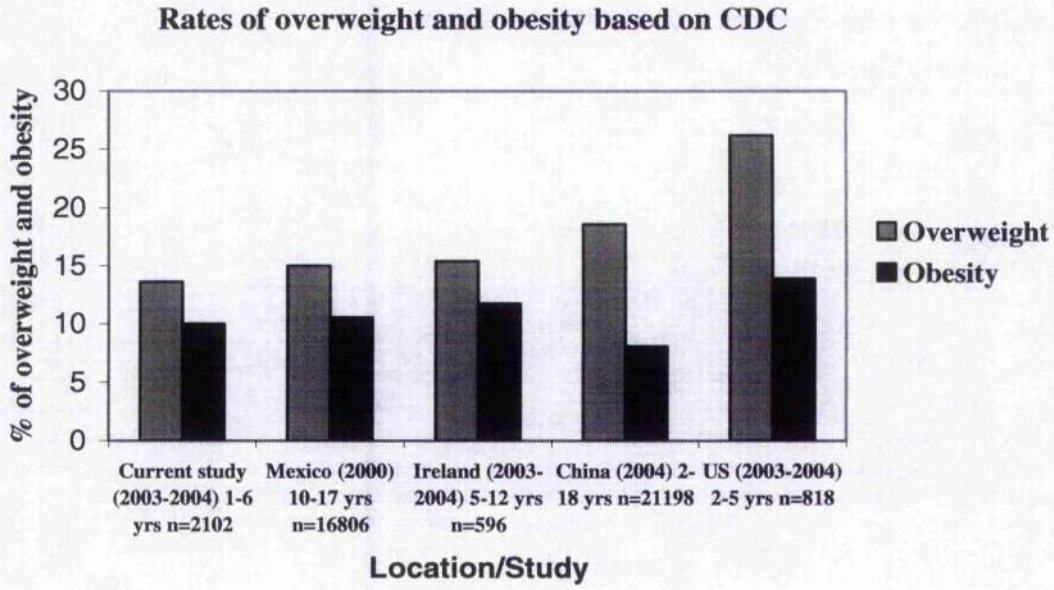


Figure 3.5 Prevalence of overweight and obesity based on the CDC 2000 reference data. Grey and black bars represent % of overweight and obesity respectively. The period during which each study was carried out, as well as the number and the age of subjects in each study are also given. Data were nationally representative for Mexico (Rio-Navarro et al., 2004), Ireland (O'Neill et al., 2007) and US (Ogden et al., 2006)and regionally representative for Beijing China (Mi et al., 2006).

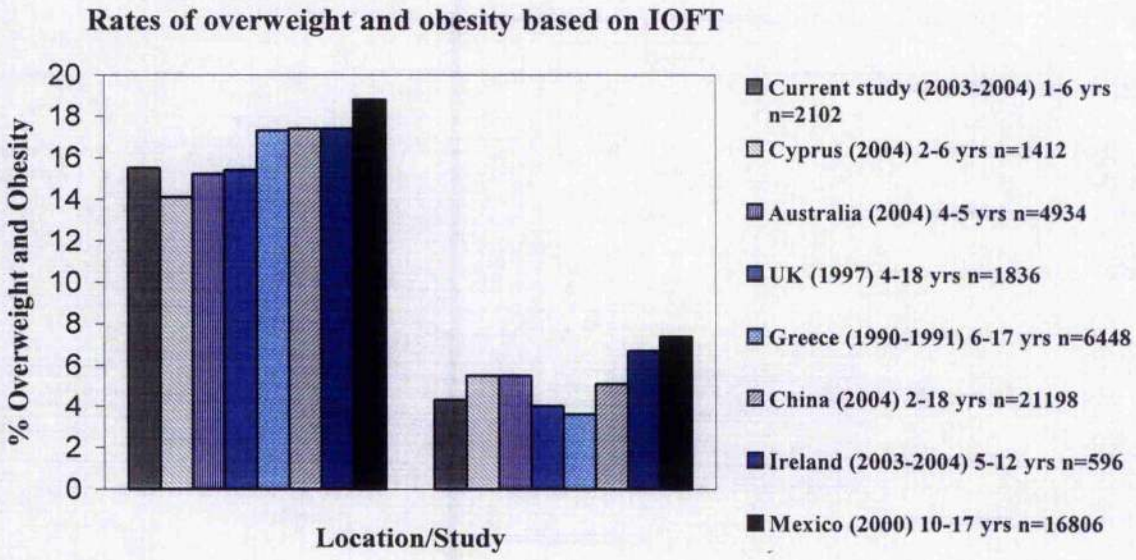


Figure 3.6 Prevalence of overweight and obesity based on the IOFT cut-off points. Group of bars on the left and on the right represents % of overweight and obesity respectively. The period during which each study was carried out, as well as the number and the age of subjects in each study are also given. Data were nationally representative for Cyprus (Savva et al., 2005), Australia (Wake et al., 2007), UK (Jebb et al., 2004), Greece (Georgiadis and Nassis, 2007), Ireland (O'Neill et al., 2007) and Mexico (Rio-Navarro et al., 2004) and regionally representative for Beijing China (Mi et al., 2006).

Chapter 4

Effect of *ACE* I/D polymorphism on adiposity-related phenotypes in Greek children

4.1 Introduction

The *ACE* gene, encodes a carboxypeptidase that is a key component of the RAS and converts angiotensin I into a biologically active hormone, angiotensin II (a potent vasoconstrictor) (Bernstein et al., 1989). *ACE* also has the ability to hydrolyze numerous other peptide substrates (Hooper and Turner, 2003). Although *ACE* gene is highly polymorphic, work is historically focused on the I/D polymorphism with alleles characterised by the presence (insertion, allele 'I') or absence (deletion, allele 'D') of a 287bp *Alu* repeat sequence in intron 16 (Rigat et al., 1990). Although *ACE* I/D is not the causative variant for serum *ACE* levels, a better candidate being rs4363 (a SNP at genomic nucleotide position 22982) (Zhu et al., 2000;Cox et al., 2002), I/D is a sufficiently good marker of circulating *ACE* activity in Caucasian populations, since it is in almost complete linkage disequilibrium with the functional rs4363 variant (Soubrier et al., 2002). Moreover, in a previous study in older Greek girls (11-18 years old), the *ACE* I/D polymorphism showed a stronger degree of association with obesity-related phenotypes that was not exceeded by other *ACE* polymorphisms (Moran et al., 2005).

Previous studies, albeit in adults, have shown somewhat contradictory findings (Cooper et al., 1997;Strazzullo et al., 2003;Um et al., 2003;Riera-Fortuny et al., 2005). It is unclear whether *ACE* exerts an appreciable influence on adiposity, although hints of such an effect are beginning to outweigh the counterarguments. Difficulties have included differences in age and ethnicity between studies, as well a lack of statistical power. There are no data on *ACE* association with adiposity in young children. With these limitations in mind the present study aimed to assess whether associations between the I/D polymorphism and adiposity-related phenotypes exist in the large population of Greek toddlers and preschoolers from the GENESIS study (Manios, 2006).

4.2 Methods

4.2.1 Subjects and phenotypes

The number of subjects that took part in the study, as well as information on the assessed adiposity-related phenotypes and the protocols used for anthropometry, are given in Chapter 2 (General Methods). The anthropometric measurements assessed in the current analysis included: BMI, arm, waist and hip circumferences, as well as sum of skinfolds.

4.2.2 DNA extraction and ACE I/D genotyping

The method used for DNA extraction from all buccal samples is given in Chapter 2 (General Methods). Details on the set up of PCR reactions and digestions for *ACE* I/D genotyping are also given in the same section.

4.2.3 Statistical analyses

Data and statistical analyses carried out are described in detail in Chapter 2 (General Methods).

4.3 Results

4.3.1 Genotyping and phenotyping

2102 children (1095 boys and 1007 girls) were included in the phenotypic analyses and marked differences between boys and girls were found (Figure 3.1), a reflection of gender-specific differences in growth and development. Genotyping for the *ACE* I/D polymorphism was successful in 2008 individuals (1037 boys and 971 girls). The population was found to be in HWE at this locus ($\chi^2_{(df=1)}$=1.64, P=0.199) and the overall allele frequencies were $f_{(I)}$=0.39 and $f_{(D)}$=0.61. Overall allele frequencies matched the frequencies ($\chi^2_{(df=1)}$=0.007, P=0.936) observed in a Greek cohort of adolescents (Moran et al., 2005;Manios, 2006). There were no differences in genotype frequencies between boys and girls ($\chi^2_{(df=2)}$=0.731, P=0.694).

4.3.2 GLM analysis for ACE I/D polymorphism

GLM analysis was carried out in the total population (boys and girls) to assess the main effect of the *ACE* I/D polymorphism on adiposity-related phenotypes and whether these genetic effects were influenced by gender and/or age. GLM analysis has shown that the *ACE* I/D individually had a significant main effect on BMI (P=0.019) and an almost significant effect on waist circumference (P=0.055). For the same polymorphism, significant interactions were observed between age and genotype for BMI (P=0.023) (Figure 4.1) and close to significance for waist circumference (P=0.074). Based on these findings, further ANOVA analysis was carried out stratified by single-year age groups but not gender. ANOVA analysis has revealed significant association between the I/D genotypes and BMI in 1-2 year old boys and girls (P=0.003) that explained 6.4% of the

phenotypic variation. In particular, boys and girls aged 1-2 years bearing the II genotype showed significantly higher BMI compared to those carrying the DD genotype (Figures 4.1 and 4.2). Boys and girls aged 2-4 years carrying the II genotype had also higher (but not significantly) BMI compared to those with the DD genotype. (Figure 4.1) However, at the age of 4 years, boys and girls with the DD rather than II genotype had higher, but not significantly, BMI indicating a change in the direction of I/D genotypic effect (Figure 4.1).

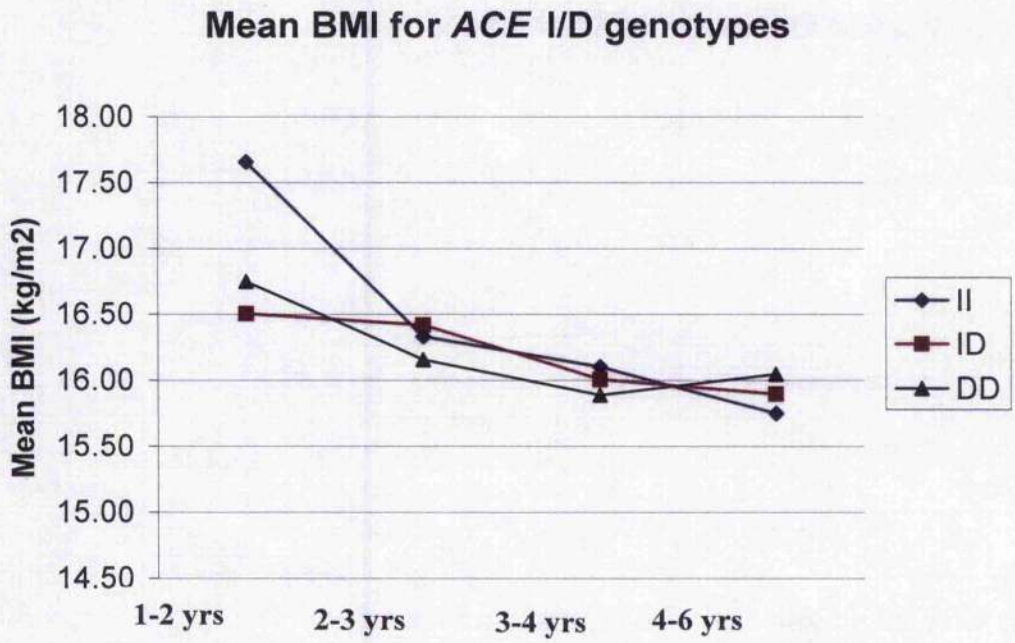


Figure 4.1 Effects of *ACE* I/D genotypes on mean BMI in boys and girls in each age group.

4.3.3 ACE I/D genotype associations with phenotypes

After performing the Dunn-Sidak correction for multiple testing for each age group, differences between the means of the I/D genotypes for BMI (α value=0.014) in boys aged 1-2 years (Figure 4.3), as well as for BMI, waist and arm circumferences (α value=0.022) in girls aged 4-6 years (Table 4.1) were found to be statistically significant. ANOVA tests revealed that boys 1-2 years old with the II genotype had significantly higher mean values for BMI (P=0.001) than did boys with either ID or DD genotypes (Figure 4.1). This finding was reflected in the main GLM analysis. At 4-6 years of age, girl carriers of the D allele had higher BMI (P=0.018), as well as higher arm (P=0.013) and waist (P=0.001) circumferences than girls with the other genotypes (Table 4.1). However, these isolated significant associations were not reflected in the GLM analysis. The mean values for all anthropometric parameters according to gender and *ACE* I/D genotypes are shown in the Appendix 1 (Tables 1 and 2).

4.3.4 Determination of the type of genetic effect observed for associations with the I/D polymorphism

To determine the genetic mechanism for the significant associations, three genetic models (additive and dominance models) were tested using correlation analysis against model genotype scores. For the GLM analysis, a D-dominant model could explain 91% (P=0.001) of the genetic variance observed for BMI in boys and girls aged 1-2 years. For the gender and age subgroup analysis, an additive model best explained the statistically significant associations observed between I/D genotype and BMI, waist and arm circumferences in girls 4-6 years accounting for 96% of the genetic variance for BMI and 99% for both circumferences. For the same analysis, a D-dominant model could explain 98% of the genetic variance for BMI in boys aged 1-2 years.

4.3.4 ACE I/D genotypic effects on the phenotypic distribution

As already mentioned, significant associations of the I/D polymorphism with adiposity-phenotypes could be due to a small number of extreme individuals influencing the ANOVA analysis, or to a distributed effect of the polymorphism on most of the population. For the GLM analysis, the influence of the genotype on the distribution of BMI in boys and girls 1-2 years was also assessed by calculating odds ratios and visualised by using probability plots. When tested by odds ratio, the DD homozygotes were underrepresented in both the lowest and highest quartiles for BMI, while the II homozygotes were almost significantly underrepresented and significantly overrepresented in the lowest and the highest quartiles, respectively (Table 4.2). The significant odds ratio in the whole range of the distribution could indicate a distributed effect of the I/D genotype on most of the population for BMI. This effect is also illustrated in Figure 4.4 where the cumulative frequency distribution of BMI within I/D genotypes is plotted in a normal probability scale. For the age and gender subgroup analysis, when tested by odds ratios, II homozygotes (n=11) were significantly underrepresented (0/11) in the lowest and overrepresented (9/11) in the highest quartiles for BMI, indicating that the effect of the I/D genotype was throughout the phenotypic range in boys aged 1-2 years, while in girls 4-6 years old DD homozygotes were significantly underrepresented in the lowest quartile for arm and waist circumferences and II homozygotes significantly overrepresented in the same quartile for arm and waist circumferences (Table 4.3). However, neither genotype (DD or II) was significantly over- or underrepresented in the highest quartile for these phenotypes indicating that the effect of the I/D genotype was mainly in the thinner end of the population distribution.

Table 4.1 Analysis of associations between I/D genotypes and adiposity-related phenotypes in girls 4-6 years.

ACE I/D	BMI	Arm Circumference	Waist Circumference
Girls 4-6 yrs (N=50, 142, 126)	II=15.41 (15.01 to 15.84) ID=15.89 (15.63 to 16.16) DD=16.16 (15.87 to 16.47) V=2.5%, P=0.018	II=16.58 (16.20 to 16.99) ID=16.93 (16.67 to 17.20) DD=17.32 (17.04 to 17.60) V=2.7%, P=0.013	II=51.52 (50.45 to 52.67) ID=52.93 (52.27 to 53.61) DD=53.97 (53.21 to 54.77) V=4.0%, P=0.001

Summary of associations between I/D genotypes and particular adiposity-related parameters assessed ANOVA in girls 4-6 years. For each test, mean values, 95% confidence intervals are given, as well percentage variance explained by ANOVA (V), probability (P), and the number (N) of individuals of II, ID a DD genotypes respectively. P values are given to three significant figures. Only age groups with at lea one significant association after Sidak correction are shown.

Boys and girls (1-2 years)

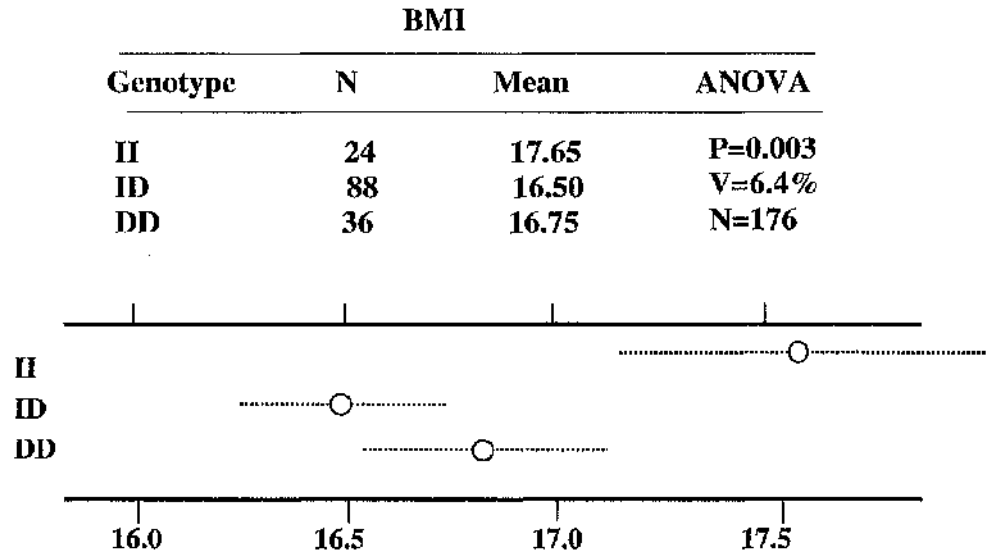


Figure 4.2 *ACE* I/D genotype differences in BMI for 1-2 year old boys and girls after GLM analysis. P=probability, V=observed variance explained, N=number within group.

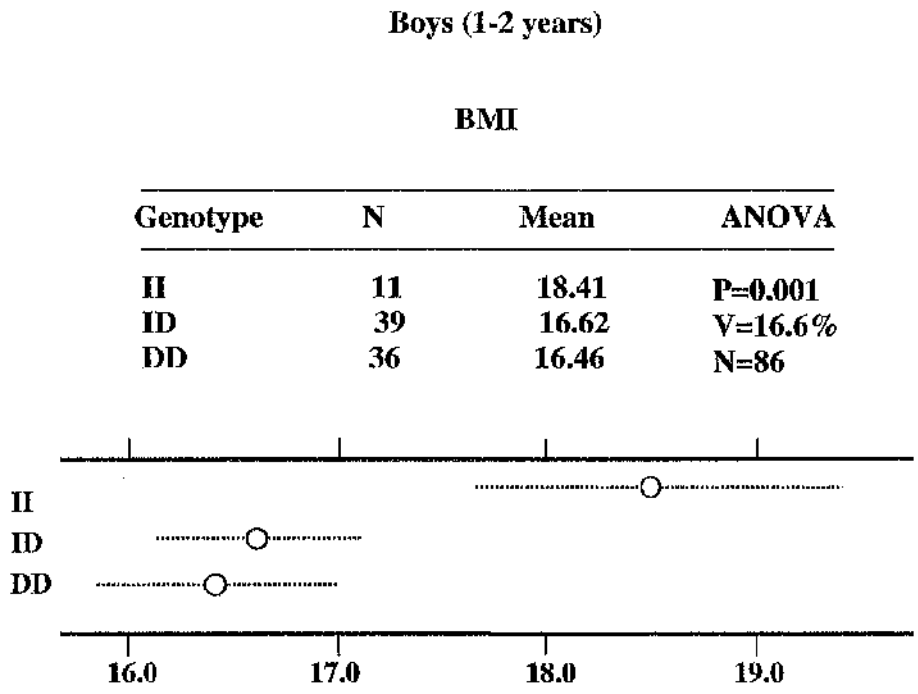


Figure 4.3 *ACE* I/D genotype differences in BMI and subscapular skinfold for 1-2 year old boys. P=probability, V=observed variance explained, N=number within group.

4.4 Discussion

Obesity is a complex disorder caused by genetic and environmental factors and interactions between them (Prentice and Jebb, 1995). It is expected that the influence of genetics is mediated through the minor contributions of many individual susceptibility genes (Marti et al., 2004). Longitudinal twin studies have suggested that these genetic determinants have a greater influence on the development of obesity than environment in young populations (Maes et al., 1997). In this study, the effects of the *ACE* I/D polymorphism on adiposity indices were investigated in a population of toddlers and preschoolers by two different approaches. GLM analysis on the total population has revealed a significant main effect of the I/D genotype on BMI (P=0.019) and a significant interaction between I/D genotype and age for BMI (P=0.023) (Figure 4.1). Further ANOVA analysis stratsfied by age has revealed that children (boys and girls) aged 1-2 years with the II genotype had singificantly higher BMI than those with the DD genotype (Figure 4.2). Employing the second approach of analysis with gender and age subgrouping has also revealed that boys aged 1-2 years bearing the I-allele showed significantly higher BMI (P=0.001) that explained 17% of the phenotypic variance (Figure 4.3). No previous studies have associated the *ACE* I allele with higher adiposity measurements and therefore these significant findings did not confirm hypothesis 1 (see Introduction). In contrast, age and gender subgrouping analysis has shown that the D-allele was significantly associated with higher BMI waist and arm circumferences in girls aged 4-6 years (Table 4.1), a finding consonant with hypothesis 1 and with previous studies in teenagers (Moran et al., 2005) and adults (Strazzullo et al., 2003;Riera-Fortuny et al., 2005). The observation that these significant findings in girls 4-6 years were not reflected in the GLM analysis could be an evidence that these associations are Type I errors. It is also possible though that gender and genotype do interact, but the study lacks the power to detect such an interaction.

Although greater genetic effects are likely to be found in younger populations due to absence of confounding environmental factors, as previously suggested (Maes et al., 1997) and as shown in the current study, it is possible that *ACE* genotype only influences adiposity during a particular set of developmental windows, presumably because of other events and processes occurring at that time. As Figure 4.1 indicates, although subjects with the II genotype show higher BMI than those with the DD genotype between the ages of 1-4 years, children aged 4-6 years signal in an opposite way with the DD rather than II genotype showing higher (but not significantly) BMI. However, by comparing the 4-6 age group of boys with the corresponding age group of girls (4-6 years), it is clear that although girls bearing the D allele show higher BMI than those with the I allele, the opposite is observed in boys (i.e. the I allele carriers shows higher BMI) (Appendix 1, Tables 1 and 2). In the population studied here, BMI decreased until the age of about 4 years (48 months) (Figure 3.1), and then began to rise in both sexes. As Figure 3.1 indicates, all the measured subphenotypes (arm and waist circumference and biceps skinfold) are rising simultaneously during this period in girls, suggesting that this is a genuine increase in adiposity. In boys, in contrast, as BMI increases after 48 months, biceps skinfold decreases and levels off, while arm circumference increases (Figure 3.1), suggesting that BMI increase at this time may be as much related to an increase in muscularity and/or bone volume as to an increase in adipose tissue volume. The finding of significant associations in girls but not in boys at this time therefore suggests that its effect is mediated though adipose tissue metabolism and growth, rather than other growth processes. The fact that these isolated significant associations are not reflected in the GLM analysis could not exclude the possibility that gender, as age, is also a significant factor in modifying genotype-phenotype interactions. However, the present study lack the power to detect such an interaction, since gender differences in the effects of I/D genotype on BMI were observed only in one age group (i.e. 4-6 years). Furthermore, in view of the

apparently contrary results obtained with younger boys and girls (Figure 4.2) and the significant interaction observed between age and I/D genotypes, the finding of altered expression of different angiotensin receptor types during the first years of life may be significant (Viswanathan et al., 2000).

The influence of the *ACE* I/D polymorphism on adiposity was found to be relatively small (see percentage of phenotypic variance in Figure 4.2), as expected for a complex trait, and it is presumed that the effect is mediated possibly through interactions with other susceptibility genes and/or environmental factors. A distributed effect of the I/D genotypes over the whole phenotypic range, as shown by odds ratios (Table 4.2, Figure 4.4), also supports the fact that the *ACE* is a polygene quantitative trait locus. Environmental factors such as nutritional status and physical activity may also modulate the extent to which the I/D polymorphism influences obesity, as previously suggested (Frederich, Jr. et al., 1992;Moran et al., 2005). In particular, the previous study in Greek teenagers (Moran et al., 2005) revealed significant associations between the DD genotype and larger skinfold thickness in inactive females, but not in inactive males or active females. The young children in the GENESIS study showed an increased tendency for reduced physical activity and increased energy intake with advancing age (Manios, 2006). Specifically, children aged 4-5 years old tended to spend more time watching television and were less physically active than children in younger age groups (1-2, 2-3 and 3-4 years old). Similar trends have previously been reported in schoolchildren aged 5-6 years (Salmon et al., 2006), although other studies in preschool children aged 3-4 years reported an increase in physical activity over a one-year period (Jackson et al., 2003). Therefore, it is possible that inactivity even at this young age (e.g. girls aged 4-6 years in the present study) may interact with the *ACE* I/D polymorphism and in doing so influence body fat accumulation in a similar manner as previously reported in Greek teenaged girls (Moran et al., 2005).

While the present study provides further support for the *ACE* I/D polymorphism being a good candidate gene for human obesity (Strazzullo et al., 2003;Moran et al., 2005;Riera-Fortuny et al., 2005), the precise mechanism by which *ACE* may produce this effect remains unclear. The primary role of *ACE* is to convert angiotensin I into angiotensin II (a potent vasoconstrictor) (Bernstein et al., 1989). The main components of the tissue RAS systems, which can generate angiotensin II and other active peptides independently of circulating RAS components, are expressed in both visceral and subcutaneous adipose tissue (Giacchetti et al., 2002). While the exact role of angiotensin II in adipose tissue is not yet clear, it has been shown to promote adipocyte growth and differentiation and can inhibit lipolysis by reducing skeletal and adipose-tissue blood flow leading to increased fat storage in normal-weight and obese subjects (Goossens et al., 2004). Furthermore, angiotensin II has been shown to increase lipid synthesis and storage in adipose cells *in vitro* (Jones et al., 1997) and, thus, may play an important role in growth and differentiation of this tissue. The DD genotype is associated with higher circulating *ACE* levels than the other I/D genotypes (Rigat et al., 1990). In the context of obesity, the I/D polymorphism (or another polymorphism in strong linkage disequilibrium with it, if it turns out not to be the functional polymorphism responsible for these associations) may modulate locally produced angiotensin II levels at particular times indevelopment, resulting in the significant associations found between the I/D polymorphism and adiposity-related phenotypes in this and other studies (Strazzullo et al., 2003;Moran et al., 2005;Riera-Fortuny et al., 2005).

In summary, the data presented here revealed that the I/D polymorphism has a stronger influence on particular adiposity-related phenotypes in young Greek children than in adult populations. The I/D polymorphism, being a sufficiently good marker of circulating *ACE*

levels, may be associated with developmental changes observed during the first stages of life. Environmental factors, such as dietary patterns and physical activity, could also mediate the age-specific effects of *ACE* I/D polymorphism in childhood obesity.

Table 4.2 Odds ratios for BMI in boys and girls aged 1-2 years.

	Genotype	Phenotype	Lowest quartile	Highest quartile
Boys and girls 1-2 years	II	BMI	*0.35 (0.10-1.24)* *P=0.09*	**5.69 (2.30-14.07)** **P=<0.001**
	ID		1.50 (0.77 to 2.95) P=0.233	**0.37 (0.18 to 0.75)** **P=0.005**
	DD		0.99 (0.49 to 1.98) P=0.974	0.99 (0.48 to 2.02) P=0.999

95% CIs are given in the parentheses. Chi-square tests were significant (P<0.05) for odds ratios and CIs indicated in bold and showed tendencies to significance in those indicated in italics. P values are given to three significant figures.

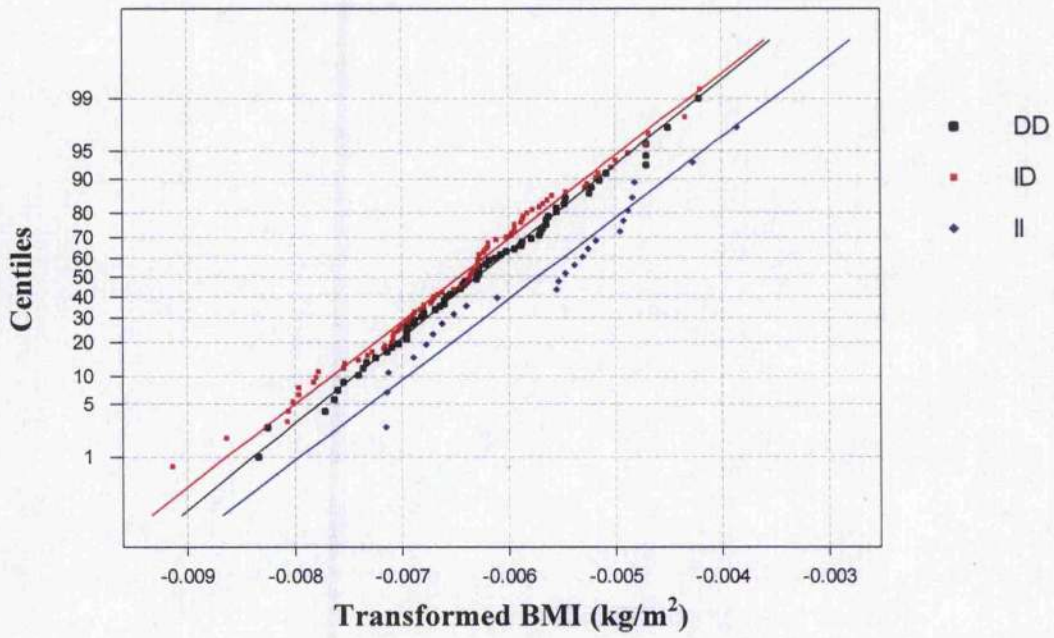


Figure 4.4 Normal probability plots for BMI (boys and girls 1-2 years) by *ACE* I/D genotype. Centiles were calculated for each genotype separately and plotted against the Box-Cox transformed data for each individual using the default method in Minitab 13.30. Higher centiles represent increased adiposity (i.e. higher adiposity measurements are further to the right).

Table 4.3 Odds ratios for phenotypes significant by ANOVA.

	Genotype	Phenotype	Lowest quartile	Highest quartile
Boys 1-2 years	II	BMI	**0.26 (0.03-2.17) P=0.045**	**24.00 (4.60-125.17) P=<0.001**
	ID		0.81 (0.30 to 2.15) P=0.835	*0.40 (0.14 to 1.16) P=0.085*
	DD		1.92 (0.72 to 5.10) P=0.120	0.45 (0.16 to 1.31) P=0.137
Girls 4-6 years	II	BMI	*1.72 (0.91 to 3.27) P=0.094*	0.61 (0.28 to 1.32) P=0.203
	ID		0.97 (0.59 to 1.60) P=0.896	1.02 (0.61 to 1.70) P=0.942
	DD		0.74 (0.44 to 1.25) P=0.265	1.26 (0.75 to 2.10) P=0.383
	II	Arm circumference	*1.90 (0.94 to 3.81) P=0.069*	0.92 (0.43 to 1.95) P=0.822
	ID		1.44 (0.83 to 2.51) P=0.193	0.74 (0.43 to 1.27) P=0.278
	DD		**0.44 (0.24 to 0.82) P=0.008**	1.41 (0.83 to 2.40) P=0.205
	II	Waist circumference	**2.80 (1.49 to 5.29) P=0.001**	0.62 (0.29 to 1.34) P=0.223
	ID		1.0 (0.60 to 1.69) P=0.987	0.92 (0.55 to 1.53) P=0.739
	DD		**0.50 (0.28 to 0.88) P=0.014**	1.39 (0.83 to 2.32) P=0.213

95% CIs are given in the parentheses. Chi-square tests were significant (P<0.05) for odds ratios and CIs indicated in bold and showed tendencies to significance in those indicated in italics. P values are given to three significant figures.

Chapter 5

Effects of *ADRB* gene polymorphisms on adiposity-related phenotypes in Greek children

5.1 Introduction

ADRB1, *ADRB2* and *ADRB3* genes, which encode for GPCRs (G-protein coupled receptor) in the plasma membrane, represent attractive candidate genes for obesity. Expression of these genes in adipose tissue promotes lipolysis and thermogenesis by mediating the stimulation by catecholamines (adrenaline and noradrenaline) of adenylyl cyclase (Lafontan and Berlan, 1993). Functional SNPs within the coding region of *ADRB* genes have been suggested to contribute to the development of several pathophysiologies, such as asthma, congestive heart disease and obesity (Small et al., 2003;Leineweber et al., 2004). Conflicting results have been published, so far, as to whether specific *ADRB* variants at different loci are associated with obesity or not. Most of these association studies have been focused mainly on adult populations and on polymorphisms within a single *ADRB* gene rather than considering haplotypes. Genotyping of the *ADRB1* Gly49Ser (rs1801252) and Arg389Gly (rs1801253) variants allows the inference of three haplotypes that represent the majority of variation along the complete length of gene. In *ADRB2* gene, genotyping of the Gly16Arg (rs1042713) and Glu27Gln (rs1042714) variants reveals the occurrence of three haplotypes that account for 95% of the genetic variance observed in *ADRB2* gene in Caucasian populations (Drysdale et al., 2000).

To obviate the limitations of previous studies, the present study aimed to investigate the effects of variations in all three *ADRB* genes on adiposity-related phenotypes in a large population of toddlers and preschoolers using both genotype and haplotype-based approaches. Young cohorts may be the study group of choice when characterising the genetic influences on complex diseases like obesity, as the environment has less time to take effect (Maes et al., 1997).

5.2 Methods

5.2.1 Subjects and phenotypes

The number of subjects that took part in the study, as well as information on the assessed adiposity-related phenotypes and the protocols used for anthropometry, are given in Chapter 2 (General Methods). The anthropometric measurements assessed in the current analysis included: BMI, arm, waist and hip circumferences, as well as sum of skinfolds.

5.2.2 DNA extraction, genotyping and haplotype inference

The method used for DNA extraction from all buccal samples is given in Chapter 2 (General Methods). Details on the set up of PCR reactions, digestions for *ADRB1* (C49 and C389), *ADRB2* (C16 and C27) and *ADRB3* (C64) genotyping, as well as *ADRB1* and *ADRB2* haplotype inference are also given in the same section.

5.2.3 Statistical analyses

Data and statistical analysis carried out are described in detail in Chapter 2 (General Methods).

5.3 Results

5.3.1 Genotyping and phenotyping

2102 children (1095 boys and 1007 girls) were phenotyped. Genotyping for *ADRB1* gene was successful in 1929 individuals at the C49 polymorphism and 1856 individuals at the C389 polymorphism. The overall allele frequencies were $f_{(Gly49)}$=0.09 and $f_{(Ser49)}$=0.91 for Gly49Ser polymorphism and $f_{(Arg389)}$=0.67, $f_{(Gly389)}$=0.33 for Arg389Gly polymorphism and the population was found to be in HWE for both C49 ($\chi^2_{(df=1)}$=0.22, P=0.638) and C389 ($\chi^2_{(df=1)}$=0.45, P=0.503) loci. Diplotypes (haplotype allelic combinations) were inferred in 1795 individuals; haplotype frequencies were $f_{(Ser49Arg389)}$=0.33, $f_{(Ser49Gly389)}$=0.59 and $f_{(Gly49Arg389)}$=0.09. Genotyping was successful in 1999 children for *ADRB2* C16 polymorphism and in 2027 subjects for *ADRB2* C27 polymorphism. The overall allele frequencies were $f_{(Gly16)}$=0.63 and $f_{(Arg16)}$=0.37 for Gly16Arg and $f_{(Glu27)}$=0.37 and $f_{(Gln27)}$=0.63 for Glu27Gln polymorphism. The population was found to be in HWE for both C16 ($\chi^2_{(df=1)}$=2.36, P=0.124) and C27 ($\chi^2_{(df=1)}$=1.99, P=0.158) loci. Diplotypes were inferred based on published literature (Drysdale et al., 2000) in 2008 individuals; haplotype frequencies were $f_{(Arg16Gln27)}$=0.37, $f_{(Gly16Gln27)}$=0.26 and $f_{(Gly16Glu27)}$=0.37. Finally, for Arg64Trp polymorphism in *ADRB3*, genotyping was successful in 2032 individuals and the overall allele frequencies were $f_{(Arg64)}$=0.05 and $f_{(Trp64)}$=0.95. The population was found to be in HWE for this polymorphism ($\chi^2_{(df=1)}$=1.03, P=0.311). There were no significant differences between boys and girls for any of the aforementioned polymorphisms (data not shown). None of the individuals above showed evidence of rare recombinant haplotypes. The population was found to be in HWE for both *ADRB1* and *ADRB2* haplotypes (Table 5.1)

Table 5.1 Haplotypes frequencies for *ADRB1* and *ADRB2* genes.

Gene	Haplotype	Haplotype Frequency	HWE for haplotypes
ADRB1	Gly49Arg389	0.09	$\chi^2_{(df=5)}=3.80$, P=0.578
	Gly49Gly389	0	
	Ser49Arg389	0.59	
	Ser49Gly389	0.33	
ADRB2	Gly16Glu27	0.37	$\chi^2_{(df=5)}=5.68$, P=0.339
	Gly16Gln27	0.26	
	Arg16Glu27	0	
	Arg16Gln27	0.37	

5.3.2 GLM analysis for ADRB polymorphisms

GLM analysis was carried out in the total population to assess the main effects of the *ADRB1*, *ADRB2* and *ADRB3* polymorphisms of interest on adiposity-related phenotypes and whether these genetic effects were influenced by gender and/or age. GLM analysis on the total population has shown that the *ADRB2* C16 polymorphism individually had a significant effect on waist (P=0.041) and hip (P=0.004) circumferences. For the same polymorphism, significant interactions were found between age and C16 genotype for waist (P=0.042) (Figure 5.1) and hip (P=0.008) (Figure 5.2) circumferences and between gender and C16 genotype for BMI (P=0.025) and arm circumference (P=0.041) (Table 5.2). Furthermore, GLM on the total population for Glu27Gln polymorphism has revealed that *ADRB2* C27 genotypes had a significant main effect on hip circumference (P=0.006), as well as total skinfolds (P=0.025). Significant interactions between age and C27 genotypes (P=0.012), as well as gender and C27 genotypes (P=0.019) were observed for hip circumference (Figure 5.3). For *ADRB1* C49 and C389, as well as *ADRB3* C64 polymorphisms, GLM analysis on the total population has shown no significant main genotypic effects or interactions with age and/or gender.

Based on these findings from the GLM analysis, futher ANOVA analysis was carried out stratisfied by age but not gender to assess associations between *ADRB2* C16 genotypes and waist and hip circumferences. ANOVA analysis revealed that although boys and girls carrying the Arg16 allele genotype had higher (but not significantly) waist (Figure 5.1) and hip (Figure 5.2) circumference measures between the ages of 1 to 4 years compared to those with the Gly16 variant, in the 4-6 years age group boys and girls homozygotes for the Gly16 allele rather than those homozygotes for the Arg16 allele had significantly higher waist (P=0.026) (Figures 5.2 and 5.3) and higher (but not significantly) hip

(P=0.129) (Figure 5.2) circumferences. Further ANOVA analysis stratisfied by gender was also carried out to assess possible associations between *ADRB2* C16 genotypes and BMI and arm circumference. This analysis revealed no significant associations between *ADRB2* C16 genotypes and BMI or arm circumference in total boys or total girls (Table 5.2). However, boys carrying the Arg16 allele showed higher measures for both BMI and arm circumference than those with the Gly16 allele, whereas girls carrying the Gly16 rather than Arg16 variant had higher (but not singificantly) measures for the same phenotypes (Table 5.2). As far as GLM analysis on the total population for *ADRB2* C27 effects on adiposity is concerned, further ANOVA analysis stratisfied by age and gender for hip circumference revealed that boys aged 2-3 years bearing the Gln27 (C) allele had significantly higher hip circumference (P=0.001) than those with the Glu27 (G) allele (Figure 5.4).

5.3.3 ADRB genotype associations with phenotypes after age and gender subgrouping

5.3.3.1 *ADRB2* C16 and C27 genotype associations with phenotypes

After performing Dunn-Sidak correction for multiple testing for each age group, differences between the means of the C16 genotypes for BMI in boys (3-4 years) (a value=0.020) (Appendix 1, Table 7), as well as the means of the C27 genotypes for hip circumference in boys (2-3 years) (P=0.001) (a value=0.017) were statistically significant (Figure 5.4). In particular, boys with the Arg16 allele had significantly higher BMI (P=0.020) than those carrying the Gly16 allele. This isolated significant finding was also reflacted in the main GLM analysis (Table 5.2) where total boys carrying the Arg16 allele had higher BMI than those with the Gly16 variant. The mean values of all anthropometric

parameters according to gender and *ADRB2* C16 and C27 genotypes are shown in Appendix 1 (Tables 7 to 10).

5.3.3.2 *ADRB1* C49 and C389 genotype associations with phenotypes

ANOVA tests for *ADRB1* Gly49Ser polymorphism revealed no significant differences in boys or girls for any adiposity-related trait (shown in Appendix 1, Tables 3 and 4). This was also reflected in the GLM analysis where no main effects of C49 genotypes on phenotypes or genotype and/or gender interactions were observed (data not shown). For the Arg389Gly polymorphism, after adjusting the P value for significance within each gender and age group using the Dunn-Sidak correction for multiple testing, differences between the means of C389 genotypes for hip circumference (P=0.004) in girls aged 2-3 years (a value=0.018) were found to be statistically significant (Appendix 1, Table 6). However, this isolated significant association was not reflected in the GLM analysis on the total population. Also, due to the fact that this association was best explained by an overdominant model as correlation analysis showed (data not shown), this finding should be interpreted with caution and seems to be a Type I error. Such genetic models (under- or over-dominance) are difficult to be explained mechanistically and were most likely caused by lack of power or by outliers shifting the Arg389Gly mean higher than both the homozygote means. The mean values of all anthropometric parameters according to gender and *ADRB1* C49 and C389 genotypes are shown in Appendix 1 (Tables 3 to 6)

5.3.3.3 *ADRB3* C64 genotype associations with phenotypes

For *ADRB3* Arg64Trp polymorphism, no statistically significant differences where observed for any adiposity-related trait tested after Sidak correction for repeated tests. This was also reflected in the GLM analysis where no main effects of C64 genotypes on

phenotypes or genotype and/or gender interactions were observed (data not shown). The Arg64 allele was found to be relatively rare in the Greek population ($f_{(Arg64)}$=0.05), as shown also in previous studies (Corella et al., 2001) and thus, only heterozygotes at this site were identified in some age groups for both genders. The mean values of all anthropometric parameters according to gender and *ADRB3* C64 genotypes are shown in Appendix 1 (Tables 11 and 12).

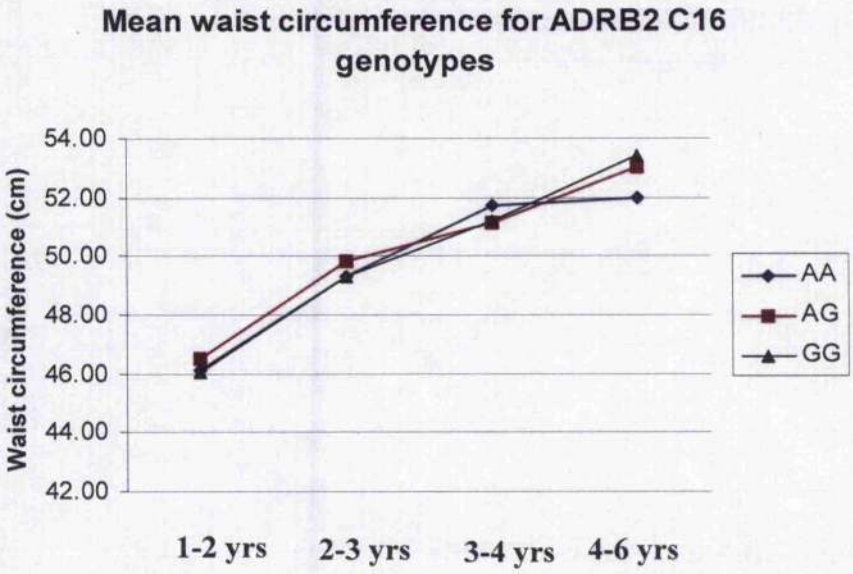


Figure 5.1 Effects of *ADRB2* C16 genotypes on mean waist circumference in boys and girls in each age group.

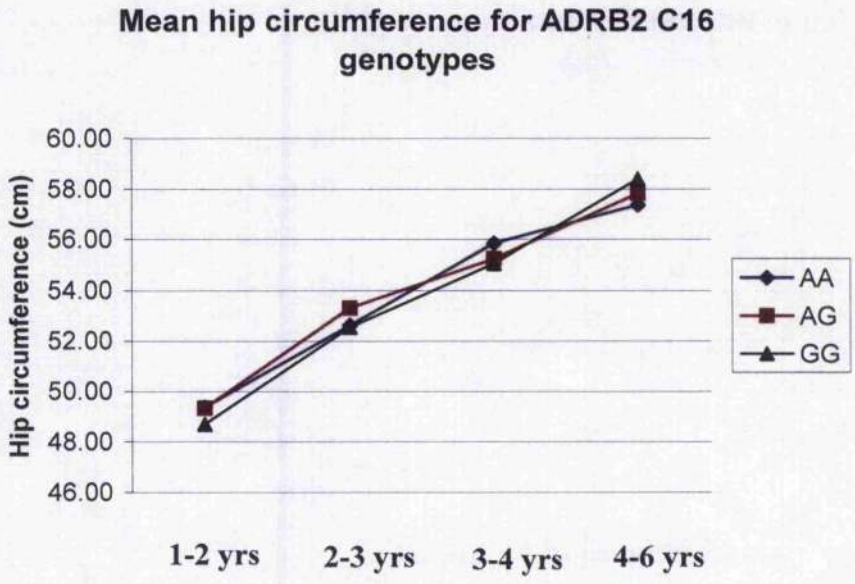


Figure 5.2 Effects of *ADRB2* C16 genotypes on mean hip circumference in boys and girls in each age group.

Table 5.2 Analysis of associations between *ADRB2* C16 polymorphism and adiposity-related phenotypes in total boys and total girls.

Total boys *ADRB2* C16 GG=407, GA=485, AA=140	**BMI** GG= 16.05 (15.91 to 16.20) GA= 16.06 (15.92 to 16.20) AA=16.33 (16.08 to 16.59) V=0.4%, P=0.139	**Arm Circumference** GG=16.24 (16.11 to 16.37) GA=16.28 (16.16 to 16.40) AA=16.43 (16.21 to 16.66) V=0.2%, P=0.350
Total girls *ADRB2* C27 GG=368, GA=480, AA=119	**BMI** GG=16.10 (15.93 to 16.27) GA=16.10 (15.97 to16.23) AA=15.81 (15.54 to 16.09) V=0.4%, P=0.153	**Arm Circumference** GG=16.52 (16.35 to 16.68) GA=16.41 (16.27 to 16.54) AA=16.27 (16.01 to 16.53) V=0.3%, P=0.267

Associations between *ADRB2* C16 genotypes and adiposity-related parameters assessed by ANOVA in total boys and girls. For each test, mean values, 95% CIs are given, as well as percentage variance explained by ANOVA (V), probability (P). P values are given to three significant figures.

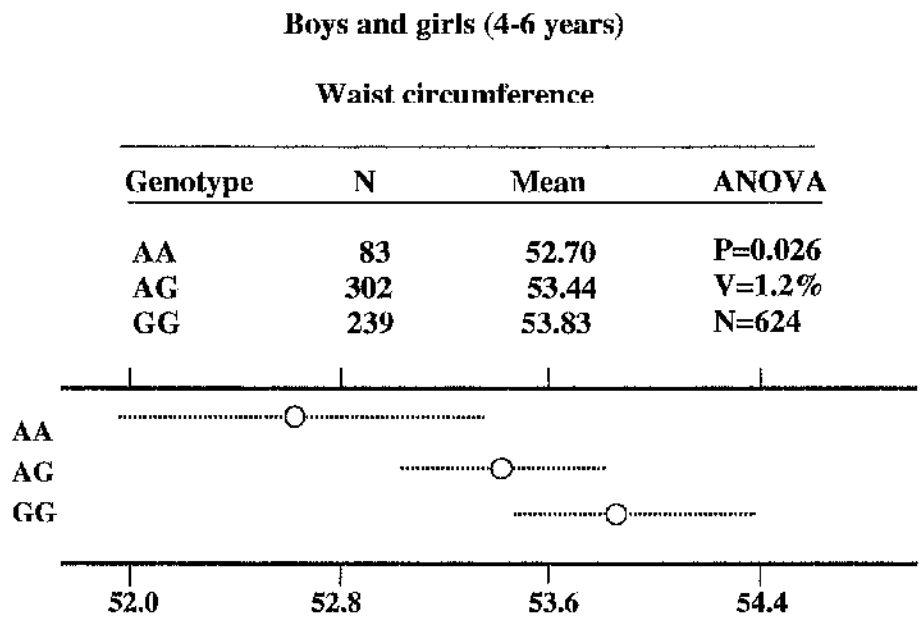


Figure 5.3 *ADRB2* C16 genotype differences in waist circumference for boys and girls aged 4-6 years. P=probability, V=observed variance explained, N=number within group.

Boys (2-3 years)

Hip circumference

Genotype	N	Mean	ANOVA
GG	29	50.94	P=0.001
GC	115	52.47	V=6.0%
CC	97	53.46	N=241

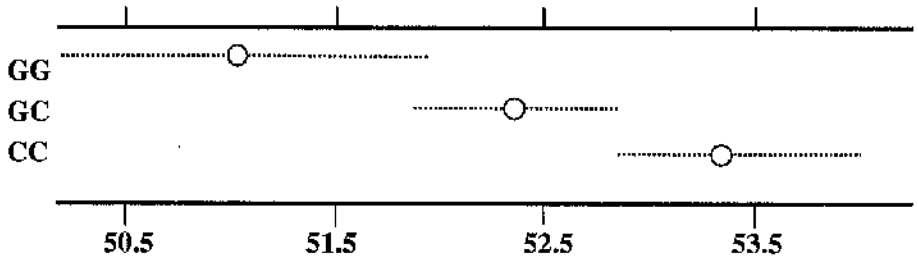


Figure 5.4 ***ADRB2*** **C27 genotype differences in hip circumference for 2-3 years boys. P=probability, V=observed variance explained, N=number within group.**

5.3.4 Determination of the type of genetic effect observed for associations with the ADRB gene polymorphisms

Three genetic models were used (additive, dominance and recessive models) to determine the genetic mechanism for the observed significant associations using correlation analysis. Under-/over-dominant models were also tested. In boys and girls aged 4-6 years, an additive model best explained the significant association between C16 and waist circumference accounting for 87% of the genetic variance (Figure 5.3). In boys aged 2-3 years, an additive genetic model best explained the statistically significant association observed between Glu27Gln genotype and hip circumference of age accounting for 97% of the genetic variance, while Glu-dominant and Glu-recessive models accounted for 63% and 0% of the genetic variance respectively (Figure 5.4). Finally, under/overdominance models accounted for 6% of the variance. In boys (3-4 years), a dominant genetic model, with the Gly16 allele completely dominant over the Arg16 allele, could explain the statistically significant associations between Gly16Arg genotypes and BMI accounting for 99% of the genetic variance (Appendix 1, Table 7).

5.3.4 Genotypic effect of ADRB1 and ADRB2 variants on the phenotypic distribution

The influence of genotype on the distribution of the measured adiposity-related phenotype was assessed by calculating odds ratios and visualised by using probability plots. The odds ratios (Table 5.3) were generally in agreement with the phenotypic means given by ANOVA and the genetic models by correlation analysis. In boys and girls aged 4-6 years, calculation of odds ratios for waist circumference has shown that homozygotes for the Arg16 allele were significantly overrepresented in the lowest quartile and underrepresented

(but not significantly) in the highest quartile, whereas homozygotes for the Gly16 allele were significantly underrepresented in the lowest quartile and overrepresented (but not significantly) in the highest (Table 5.3). This could suggest that the Gly16Arg polymorphism exerts a larger effect on the thinner end of the distribution. In boys (2-3 years), the difference between the means of the three *ADRB2* C27 genotypes for hip circumference is not uniform throughout the distribution, as illustrated by the normal probability plot and this difference becomes more pronounced in the fatter end of the distribution (Figure 5.5). This uneven effect of the Glu27Gln polymorphism throughout the phenotypic range is also highlighted by the odds ratios for individuals of different C27 genotypes falling into the low or high quartiles (Table 5.3). According to these odds ratios, Glu27 homozygotes (n=29) were significantly overrepresented in the lowest quartile (14/29) and significantly underrepresented in the highest quartile (0/29) for hip circumference, whereas Gln27 homozygotes (n=97) were significantly underrepresented (9/97), but not significantly overrepresented (23/97) in the highest quartile, suggesting that the Gln27Glu polymorphism exerts a larger effect on the leaner end of the distribution (Table 5.3).

5.3.5 ADRB2 diplotypes association with phenotypes

Significant associations by ANOVA were further assessed by haplotype-based analysis for the *ADRB2* gene. The extra portion of the phenotypic variance accounted for by using *ADRB2* diplotypes instead of the individual C16 or C27 genotypes was not significantly larger for any of the measured phenotypes. However, in boys 2-3 years old, the Arg16Gln27/Gly16Gln27 and Gly16Gln27/Gly16Gln27 diplotypes were associated with higher hip circumference measurements compared to the Gly16Glu27/Gly16Glu27 diplotype (Figure 5.6). Correlation analysis for possible genetic models underlying these associations in boys revealed that a Gly16-dominant model could explain the data best

when assessing the effect of the *ADRB2* C16 genotypes on adiposity, in the absence of variation in C27 (a fixed Gln27 allele at this locus), while a Glu27-recessive model could best predict the phenotype when assessing the effect of the *ADRB2* C27 genotypes on adiposity, in the absence of variation in C16 (a fixed Gly16 allele at this locus) (Figure 5.6). The mean values of all anthropometric parameters according *ADRB2* diplotypes for both genders are shown in Appendix 2 (Tables 1 and 2).

Table 5.3 *ADRB2 C16 and C27* genotype odds ratios for phenotypes significant by ANOVA.

	Genotype	Phenotype	Lowest quartile	Highest quartile
Boys and girls 4-6 years C16	AA	Waist circumference	**1.86 (1.14-3.04) P=0.011**	0.96 (0.55-1.68) P=0.889
	AG		1.13 (0.78-1.62) P=0.517	0.95 (0.65-1.39) P=0.806
	GG		**0.62 (0.42-0.91) P=0.015**	1.07 (0.72-1.58) P=0.727
Boys 2-3 years C27	GG	Hip circumference	**3.67 (1.65 to 8.18) P=0.001**	**P=0.005**
	GC		**1.88 (1.03 to 3.44) P=0.039**	1.60 (0.84 to 3.04) P=0.150
	CC		**0.20 (0.09 to 0.44) P=<0.001**	1.48 (0.78 to 2.80) P=0.226

95% CIs are given in the parentheses. Chi-square tests that were significant (P<0.05) for odds ratios and CIs are indicated in bold. P values are given to three significant figures. Note that in the highest quartile, no individuals with the GG genotype falling into the highest quartile for hip circumference were identified, so calculation of odds ratio was not possible.

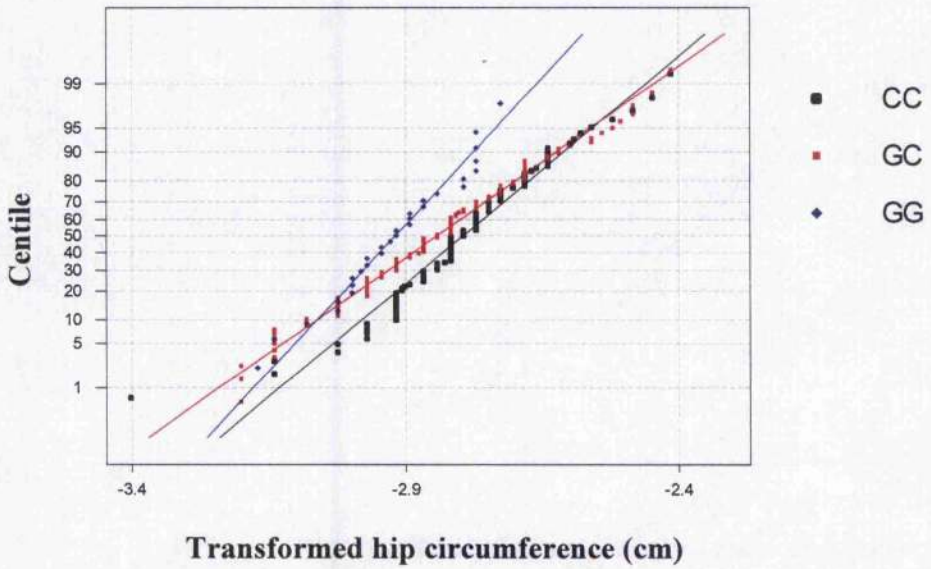


Figure 5.5 Normal probability plots for hip circumference by Glu27Gln genotype in boys 2-3 years. Centiles were calculated for each genotype separately and plotted against the Box-Cox transformed data for each individual using the default method in Minitab 13.30. Higher centiles represent increased adiposity (i.e. higher adiposity measurements are further to the right).

5.4 Discussion

In the present study (GENESIS), variation within the *ADRB2* (C16 and C27) was significantly associated with specific adiposity-related phenotypes. The GLM analysis on the total population has revealed a significant main effect of *ADRB2* C16 genotypes on waist (P=0.041) (Figure 5.1) and hip (P=0.004) (Figure 5.2) circumferences, as well as significant interactions between age and C16 genotype for waist (P=0.042) and hip (P=0.008) circumferences and between gender and C16 genotypes for BMI (P=0.025) and arm circumference (P=0.041). Further ANOVA analysis stratisfied by age for waist and hip circumference has revealed that boys and girls with the Gly16 allele had significantly higher waist circumference (P=0.026) than those with the Arg16 allele (Figures 5.1 and 5.3). This was in agreement with hypothesis 2 according which homozygotes for the Gly16 allele would be expected to show higher adiposity than those with the Arg16 allele. Previous studies, albeit in adolescents and adults, on the effect of the Gly16Arg polymorphism on adiposity are in agreement or in contrast to the present findings. For example, a strong association was observed in Greek adolescents between C16 and BMI, as well as skinfold thickness with homozygotes for the Gly16 allele showing higher adiposity (unpublished data). Furthermore, the Gly16 variant was associated with lower BMI in French-Canadian men (Ukkola et al., 2000) and Japanese women but no association was found in Japanese men (Ishiyama-Shigemoto et al., 1999). Based on the GLM analysis for the Glu27Gln polymorphism, a significant main effect of this variant on hip circumference and significant interactions between both age and gender with the same phenotype were observed. Boys 2-3 years bearing the Gln27 allele showed significantly higher hip circumference measures (P=0.001) than those with the Glu27 allele (Figure 5.4). These findings are consistent with a previous published report where the Gln27 allele was associated with higher scores for adiposity-related phenotypes in French men, but not in

women (Meirhaeghe et al., 2000b). Furthermore, the Glu27 variant frequency was found to be significantly lower in obese Swedish males but not females (Hellstrom et al., 1999), while the Glu27 allele was associated with increased adiposity in Swedish women (Large et al., 1997) and reduced lipolysis in Spanish women (Macho-Azcarate et al., 2002). These discrepancies in the findings could suggest that ethnicity, age (Ellsworth et al., 2002) and gender differences (Hellstrom et al., 1999;Meirhaeghe et al., 2000b;Gonzalez Sanchez et al., 2003), as well as environmental factors, such as physical activity (Meirhaeghe et al., 1999) and diet (Ukkola et al., 2001) can modify the possible effects of *ADRB* genotypes on adiposity.

Haplotypes have been advocated to be a more powerful tool for detecting associations between genetic variation and phenotypes than single SNPs in complex traits (Stephens et al., 2001), since SNPs predispose to a disease in combination with other variants. In the present study, genotyping of *ADRB1* (rs1801252 and rs1801253) and *ADRB2* (rs1042713 and rs1042714) polymorphisms that help define the major haplotypes in European populations (current study for *ADRB1* and (Drysdale et al., 2000)study for *ADRB2*) did not produce phenotypic associations stronger than those with the individual SNPs (Appendix 2, Tables 1 and 2) and the proportion of variance explained by haplotypes, although generally larger (due to increased degrees of freedom, after splitting the data into more groups), was not significantly greater than that explained by C49 or C389 and C16 or C27 genotypes analysed alone. This could suggest that most of the genetic effect observed is accounted for either by the individual SNPs or by variants, which are in strong linkage disequilibrium with them. However, this is not a proof against the higher analytical power and robustness of the haplotypes over the single markers. Nevertheless, by using haplotype-based tests in the present study, In boys 2-3 years old, the Arg16Gln27/Gly16Gln27 and Gly16Gln27/Gly16Gln27 diplotypes were associated with

higher hip circumference measurements compared to the Gly16Glu27/Gly16Glu27 (Figure 5.6), which could suggest a dominant effect of the Glu27Gln polymorphism on the function of the receptor.

Although previous work has mainly focused on cardiovascular diseases (Drysdale et al., 2000;Small et al., 2003), a few studies have attempted to associate *ADRB1* polymorphisms with obesity with somewhat conflicting results (Ryden et al., 2001;Dionne et al., 2002). In the present study, no significant associations were identified between Gly49Ser genotypes and adiposity-related phenotypes (Appendix 1, Tables 3 and 4) Furthermore, no significant associations were observed between *ADRB3* C64 genotypes for any of the adiposity-related phenotypes for either gender (Appendix 1, Tables 11 and 12). It is known that *ADRB3* expression is largely confined to adipose tissue, where it regulates noradrenaline-induced changes in energy metabolism (white adipose tissue) and thermogenesis (brown adipose tissue) (Leineweber et al., 2004). The Trp64Arg polymorphism has been studied mainly in obese subjects from several populations, such as Prima Indians (Walston et al., 1995), Finns (Widen et al., 1995), French Caucasians (Clement et al., 1995), as well as Japanese subjects (Kim-Motoyama et al., 1997) with different allelic frequencies being reported reflecting ethnic variability. In the present study, the low prevalence of the Gly49 ($f_{(Gly49)}$=0.09) and the Arg64 ($f_{(Arg64)}$=0.05) alleles coupled to the small number of subjects may have influenced the accurate assessment of any small effects of this polymorphism on adiposity. However, the little significance of these variants to the Greek population does not preclude them from having significant effects in other populations where they are more common (Kawamura et al., 2001).

Although the study of younger populations may be a more powerful approach to investigate complex traits than adult populations, since the environmental influence is less (Maes et al., 1997), the effect of *ADRB* genes in the present population of toddlers and

preschoolers (Figures 5.3 and 5.4) was not generally stronger than that observed in adolescents for the Gly16Arg polymorphism (unpublished data) and in adults for the Glu27Gln polymorphism (Meirhaeghe et al., 2000b). These results, as well as the significant interactions between age and both C16 and C27 could suggest that the effects of *ADRB2* polymorphisms on adiposity are possibly age-dependent. This notion is also supported by a previous longitudinal study, which has shown that, although children (4-9 years) with the Gly16Gly genotype had higher adiposity-related measures than those with the other Gly16Arg genotypes, these differences became more pronounced with age and reached statistical significance by the age of 20 (Ellsworth et al., 2002). Furthermore, given the fact that plasma catecholamine levels increase with advancing age (approximately 10-15% per decade in adults) (Seals and Esler, 2000) and they have been shown to have no or little effect on lipolysis during the first years of life due to a prominent antilipolytic effect of a2A-adrenoceptor (Arner, 2005), the effects of variation in the *ADRB* genes are expected to be larger in adult populations.

The modest effects of *ADRB* genes on adiposity observed in the present study are the ones expected for the development of complex trait, such as obesity and are manifested possible through interactions with other variants of functional significance and/or environmental factors, such as physical activity (Meirhaeghe et al., 1999) and diet (Ukkola et al., 2001). For example, in Greek adolescents (unpublished data), the association of Gly16Gly genotype with larger triceps skinfold was stronger in active (P=0.005, V=2.7%) than in inactive males (P=0.986, V=0%), while physically inactive French men with the Gln27Gln genotype had increased BMI compared to inactive carriers of the Glu27 allele or active men of any genotype (Meirhaeghe et al., 1999). Children in the GENESIS study showed an increased tendency for reduced physical activity with advancing age (Manios, 2006). Given the fact that the adrenergic system responds to physical activity by increasing

plasma catecholamines (Strobel et al., 1999), it is possible that different alleles of *ADRB* genes will respond differently to this catecholamine activation. Therefore, it is possible that inactivity even at a very young age can modify or mask the possible effects of *ADRB* variants on fat accumulation.

In summary, variants of the *ADRB2* gene were associated with subtle influences on adiposity-related phenotypes in early childhood and these effects were manifested in a gender-specific and age-related manner. Although haplotype-based analysis did not reveal stronger associations compared to individual SNPs, Gly16Gln27/Gly16Gln27 and Arg16Gln27/Gly16Gln27 diplotypes were associated with higher hip circumference measurements compared to the Gly16Glu27/Gly16Glu27 suggesting a possible dominant effect of the *ADRB2* Glu27Gln polymorphism on the receptor's function.

Boys 2-3 years

Hip Circumference			
***ADRB2* Diplotypes**	**N**	**Mean**	**ANOVA**
AC/AC	37	52.92	
AC/GC	44	53.64	P=0.011
AC/GG	61	52.21	V=6.3%
GC/GC	14	53.41	N=232
GG/GC	50	52.77	
GG/GG	26	51.15	

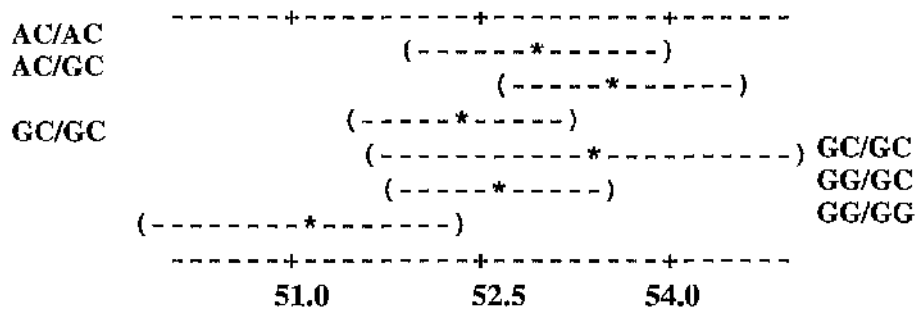


Figure 5.6 *ADRB2* diplotype differences in hip circumference for 2-3 year old boys. P=probability, V=observed variance explained, N=number within group. For haplotypes, AC=Arg16Gln27, GC=Gly16Gln27 and GG=Gly16Glu27.

Chapter 6

Effects of *PPARγ* polymorphisms on adiposity-related phenotypes in Greek children

6.1 Introduction

The nuclear receptor, *PPARγ*, which is abundantly expressed in adipose tissue, has a pivotal role in adipose tissue differentiation, fatty acid metabolism and insulin sensitization, as well as in the regulation of transcription of several adipocyte genes (Martin et al., 1998;Gurnell, 2005). The most frequently occurring *PPARγ* polymorphism, the Pro12Ala, has been extensively studied in association with obesity, insulin resistance and type 2 diabetes with inconsistent results being reported (Martin et al., 1998;Meirhaeghe and Amouyel, 2004). Conflicting findings have prompted a meta-analysis based on data from 30 independent studies, which has shown that the Ala12 allele was significantly associated with higher BMI than the Pro12 variant in obese but not in lean subjects (Masud and Ye, 2003). The next commonest *PPARγ* polymorphism, the C1431T, has been studied in relation to glucose intolerance and obesity (Meirhaeghe et al., 1998;Valve et al., 1999;Poulsen et al., 2003). Although not in the promoter region and being a synonymous polymorphism, the C1431T has been shown to interact with the Pro12Ala polymorphism, resulting in opposing associations with body weight (Valve et al., 1999;Doney et al., 2002) and highlighting the complex nature of these polymorphisms *in vivo*.

In view of the contradictory associations between the *PPARγ* polymorphisms and obesity, the aim of this study was to clarify the situation by carrying out an association analysis using a large population of toddlers and preschoolers. Assessing associations between Pro12Ala and C1431T polymorphisms and adiposity-related phenotypes using genotype- and haplotype-based methods in a younger population may reveal stronger genotype-phenotype associations than in older populations, since the amount of opportunity for environmental influence is reduced.

6.2 Methods

6.2.1 Subjects and phenotypes

The number of subjects that took part in the study, as well as information on the assessed adiposity-related phenotypes and the protocols used for anthropometry, are given in Chapter 2 (General Methods). The anthropometric measurements assessed in the current analysis included: BMI, arm, waist and hip circumferences, as well as biceps, triceps, subscapular and suprailiac thickness.

6.2.2 DNA extraction, genotyping and haplotype inference

The method used for DNA extraction from all buccal samples is given in Chapter 2 (General Methods). Details on the set up of PCR reactions, digestions for Pro12Ala and C1431T genotyping, as well as *PPARγ* haplotype inference are also given in the same section.

6.2.3 Statistical analyses

Data and statistical analysis carried out are described in detail in Chapter 2 (General Methods).

6.3 Results

6.3.1 Genotyping

2102 children (1095 boys and 1007 girls) were included in the final analysis after excluding subjects with missing phenotypic data or DNA sample. Genotyping for *PPARγ* Pro12Ala and C1431T polymorphisms was successful in 1888 (981 boys and 907 girls) and 1956 (1019 boys and 937 girls) individuals, respectively. Allele frequencies and the observed genotype frequencies for Pro12Ala and C1431T are given in Table 6.1. The population was found to be in HWE at both Pro12Ala ($\chi^2_{(df=1)}$=2.29, P=0.129) and C1431T ($\chi^2_{(df=1)}$=3.34, P=0.068) loci. The frequency of the Ala12 was 0.07, which was lower than that reported for French (0.11) (Valve et al., 1999;Doney et al., 2002;Meirhaeghe et al., 2005b)and Finnish (0.12) (Deeb et al., 1998) cohorts and for Danish twins (MZ 0.14 and DZ 0.12) (Poulsen et al., 2003), but the same as the one reported in school-aged chidren from Greece (0.07) (Dedoussis et al., 2007) and in adults from Southern Europe (0.07) (Poirier et al., 2000). Similarly, the frequency of the T1431 variant was 0.09, which was also lower than that reported for the French cohort (0.13) (Meirhaeghe et al., 2005b) and Danish twins (MZ 0.14 and DZ 0.12) (Poulsen et al., 2003). There were no significant differences in genotype frequencies between boys and girls for both Pro12Ala ($\chi^2_{(df=2)}$=0.706, P=0.706) and C1431T ($\chi^2_{(df=2)}$=0.831, P=0.660) polymorphisms (data not shown). The Pro12Ala and C1431T polymorphisms were found to be in LD, as it has previously demonstrated (Valve et al., 1999;Doney et al., 2002)(Table 6.2).

6.3.2 GLM and one-way ANOVA analysis for PPARγ Pro12Ala and C1431T polymorphisms

GLM analysis was carried out in the total population to assess the main effects of the *PPARγ* Pro12Ala and C1431T polymorphisms on adiposity-related phenotypes and whether these genetic effects were influenced by gender and/or age. GLM analysis on the total population has shown no significant main effects of the Pro12Ala and C1431T genotypes on adiposity were observed. In addition to that, no interactions between age *PPARγ* genotypes and age and/or gender were revealed.

6.3.3 PPARγ genotypes and associations with obesity-related phenotypes after age and gender subgrouping

After performing the Dunn-Sidak correction for multiple testing for each age group in each gender, differences between the means of the Pro12Ala genotypes for mid-upper arm (P=0.010) and hip (P=0.005) circumferences, as well as sum of skinfolds (P=0.011) (a value=0.020) in girls aged 3-4 years were found to be statistically significant and these associations accounted for 2.0%, 3.7% and 1.9% of the variance respectively (Appendix 1, Table 14). In particular, girls carrying the Ala12 allele had significantly higher mean values for arm (P=0.010) and hip (P=0.005) circumferences, as well as sum of skinfolds (P=0.011) at the age of 3-4 years (Appendix 1, Table 14) than those carrying the Pro12 allele. For C1431T polymorphism, girls bearing the T1431 allele showed significantly higher waist circumference (P=0.018) (a value=0.020) than those carrying the C1431 variant and this association explained 1.6% of the phenotypic variance (Appendix 1, Table 16). However, these isolated significant findings were not reflected in the GLM analysis on the total population and as such they might be cases of Type I errors. The mean values

of all anthropometric parameters for *PPARγ* genotypes are shown in the Appendix 1 (Tables 13 to 16).

Table 6.1 Observed *PPARγ* genotype and allele frequencies

Polymorphism	**Pro12Ala**		**C1431T**	
Observed Genotypes	Pro/Pro	1628	C/C	1616
	Pro/Ala	255	C/T	331
	Ala/Ala	5	T/T	9
Total		**1888**		**1956**
Allele frequencies	Pro12	0.930	C1431	0.911
	Ala12	0.070	T1431	0.089

Table 6.2 Frequencies of combined *PPARγ* genotypes and estimated haplotypes.

Diplotypes	Diplotype Distribution	Haplotype	Haplotype Distribution	Haplotype Frequencies
Pro-C/Pro-C	1396			
Pro-C/Pro-T	168			
Pro-T/Pro-T	3	**Pro-C**	3203	0.88
Pro-C/Ala-C	105	**Pro-T**	179	0.05
Pro-C/Ala-T	138	**Ala-C**	110	0.03
Pro-T/Ala-T	5	**Ala-T**	148	0.04
Ala-C/Ala-C	1			
Ala-C/Ala-T	3		**D'=0.529**	
Ala-T/Ala-T	1			
Total	**1820**			

Individuals with the Pro-C/Pro-C diplotype are homozygotes for the **Pro-C** haplotype. Invididuals with the Pro-C/Pro-T and Pro-T/Pro-T diplotypes are heterozygotes and homozygotes for the **Pro-T** haplotype respectively, while those with the Pro-C/Ala-C and Ala-C/Ala-C are heterozygotes and homozygotes for the **Ala-C** haplotype respectively. The **Ala-T** haplotype group includes individuals with Pro-C/Ala-T, Pro-T/Ala-T, Ala-C/Ala-T, Ala-T/Ala-T diplotypes. The level of LD between the two polymorphisms is also shown.

6.3.3 PPARγ interactions with ADRB3 Arg64Trp polymorphism

Interactive effects of the *PPARγ* Pro12Ala polymorphism with the *ADRB3* Arg64Trp variant were assessed on the total population by ANOVA analysis. However, no significant interactions between these two polymorphisms were observed (data not shown).

6.3.4 Moduiating effect of BMI on PPARγ genetic influence

Subjects of both genders were classified as overweight/obese if their BMI was more than the 85th percentile based on the UK reference data. Separate ANOVA analysis for lean and obese boys and girls has revealed that BMI had no significant modulating effect on the influence of *PPARγ* Pro12Ala polymorphism on adiposity (data not shown)

6.3.5 Associations of PPARγ diplotypes with adiposity-related phenotypes

Haplotype-based analysis for the *PPARγ* polymorphisms did not reveal stronger associations than genotype-based analysis and the portion of variance explained by the diplotypes, although generally higher than that explained by individual genotypes (based on the increase in the sum of squares and as a result of the increased degrees of freedom) (data not shown), was not significantly larger, suggesting that most of the genetic effect observed is accounted for either by the individual SNPs or by variants which are in strong LD with these SNPs.

6.4 Discussion

In the present study, no significant main effects of the *PPARγ* Pro12Ala and C1431T polymorphisms on adiposity-related phenotypes were observed in the total population. A significant interactive effect of the *PPARγ* genotypes with age and/or gender was not shown by GLM analysis. Previous reports mainly in adults Ala12 carriers showed increased adiposity (Beamer et al., 1998;Valve et al., 1999;Poirier et al., 2000;Meirhaeghe et al., 2000a) are in accordance with hypothesis 4, whereas others where the Ala12 allele was associated with lower BMI (Deeb et al., 1998;Ek et al., 1999) are contradictory. Previous studies, albeit in overweight subjects, have also reported significant associations between the T1431 allele and higher adiposity-related traits (Valve et al., 1999;Doney et al., 2002) and as such they are in agreement with hypothesis 4.

Haplotype-based analysis did not reveal stronger associations than genotype-based analysis and the proportion of variance explained by the diplotypes, although generally higher than that explained by individual genotypes (based on the increase in the sum of squares and as a result of the increased degrees of freedom) (data not shown), was not larger than that expected by random sampling, suggesting that most of the genetic effect observed was accounted for either by the individual SNPs or by variants in strong LD with them. Further splitting of the data according to *PPARγ* diplotypes might have reduced the statistical power to detect any significant, though modest, influence of the *PPARγ* variants on adiposity in the Greek population.

The frequency of the Ala12 allele in the present study was 0.07 (Table 6.1), which is lower than that reported for French (0.11) and Danish twins (MZ 0.14 and DZ 0.12) (Poulsen et al., 2003;Meirhaeghe et al., 2005b), but similar to other Caucasian populations. Similarly, the T1431 allele was 0.09 (Table 6.1), which is lower than that reported for French (0.13)

and Danish twins (MZ 0.13 and DZ 0.09). These results, however, are consistent with a previous study involving 11 European countries that reported an apparent north-to-south gradient in the Ala12 allele frequency through Europe, decreasing from 0.21 in Baltic countries to 0.07 in Mediterranean countries (Poirier et al., 2000). However, the hypothesis for that the prevalence of the Pro12Ala, ranging between 0.02 to 0.23 in different ethnic groups, (Altshuler et al., 2000;Poirier et al., 2000;Stumvoll and Haring, 2002) might affect the statistical power of comparisons, could not explain the inconsistent results. Similar frequencies for the rare Ala12 allele were reported in British and French populations yet contrasting associations were found with BMI (Doney et al., 2002;Meirhaeghe et al., 2005b). Similar associations were reported with BMI in Chinese and Indian populations, although they possessed different Ala12 allele frequencies (Tai et al., 2004). Similarly, comparable T1431 allele frequencies in British and French populations found opposing associations with BMI (Meirhaeghe et al., 1998;Doney et al., 2002).

Although genetic influences on adiposity-related phenotypes are easier to detect and measure in young individuals, as the environment has less time to exert its confounding effects that at later ages (Maes et al., 1997), in the present study, the Pro12Ala seemed to have no significant effect on adiposity-related phenotypes in early childhood, whereas in adults variation in *PPARγ* was significantly associated with changes in adiposity (Masud and Ye, 2003). A previous study in children (4-10 years old) has also revealed that this polymorphism had smaller effects on BMI in younger ages compared to adult populations (Cecil et al. 2005), whereas Pro12Ala variant was found not to contribute significantly to early onset obesity in a German population (Hamann et al., 1999). These modest effects of genes on the development of obesity are possibly attributed to gene-gene or gene-environment interactions. This notion is supported by two studies reporting an interaction effect of the Pro12Ala polymorphism with the Trp64Arg variant in *ADRB3* gene (which

individually was not associated with higher body weight) (Hsueh et al., 2001;Ochoa et al., 2004). In the present study, ANOVA tests for assessing the synergistic effect of Pro12Ala and Trp64Arg polymorphisms on adiposity-related phenotypes revealed no significant associations (data not shown). Furthermore, an interaction between nutrient and Pro12Ala polymorphism on BMI has been observed with carriers of the Ala12 allele showing higher BMI when the dietary polyunsaturated fat-to-saturated fat (PUFA/SFA) ratio was low and lower BMI when PUFA/SFA was high (Luan et al., 2001). Based on these findings, it is possible that in early childhood dietary composition is not similar to that in adults and especially differences in fat intake can mask potential significant effects of the *PPARγ* gene on adiposity at young ages. Previous reports have also shown that weight has a modulating effect on the genetic influence of *PPARγ* (Meirhaeghe et al., 1998;Ek et al., 1999;Valve et al., 1999), with the Ala12 allele being associated with higher BMI only in obese subjects. In the present study, separate ANOVA analysis for lean and overweight/obese boys and girls (defined as a BMI for age more than the 85th percentile based on the UK 1990 reference data) (Chapter 3) revealed no stronger associations between the Ala12 variant and BMI in overweight/obese compared to those in lean subjects (data not shown) and this could be attributed to BMI being a less direct measurement of adiposity in childhood.

In conclusion, the present study revealed that Pro12Ala and C1431T polymorphisms in *PPARγ* had no effect on adiposity and showed no interaction with age or gender in early childhood. An interactive effect of the Pro12Ala and Arg64Trp polymorphisms, as well as a modulating influence of BMI was not confirmed. Variation in the *PPARγ* seems not to contribute significantly to the high prevalence of early-onset obesity.

Chapter 7

General Discussion

The main objective of these series of experiments was to assess whether variation in selected candidate genes had an effect on adiposity-related phenotypes. In particular, the possible effects of polymorphic alleles in *ACE*, *ADRB1*, *ADRB2*, *ADRB3* and *PPARγ* on specific adiposity-related traits were investigated in a large, representative and genetically homogeneous population of toddlers and preschoolers by using genotype- and haplotype-based approaches. Although more than 600 genes, markers and chromosomal regions have been associated with adiposity-related traits (Ek et al., 1999;Rankinen et al., 2006), the aforementioned genes in the present study were selected because of their biological significance in metabolic pathways. Previous studies on these variants have been focused mainly in adult populations, which were selected in most of the cases on the basis of specific phenotypic characteristics. Reported findings on the effects of these genetic variants on obesity are not, however, consistent and these disparities may be attributed to gender, age and ethnicity differences, insufficient power, genotyping errors, as well as unconsidered multiple testing effects and regional variations in allele frequencies. Limited or no data on genes association with adiposity is available in young children, although there is evidence that genetic influences may be stronger in young individuals, as the environment has had less time to exert the confounding effects. Therefore, the series of the SNP-based association studies described in Chapters 4-6 are one of the first to investigate such associations and to assess the effect of these variants on early childhood development. In the current chapter, findings from these series of experiments are described and conclusions are discussed along with the strengths and limitations of the present study.

7.1 Growth and adiposity in Greek children: a comparison with British norms

The prevalence of overweight and obesity was found to be high in the Greek children; 13.6% and 10% of children were overweight and obese respectively (Table 3.1). The rates of overweight and obesity were comparable to those reported in similarly aged populations throughout the world (Figures 3.5 and 3.6), but lower than those reported in UK or US (Figures 3.4 and 3.5). These results were in agreement with previously studies where increasing rates of obesity were recorded in Greek children and adolescents (Ek et al., 1999;Krassas et al., 2001;Lissau et al., 2004;Magkos et al., 2005;Rankinen et al., 2006). Furthermore, children of the present study, irrespective of gender, were found to be significantly taller and heavier than their British counterparts, with these differences being more prominent with advancing age (Table 3.3 and 3.4). Compared to anthropometric data from similarly aged children from the USA, Greek children showed similar growth patterns (Figure 3.3). Sex-specific differences in the evolution of anthropometric indices were observed in both the Greek and US populations.

Due to scarcity of studies on similarly aged-children in Greece, the explanation for the present high rates of overweight and obesity becomes somewhat troublesome. Environmental factors are likely to account for the observed differences between the present and the reference population of a generation ago. Previous studies have indicated that Greek families tend to overfeed or force-feed their children, while increased adiposity in school-aged Greek children was attributed to reduced physical activity and increased sedentary behaviours rather than increased energy intake (Mamalakis et al., 2000). Furthermore, the fact that the average age of adiposity rebound in the present population is around the 4th year of age for both genders and much lower than those reported in studies

initiated several decades ago (Rolland-Cachera et al., 1984) could suggest that modern environmental factors, such as TV and video games, are major contributors to the development of obesity. Nevertheless, (Manios, 2006) has reported for the present population that the mean daily intake of energy and macronutrients was found to be increasing with advancing age but was similar to those reported for Greek children of a decade ago. On the other hand, Greek children showed an increased tendency with advancing age for sedentary lifestyles, further supporting previous suggestions of an association between overweight and reduced physical activity during preschool years (Trost et al., 2003). In light of the alarming rates of overweight and obesity in toddlers and preschoolers of the present cohort, the significant differences in the mean values of BMI observed at 4-6 years of age relative to the British normative data (Table 3.3, Table 3.4) could be reflective of an increased adiposity, as the excess BMI SDS was comparable to that reported on the specific age group in previous studies (Stenhouse et al., 2004;Reilly et al., 2006).

7.2 Effects of polymorphisms in the selected candidate genes on adiposity-related phenotypes in Greek toddlers and preschoolers

7.2.1 ACE I/D polymorphism and adiposity-related phenotypes

According to the findings in Chapter 4, a significant main effect of the *ACE* I/D polymorphism on BMI and significant interactions between I/D genotype and age for the same phenotype were revealed (Figure 4.1). The *ACE* I-allele, being associated with lower circulating *ACE* levels, was significantly associated with higher BMI in boys and girls 1-2 years (Figure 4.2). The findings in boys and girls aged 1-2 years are not in agreement with

therefore, variation in these genes can have functional consequences that influence adiposity. In recombinant cells, the *ADRB2* Gly16 allele was found to undergo enhanced agonist-promoted down-regulation compared to the Arg16 allele and hence reduced receptor efficiency and lower lipolysis, whereas the Glu27 allele was found to undergo very little agonist-promoted down-regulation as compared to the Gln27 allele (Green et al., 1994;Bachman et al., 2002). This effect of Gly16 allele on adiposity is consistent with the present finding in boys and girls 4-6 years where the Gly16 allele was associated with higher waist circumference (Figure 5.3). Furthermore, based on the observation that in 1-2, 2-3 and 3-4 years age groups, boys and girls with the Arg16 rather than Gly16 variant had higher hip circumference, it could be postulated that changes in the environment at the 4^{th} year of age may be responsible for the altered effect of the C16 polymorphism at older children (4-6 years age group). According to hypothesis 3, individuals with different alleles will respond differently to environmental stimuli, such as exercise and feeding For example, homozygotes for the Gly16 allele will lose less weight during physical activity since they will respond less to the presence of catecholamines than those homozygotes for the Arg16 allele. Furthermore, inactivity may influence the effect of Glu27Gln polymorphism on adiposity in the present population. As such, although according to hypothesis 2 (based on *in vitro* studies) individuals with the Gln27 variant will be expected to show lower adiposity, the opposite effect observed in boys 2-3 years (Figure 5.4) (i.e. boys bearing the Gln27 allele had higher hip circumference) and in a previous study in adults (Meirhaeghe et al. 1999) could suggest that physical activity may be a significant factor in modifying genotype-phenotype relationships reported here.

In the present study no apparent effect of the *ADRB1* Ser49Gly and Arg389Gly polymorphisms and inferred haplotypes, as well as *ADRB3* Trp64Arg polymorphism on adiposity was observed. In recombinant cells, the *ADRB1* Arg389 allele has been

hypothesis 1 which was based on previous studies albeit in adolescents or adults. However, at 4-6 years of age, subjects bearing the *ACE* D-allele (associated with higher circulating *ACE* levels) rather than I-allele had higher (but not significantly) BMI. This change in the direction of effect of I/D polymorphism was apparent mainly in girls (Table 4.1) and was in accordance with previous studies in teenagers and adults that have reported increased adiposity in DD individuals compared to those with the II or ID genotypes **(Hypothesis 1)**. The age, at which this alteration in the effect of *ACE* I/D polymorphism on adiposity was observed, coincided with the age at which BMI increased after its developmental nadir. These results suggested that the *ACE* I/D polymorphism is associated with developmental changes in adiposity during early childhood with age and possibly gender being important factors in modifying genotype-phenotype relationships.

There is evidence that a local angiotensin II generating system independent of circulating RAS components exists in several tissues, such as heart, brain, kidney, pancreas and adipose tissue (Lavoie and Sigmund, 2003), where production of this potent vasoconstrictor results in decreased blood flow locally (Goossens et al., 2003). Although an increase in local blood flow due to angiotensin II remains controversial (Goossens et al., 2004), such an effect would be in contrast with the present finding of the I-allele being associated with higher adiposity, as higher levels of circulating ACE (associated with the D-allele) would result in an excess secretion of angiotensin II, which will decrease adipose-tissue blood flow and consequently lead to increased lipogenesis and fat storage in adipocytes (Jones et al., 1997). Furthermore, it is known that angiotensin II induces the release of noradrenaline (Goossens et al., 2004), which stimulates the a2-adrenergic receptors that inhibit lipolytic action (Lafontan and Berlan, 1993). Increased bradykinin inactivation could also play a role in adipocyte metabolism. This finding in very young boys supports the notion of a possible lipolytic action of angiotensin II, which is based on

the fact that stimulation of AT1 receptor by angiotensin II leads to increased levels of cellular calcium concentrations associated in adipocytes with increased lipolysis (Strazzullo and Galletti, 2004). However, the hypothesis of a putative role of angiotensin II in lipolysis has not yet been confirmed in humans (Townsend, 2001). Furthermore, it has been shown that the two angiotensin II receptor subtypes (AT1 and AT2) are differentially expressed in renal and vascular tissues during the first 2 years of life (Viswanathan et al., 2000) and that AT2 is a natural antagonist for AT1 (Danser, 2003). Therefore, it could be postulated that overexpression of AT2, which results in downregulation of AT1 expression, could induce vasodilation and concomitantly have an effect in fat accumulation. This notion could explain in part the association of the I-allele (lower levels of ACE) with increased adiposity in very young boys and girls in the present study, since the actions of AT1, through which angiotensin II exerts its effects, may be counteracted by AT2 receptor.

The *ACE* I/D polymorphism has been associated in an additive manner with almost half of the variance in serum *ACE* activity (Rigat et al., 1990) in Caucasian populations (Tiret et al., 1992). The majority of the genetic effects observed in the present study are accounted for either by the I/D polymorphism or a variant in strong LD with it (Tiret et al., 1992). Two previous studies (Zhu et al., 2000;Cox et al., 2002) have suggested that such a functional polymorphism is the rs4363 (A22982G). This variant has been shown to be a better candidate for circulating *ACE* levels than the I/D polymorphism in African populations, where it was found not to be in complete LD with the I/D polymorphism. In Caucasian populations, though, the I/D polymorphism is in almost complete LD with this functional variant (Soubrier et al., 2002) and as such, is a sufficiently good marker of circulating *ACE* activity. A previous study on Greek teenagers has also shown the degree

of association between the *ACE* I/D polymorphism and adiposity-related phenotypes, was not exceeded by other ACE polymorphisms (Moran et al., 2005).

7.2.2 ADRB polymorphisms and adiposity-related phenotypes

The results of the experiments described in Chapter 5 indicated a significant main effect of the *ADRB2* Gly16Arg polymorphism on waist and hip circumferences and a significant interaction of the C16 genotypes with age for the same phenotypes. The *ADRB2* Gly16 allele (the receptor with this allele showing enhanced agonist-promoted downregulation) was associated with significantly higher waist circumference at the age of 4 years in boys and girls (Figures 5.1 and 5.3) and this finding was in accordance with **hypothesis 2** and with previous studies in adolescents and adults. Furthermore, a significant main effect of *ADRB2* Glu27Gln polymorphism on hip circumference and significant interactions with both age and gender for the same phenotype were revealed. In boys aged 2-3 years, the Gln27 allele was significantly associated with higher hip circumference (Figure 5.4); however this association was not in agreement with **hypothesis 2**, which was based on *in vitro* studies and previous association studies in adults. These results suggested that the effects of *ADRB2* C16 and C27 polymorphisms are influenced by age and gender, while variation in *ADRB1* C49 and C389, as well as in *ADRB3* C64 has no effect on adiposity-related phenotypes in early childhood.

The biological mechanisms through which variation in the *ADRB* genes influence adiposity have yet to be elucidated. Catecholamines effects are mediated by binding to lipolytic beta-adrenoceptors and anti-lipolytic alpha2-adrenoceptors of white adipose cells (Lafontan and Berlan, 1993). Stimulation of *ADRBs* in adipose tissue leads to activation of the adenylyl cyclase and subsequent increase in cAMP, which promotes lipolysis and thermogenesis (Bachman et al., 2002;Gonzalez Sanchez et al., 2003;Lowell and Bachman, 2003) and

associated with increased adenylyl cyclase activity and intracellular concentration of cAMP due to enhanced receptor-Gs interaction compared to the Gly389 receptor (Maqbool et al., 1999;Mason et al., 1999). Despite the reported effects in cell systems, this polymorphism has not been associated with obesity (Ryden et al., 2001;Tafel et al., 2004;Ellsworth et al., 2005), consistent with the present findings, and had no apparent effect on catecholamine-induced lipolysis (Ryden et al., 2001). Furthermore, the *ADRB3* Trp64Arg polymorphism is located either in the first transmembrane domain or the first intracellular loop of the *ADRB3* receptor (Small et al., 2003), where aminoacids residues are thought not to be important for the trafficking of the receptor to the cell surface or its coupling to Gs proteins (Strosberg, 1997). In addition to that, *in vitro* studies have shown conflicting results regarding the effects of this polymorphism on the pharmacological and functional properties of the receptor raising arguments for or against the involvement of the Arg64 receptor in obesity (Candelore et al., 1996;Pietri-Rouxel et al., 1997). Finally, it is known that adipocytes from normal children have a higher content of alpha2-adrenoceptors than adipocytes from adults (Lafontan and Berlan, 1993) and therefore, a preferential stimulation of these anti-lipolytic receptors rather than *ADRBs* can inhibit lipolysis and mask the possible effects of *ADRBs* on adiposity in early childhood.

7.2.3 PPARγ polymorphisms and adiposity-related phenotypes

According to the findings in Chapter 6, no significant effects of *PPARγ* Pro12Ala and C1431T polymorphisms (i.e. Ala12 and T1431) on adiposity or significant interactions with age and/or gender were revealed in the total population. These findings were consistent with previous studies in children (Hamann et al., 1999;Cecil et al., 2006), where these polymorphisms had smaller or no effect on adiposity and contradictory to other studies, mainly in adult populations, where the Ala12 and/or the T4131 alleles were associated with increased adiposity (Beamer et al., 1998;Valve et al., 1999;Meirhaeghe et

al., 2000a;Doney et al., 2002). Furthermore, a previously reported modulating effect of weight on Pro12Ala polymorphism (Meirhaeghe et al., 1998;Ek et al., 1999;Valve et al., 1999) or an interactive effect of this polymorphism with the *ADRB3* Arg64Trp variant was not cofirmed in the present population. These findings collectively could indicate that variation in *PPARγ* does not contribute to increased adiposity observed in early childhood but it is possible that the impact of these variants on adiposity-related phenotypes becomes more apparent later in life.

Although functional differences between the Pro12 and Ala12 *PPARγ* receptors (Deeb et al., 1998) could suggest that the Pro12Ala polymorphism is the causative variant, the observed effects of this polymorphism on adiposity in the present and previous studies may be due to a variant in LD with it. A polymorphism in the promoter of $PPAR_{\gamma 2}$, which has been previously tested along with the Pro12Ala polymorphism by haplotype-analysis for significant associations with obesity (Deeb et al., 1998;Meirhaeghe et al., 2005b) and metabolic syndrome (Meirhaeghe et al., 2005a), was found to be in almost complete LD with the Pro12Ala in a Caucasian population (Meirhaeghe et al., 2005b). In the same population, the synonymous C1431T polymorphism was in LD at 66-69% with the $PPAR_{\gamma 2}$ promoter and Pro12Ala polymorphisms (Meirhaeghe et al., 2005b). The $PPAR_{\gamma 2}$ promoter and Pro12Ala polymorphisms were associated with increased risk for metabolic syndrome only in the presence of a C1431 allele in the haplotype (Meirhaeghe et al., 2005a). Furthermore, C1431T was found to be a better predictor of fasting insulin levels and insulin sensitivity than the Pro12Ala polymorphism (Moffett et al., 2005), while an opposing interaction of these two polymorphisms with BMI has been observed in adults (Doney et al., 2002). Collectively these findings could suggest that the non-functional C1431T polymorphism may in tight LD with a functional variant in the *PPARγ* or in a linked gene. In the present study, haplotype analysis revealed no interaction between the

Pro12Ala and C1431T polymorphisms with any of the adiposity-related phenotypes. Nevertheless, a systematic screening of 70 diabetic individuals did not reveal any additional missense mutations in the *PPARγ* gene suggesting that a functional variant may be in a nearby gene (Altshuler et al., 2000).

7.2.4 Common findings for the effects of variants in the selected candidate genes on adiposity-related phenotypes

The series of experiments described in Chapters 4-6 assessed the effect of individual polymorphisms in selected candidate genes on a number of adiposity-related phenotypes. A large number of familial and twin studies have shown that there is a strong contribution of heredity in obesity (Bouchard and Perusse, 1988), which is likely to be the result of a number of predisposing alleles, each conferring a small increase in the risk to the individual but a significant cumulative contribution to the development of obesity (Marti et al., 2004;Rankinen et al., 2006). In the present study, genetic variations in the selected candidate genes accounted for 1-6% (for example Figures 4.2 and 5.3) of the observed variance in the adiposity-related phenotypes for significant gene associations (as determined by ANONA). Thus, the influence of these polymorphisms on adiposity was found to be relatively small, as expected for a complex disease and such a polygenic effect was also reflected by the distributed effect of the genotypes on the whole phenotypic range (for example Tables 4.2 and 4.4). In the present study, a cohort of toddlers and preschoolers was chosen in an attempt to characterise the genetic influence on adiposity, since longitudinal twin studies have shown that this influence is easier to detect and measure in younger populations, as the environment has less time to exert confounding effects than at later ages (Maes et al., 1997). Findings of the present study further supported the notion of a more pronounced genetic effect on adiposity in young children.

For example, the *ACE* I/D polymorphism explained a larger proportion of variance for BMI in the younger boys and girls (Figure 4.2; *ACE* I/D genotype explaining 6.4% of phenotypic variance) than in Greek adolescents (the proportion of variance explained in that study was 0.8% for BMI) (Moran et al., 2005). However, a stronger effect of polymorphisms in *ADRB* and *PPARγ* in the present young cohort compared to that in adult populations was not observed, offering support to previous suggestions that the effect of these genes may be age-specific and dependent on their age-related variability in exposure to environmental factors that predispose to their expression (Maes et al., 1997).

Previous reports have revealed gender-specific effects of the selected variants on adiposity (Beamer et al., 1998;O'Donnell et al., 1998;Meirhaeghe et al., 2000b) and marked differences in body composition during the first years of life (Fomon et al., 1982). The present study attempted to assess possible interactions of the genotypes of interest with gender by carrying out GLM analyses in the whole series of experiments. Gender-specific effects were demonstrated mainly for the *ADRB2* C16 and C27 polymorphisms assessed in the present population. Gender-specific effects may be the result of the dependency of certain genetic determinants on other cofactors such as hormones or other genes for their function and the influence of these cofactors may become detectable only later in life. Nevertheless, previous studies have shown that there are gender-specific differences in sympatho-adrenal activity in preschool children, with boys excreting higher levels of both catecholamines than girls (Lundberg, 1983) and these differences could account for the gender-specific effects of the *ADRB* variants in adiposity regulation.

Haplotype-based analysis did not reveal stronger associations than genotype-based analysis and the portion of variance explained by the diplotypes, although generally higher than that explained by individual genotypes (based on the increase in the sum of squares and as a result of the increased degrees of freedom) (data not shown), was not significantly larger.

This could suggest that the haplotypes mediate the majority, if not all, of any effect they have through the individual SNPs and that if the haplotypes have a significantly increased effect on the phenotypes of interest it is small enough that this study did not have the statistical power to identify, after splitting in many genetic sub-groupings. Several studies have advocated that genotyping of more than one polymorphism in a gene allows the inference of haplotypes that represent the majority of variation along the complete length of gene and they are better tools for assessing genetic effects on the development of complex traits (Akey et al., 2001). The present study did not provide further evidence for the greater analytical power of haplotypes. However, the present findings did suggest a dependency of haplotype-based approaches on sample size that is proportional to the strength of the effect under investigation.

7.3 Strengths and weaknesses of the study

Validity is the "degree to which the inference drawn from a study is warranted when account is taken of the study methods, the representativeness of the study sample and the nature of the population from which it is drawn". Minimizing bias and accounting for confounding factors are crucial for the validity of association studies (Akey et al., 2001;Zaccai, 2004).

7.3.1 Selection of sample and reliability of the measurements

7.3.1.1 Measurement error

Measurement bias can affect validity and can arise from the choice of the instruments used for this purpose, as well as the assessor's experience and the subject's cooperation. In the present study, all study sites used the same measuring equipment and procedures.

Measurements were taken by two well-trained members, which were referred as leading and assisting observer, respectively. The role of the assisting observer was to help position the child correctly, while the leading observer recorded the measurements (Manios, 2006). In that way, measurements errors due to lack of experience of the assessor and incorrect position of the subject were limited. Furthermore, the measuring equipment used was highly accurate to avoid the systematic error arising from inaccurate measurement of the different subphenotypes of interest and reduce the contribution of noise to variability (i.e. increase the fraction of variation explained by genetic factors). In the present population, children were found to be significantly taller compared to British counterparts (Tables 3.3 and 3.4, Figure 3.2). Although it could be argued that there was a systematic error in the measurement of this subphenotype in the present cohort, comparison of the mean height values in Greek children with those reported in a representative sample of preschool children (2-6 years) from Cyprus (Akey et al., 2001;Savva et al., 2005) revealed no differences in height between these two populations. Furthermore, the similarities in the evolution of anthropometric indices (waist and arm circumferences, as well as triceps and subscapular skinfolds) between Greek children of the present study and US children (Figure 3.3) could provide more evidence for the plausibility of these measurements.

7.3.1.2 Choice of study population

Selection of subjects is known to have an impact on the conclusions of a study (Zaccai, 2004). In the present study, subjects were sampled from randomly selected public and private nursery schools, as well as day-care centers within five counties in Greece (Manios, 2006). Given the fact that these counties are widely distributed across Greece and their overall local population comprises 70% of the total Greek population (Manios, 2006), the present cohort could be considered as a representative sample of Greek toddlers and preschoolers. However, not all Greek children attend nursery or day-care centers at that

age and as such children included in the study are likely to differ from non-participants (children that do not attend nursery) in a number of important ways, such as levels of physical activity (organized activities in the school), as well as differences in the health knowledge and practices between parents and caregivers that exert an increased control in food intake at that age. Therefore, it is possible that the sample of the present study is representative of the nursery/carecentre preschoolers rather than the total Greek population of this age. Furthermore, the higher rates (95%) of participation for rural areas compared to those in urban areas in the present study may have also introduced bias. However, a previous study in preschoolers (2-6 years) has shown that area of residence or parental education level was not significantly associated with obesity status at that age (Savva et al., 2005). Finally, the population studied here is genetically homogeneous (by ethnicity) and as such pitfalls due to population stratification are diminished.

7.3.2 GLM analysis on total population versus gender- and age-stratified ANOVA analysis: power of the study

Previous studies have shown effects of gender and/or age on the association of *ACE* I/D, *ADRB2* C16 and C27, as well as ***PPARγ*** Pro12Ala and C1431T polymorphisms on adiposity-related phenotypes. For example, Ellsworth et al. (2002) has used gender and age-stratified analysis to account for growth and developmental effects in the investigation of the effects of the *ADRB2* gene on childhood obesity. In the present study, GLM analysis has been carried out initially in the total population (boys and girls together) to assess possible associations between genetic variants and adiposity-related phenotypes and to test for possible interactions between gender and/or gender and genotypic effects on phenotype. Results from this GLM ANOVA analysis were compared to those found in the one-way ANOVA analysis for each gender and age group.

A key determinant in the quality of an association study is sample size. Studies should be powered to detect the small effects of common variants involved in complex traits, such as obesity (Hattersley and McCarthy, 2005). Based on this fact, GLM ANOVA analysis was carried out in the total population (2102 children) and provided evidence of genotype interactions with age and/ or gender which could indicate that carrying out ANOVA analysis in the total population can mask potential significant genotype-phenotype interactions. For example, GLM analysis has shown that the C27 genotypes interact with both age and gender for hip circumference and, as such, dividing the population in boys and girls, as well as age groups becomes essential. As such, significant associations between C27 genotype and hip circumference were observed only in boys aged 2-3 years rather than the total population. Therefore, although examining the total population increases the power of the study, sub-grouping is necessary when age and/or gender are shown to be significant factors in modifying the genotype-phenotype associations. On the other hand, when no significant interactions between gender and/or age are found by GLM-ANOVA analysis, dividing the population in genders and age groups for further analysis may lead to false positive genotype-phenotype associations. For example, although no significant interactions were found between age and/or gender and ***PPARγ*** C1431T variants, further one-way ANOVA analysis stratified by gender and age revealed significant associations between the T1431 allele and higher adiposity measurements only in girls 3-4 years suggesting that these may be the result of a Type I error. In some cases, however, it may be that two variables do interact, but the study lacks the power to detect an interaction. For example, it has been shown that the *ACE* I/D polymorphism has a gender effect on the influence of genotype in children 11-18 years (Moran et al., 2005). In the present study, GLM analysis has shown an interaction between age and I/D genotype but no interaction between gender and genotype. This could suggest that either the relationship

between gender and I/D genotype is truly insignificant or that the present study failed to detect the significance of gender (a Type II error).

Association studies should take into consideration possible age and/or age effects that can influence genetic effects on phenotypes. Furthermore, subdividing a cohort by gender or age could easily mask the small effects of genes on the phenotype in question and the presence of such effects may have contributed to the shortcomings of previous investigations. Although gene association studies require, by design, large sample sizes for genuine findings (Clayton and McKeigue, 2001), analyzing the total population instead of gender and/or age subgroups can increase the likelihood of type II errors so that real associations are missed.

Despite the limitations mainly associated with the design of the study, association studies based on candidate-gene approaches offer a potentially powerful tool in identifying genetic variants that influence susceptibility to obesity (Risch, 2000). According to the human Obesity Map, the number of studies reporting associations between genetic variants in specific genes and obesity-related phenotypes increases each year with 358 findings of positive associations with 113 candidate genes being reported in the 2004 update (Perusse et al., 2005) and 429 findings of associations with 127 candidate genes in the 2005 update (Rankinen et al., 2006). Studying the impact of these genetic variants on phenotypic outcomes of interest enhances the evidence of causality, while knowledge of the functionally significant variants may also aid in prediction and prognosis, as well as in designing drug therapies and intervention. Results from candidate-gene based approaches complemented by analyses quantifying protein expression in several tissues under different environmental influences can provide new insights into the understanding of energy homeostasis and management of obesity.

7.4 General conclusions

From the findings of the aforementioned experiments the following conclusions can be made:

I. From the results of the study assessing the developmental changes in adiposity in a large, representative sample of Greek toddlers and preschoolers, as described in Chapter 3, the following conclusions can be made:

   a. The present study was carried out on a large cohort of toddlers and preschoolers from Greece and a wide range of adiposity-related phenotypes were included in the analyses. As such, this is the first study with such a wide range of adiposity-related phenotypes available on toddlers and preschoolers.

   b. The rates of overweight in the present population were found to be comparable to the high rates reported in other studies conducted in representative populations worldwide. Considering overweight and obesity prevalence, the present population had comparable rates with those in other European and Australian preschool children, but still lower than those reported in UK and the USA. Relative to normative data from British children of two decades ago, Greek children were much taller and concomitantly heavier suggestive of improved nutritional status and accelerated growth and they showed a tendency for increasing BMI with advancing age. The excess BMI levels were similar to those found in modern preschoolers and most likely attributed to increased adiposity.

c. Greek children of the present population were characterised by an early adiposity rebound possibly attributed to increased body fat in girls and increased bone and/or muscle mass in boys. The age- and gender- related differences in the evolution of several adiposity-related phenotypes were similar to those observed in similarly aged US children, suggesting similar growth patterns among preschoolers.

II. From the series of experiments assessing the influence of variation in *ACE*, *ADRB* and *PPARγ* genes on adiposity-related phenotypes, as described in Chapters 4-6, the following conclusions can be made:

a. The present findings advocated a stronger effect of the *ACE* I/D polymorphism in Greek toddlers and preschoolers than that reported in adolescent and adult populations. The *ACE* I-allele was significantly associated with higher scores for BMI in younger boys and girls in contrast to hypothesis 1, but with lower measures at the age of 4 confirming the significant interaction between I/D genotypes and age. The I/D polymorphism, being a sufficiently good marker of circulating *ACE* activity, was associated with developmental changes observed during early childhood. The age- and possibly gender- related significant associations suggested that the effect of *ACE* I/D polymorphism on adiposity is possibly modulated by gene-environment interactions.

b. The effects of *ADRB2* variants on adiposity were modest and manifested in a gender- and age-specific manner, whereas polymorphic variants of *ADRB1* and *ADRB3* genes were found to have no influence on adiposity-related phenotypes or to interact with age/gender in early childhood. A significant

interaction between C16 genotypes and age was observed with Gly16 carriers showing higher adiposity. This effect was in accordance with hypothesis 2. A significant interaction was also revealed between C27 genotypes and age, as well as gender with the Gln27 allele being associated with higher adiposity only in boys 2-3 years. This effect was in contrast to hypothesis 2, but it could be explained by hypothesis 3, which supports an age-related variability to environmental stimuli important for the function of ADRB2 receptor. Although haplotype-based analysis did not reveal stronger associations compared to individual SNPs, Gly16Gln27/Gly16Gln27 and Arg16Gln27/Gly16Gln27 diplotypes were associated with higher adiposity compared to the Gly16Glu27/Gly16Glu27 suggesting a possible dominant effect of the *ADRB2* Glu27Gln polymorphism on the receptor's function.

c. Polymorphic variants of the *PPARγ* gene had no significant effect on adiposity in early childhood. A synergistic effect of *PPARγ* Pro12Ala and *ADRB3* Arg64Trp polymorphisms or a modulating effect of BMI on the genetic influence of the Pro12Ala on adiposity-related phenotypes was not confirmed. Haplotype-based analysis revealed no stronger associations compared to individual genotypes. Variation in *PPARγ* is not a significant contributor to childhood obesity. The lack of significant associations in children, but not in adults, could be attributed to age-related differences in fat intake, since fatty acids derived from nutrition are the main governors of *PPARγ* activity.

d. Findings from the present study further supported previous suggestions of a more pronounced genetic effect on adiposity in young children than adults. This effect was only observed for *ACE* I/D polymorphism, but not for *ADRB*

and *PPARγ* variants indicating that genetic influence can be age-specific and expression of these genes may be dependent on the age-related variability in exposure to environmental factors. The observed gender-specific effects of the selected genetic variants on adiposity may also reflect gender-specificity in gene expression that results in differences in adipose tissue metabolism between boys and girls.

e. Haplotype-based analysis did not produce markedly stronger associations relative to individual SNPs and the proportion of variance explained by haplotypes, although generally larger, was not significantly greater than that expected by random-sampling effects, suggesting that most of the genetic effect observed accounted for either by the individual SNPs or by variants in strong LD with them. This is not, however, against the robustness of haplotypes in analysing complex traits and it could indicate that the present population was probably not large enough to assess the modest genetic effects on phenotypes when so many different genetic subgroups were created, suggesting a dependency of haplotype-based approaches on sample size.

f. Association studies can lead to misleading results, if not designed well. Bias and confounding factors should be taken into consideration for maximizing the study's validity. Dividing the population in many subgroups when not necessary can increase the risk of Type I errors, while examining the total population without taking into consideration confounding factors, such as age and gender can result in missing genuine associations. Discussing significant associations with respect to their biological plausibility, as well as reporting any potential impact of limitations on the findings is in keeping with good

scientific practice and can help assess the validity or bias of significant findings.

Appendices

List of appendices

Appendix 1: ANOVA analysis for associations between adiposity-related phenotypes and variants of the *ACE*, *ADRB1*, *ADRB2*, *ADRB3* and *PPARγ* gene polymorphisms in different age groups for boys and girls.

Mean values and 95% CIs are given for the measured parameters for each genotype at different ages in boys and girls. Associations between the genotypes and the adiposity-related phenotypes in different age groups were analysed by ANOVA. Data have been normalised, then analysed, then back-transformed to values appropriate for each age group. For each test, the percentage of variance explained by ANOVA (V) and the probability (P) are given. P values are given to three significant figures. Tests significant before ($P<0.05$), but not after Sidak correction are indicated in italics; tests that remained significant after Sidak correction for each age group are indicated in bold. N values indicate group sizes for the individual genotypes.

Table 1 Associations of adiposity-related phenotypes with *ACE* I/D polymorphism in boys.

	BMI	Arm Circumference	Waist Circumference	Hip Circumference
Boys 1-2 yrs (N=11, 39, 36)	**II=18.41 (17.51 to 19.50)** **ID=16.62(16.25 to 17.02)** **DD=16.46 (16.10 to 16.85)** **V=16.6%, P=0.001** **Sum of Skinfolds** II=29.80 (25.72 to 34.14) ID=25.59 (24.01 to 27.22) DD=26.14 (24.24 to 28.11) V=0%, P=0.109	II=15.90 (15.08 to 16.64) ID=15.57 (15.30 to 15.84) DD=15.59 (15.23 to 15.94) V=1.1%, P=0.627	II=47.50 (46.02 to 48.99) ID=46.52 (45.47 to 47.58) DD=46.74 (45.98 to 47.51) V=1.2%, P=0.612	II=50.51 (49.26 to 51.75) ID=49.31 (48.19 to 50.42) DD=49.09 (48.33 to 49.85) V=2.4%, P=0.368
Boys 2-3 yrs (N=29, 108, 97)	**BMI** II=16.34 (15.76 to 16.95) ID= 16.43 (16.16 to 16.69) DD=16.27 (16.02 to 16.53) V=0.3%, P=0.725 **Sum of Skinfolds** II=25.39 (22.91 to 26.31) ID=25.61 (24.55 to 26.76) DD=25.88 (24.81 to 27.05) V=0%, P=0.487	**Arm Circumference** II=16.00 (15.62 to 16.41) ID=16.03 (15.82 to 16.24) DD=15.93 (15.71 to 16.16) V=0.2%, P=0.821	**Waist Circumference** II=49.89 (48.66 to 51.19) ID=49.70 (49.13 to 50.29) DD=49.22 (48.65 to 49.80) V=0.8%, P=0.415	**Hip Circumference** II=53.21 (52.19 to 54.27) ID=52.87 (52.24 to 53.53) DD=52.45 (51.85 to 53.06) V=0.7%, P=0.446
Boys 3-4 yrs (N=72, 196, 145)	**BMI** II=15.88 (15.55 to 16.23) ID=16.02 (15.94 to 16.24) DD=15.97 (15.74 to 16.21) V=0.1%, P=0.777 **Sum of Skinfolds** II=23.43 (22.10 to 24.89) ID=25.21 (24.85 to 26.14) DD=24.79 (23.95 to 25.68) V=0%, P=0.084	**Arm Circumference** II=16.30 (15.99 to 16.64) ID=16.41 (16.33 to 16.61) DD=16.26 (16.05 to 16.47) V=0.3%, P=0.587	**Waist Circumference** II=51.06 (50.23 to 51.93) ID=51.44 (51.22 to 51.98) DD=51.04 (50.51 to 51.59) V=0.3%, P=0.556	**Hip Circumference** II=54.89 (54.08 to 55.73) ID=55.18 (54.95 to 55.75) DD=54.67 (54.06 to 55.30) V=0.3%, P=0.484
Boys 4-6 yrs (N=49, 143, 112)	**BMI** II=15.98 (15.59 to 16.40) ID=15.90 (15.65 to 16.16) DD=15.90 (15.62 to 16.19) V=0%, P=0.948 **Sum of Skinfolds** II=23.76 (22.32 to 25.39) ID=23.46 (22.60 to 24.38) DD=23.55 (22.53 to 24.65) V=0%, P=0.947	**Arm Circumference** II=16.79 (16.38 to 17.23) ID=16.65 (16.43 to 16.89) DD=16.83 (16.61 to 17.07) V=0.4%, P=0.553	**Waist Circumference** II=53.27 (52.21 to 54.40) ID=52.96 (52.31 to 53.64) DD=52.82 (52.09 to 53.58) V=0.1%, P=0.804	**Hip Circumference** II=57.58 (56.27 to 58.98) ID=57.29 (56.58 to 58.03) DD=57.45 (56.71 to 58.23) V=0.1%, P=0.911

Table 2 Associations of adiposity-related phenotypes with *ACE* I/D polymorphism in girls.

Girls 1-2 yrs (N=13, 49, 28)	**BMI** II=17.04 (16.31 to 17.87) ID=16.40 (16.02 to 16.80) DD=17.08 (16.54 to 17.67) V=5.4%, P=0.092 **Sum of Skinfolds** II=25.24 (22.93 to 27.85) ID=25.62 (23.95 to 27.43) DD=24.66 (22.85 to 26.64) V=0.3%, P=0.771	**Arm Circumference** II=15.38 (14.83 to 15.92) ID=15.38 (15.05 to 15.70) DD=15.29 (14.68 to 15.88) V=0.1%, P=0.951	**Waist Circumference** II=47.05 (45.35 to 48.91) ID=45.73 (44.87 to 46.62) DD=46.14 (44.86 to 47.51) V=1.8%, P=0.444	**Hip Circumference** II=48.28 (45.71 to 50.95) ID=49.29 (48.22 to 50.39) DD=49.00 (47.61 to 50.42) V=0.8%, P=0.715
Girls 2-3 yrs (N=30, 86, 78)	**BMI** II=16.27 (15.75 to 16.81) ID=16.36 (16.03 to 16.70) DD=15.96 (15.69 to 16.25) V=1.6%, P=0.204 **Sum of Skinfolds** II=28.87 (26.00 to 31.98) ID=26.50 (25.16 to 27.94) DD=27.32 (26.00 to 28.74) V=0%, P=0.286	**Arm Circumference** II=16.32 (15.78 to 16.89) ID=15.94 (15.64 to 16.25) DD=16.06 (15.80 to 16.32) V=0.9%, P=0.424	**Waist Circumference** II=50.14 (48.74 to 51.56) ID=49.35 (48.53 to 50.17) DD=49.51 (48.84 to 50.18) V=0.6%, P=0.578	**Hip Circumference** II=53.02 (51.55 to 54.62) ID=53.19 (52.40 to 54.01) DD=52.66 (52.06 to 53.28) V=0.5%, P=0.622
Girls 3-4 yrs (N=59, 160, 150)	**BMI** II=16.39 (16.03 to 16.76) ID=15.99 (15.77 to 16.22) DD=15.83 (15.58 to 16.09) V=1.5%, P=0.064 **Sum of Skinfolds** II=28.82 (27.02 to 30.79) ID=27.91 (26.85 to 29.03) DD=27.20 (26.18 to 28.27) V=0.6%, P=0.295	**Arm Circumference** II=16.72 (16.36 to 17.09) ID=16.40 (16.18 to 16.61) DD=16.30 (16.07 to 16.52) V=1.0%, P=0.147	**Waist Circumference** II=52.34 (51.25 to 53.47) ID=51.48 (50.87 to 52.11) DD=51.03 (50.36 to 51.71) V=1.1%, P=0.125	**Hip Circumference** II=56.73 (55.56 to 57.97) ID=55.25 (54.68 to 55.84) DD=55.63 (55.01 to 56.26) V=1.6%, P=0.056
Girls 4-6 yrs (N=50, 142, 126)	**BMI** **II=15.41 (15.01 to 15.84)** **ID=15.89 (15.63 to 16.16)** **DD=16.16 (15.87 to 16.47)** **V=2.5%, P=0.018** **Sum of Skinfolds** II=26.21 (24.88 to 27.07) ID=28.01 (26.86 to 29.26) DD=29.00 (27.56 to 30.58) V=1.7%, P=0.069	**Arm Circumference** **II=16.58 (16.20 to 16.99)** **ID=16.93 (16.67 to 17.20)** **DD=17.32 (17.04 to 17.60)** **V=2.7%, P=0.013**	**Waist Circumference** **II=51.52 (50.45 to 52.67)** **ID=52.93 (52.27 to 53.61)** **DD=53.97 (53.21 to 54.77)** **V=4.0%, P=0.001**	**Hip Circumference** *II=57.47 (56.36 to 58.64)* *ID=58.25 (57.50 to 59.03)* *DD=59.26 (58.42 to 60.14)* *V=1.9%, P=0.046*

Table 3 Associations of adiposity-related phenotypes with *ADRB1* C49 polymorphism in boys.

Boys 1-2 yrs GG=1, GA=7,AA=75	**BMI** (GG=16.62 (16.62 to16.62)) GG+GA=16.94 (15.67 to 18.46) AA=16.75 (16.42 to 17.10) V=0.1%, P=0.749 **Sum of Skinfolds** (GG=27.00 (27.00 to 27.00)) GG+GA=25.12 (24.19 to 26.25) AA=25.64 (25.29 to 26.01) V=1.0%, P=0.349	**Arm Circumference** (GG=15.69 (15.69 to 15.69)) GG+GA=15.21 (14.27 to 16.09) AA=15.51 (15.27 to 15.74) V=0.7%, P=0463	**Waist Circumference** (GG=48.00 (48.00 to 48.00)) GG+GA=48.52 (46.67 to 50.38) AA=46.55 (45.91 to 47.20) V=4.1%, P=0.067	**Hip Circumference** (GG=50.98 (50.98 to 50.98)) GG+GA=50.31 (46.38 to 53.81) AA=49.29 (48.66 to 49.92) V=1.0%, P=0.376
Boys 2-3 yrs GG=0, GA=40,A=180	**BMI** GA=16.40 (16.00 to 16.82) AA=16.42 (16.21 to 16.62) V=0%, P=0.995 **Sum of Skinfolds** GA=25.36 (24.89 to 25.87) AA=25.49 (25.25 to 25.73) V=0.1%, P=0.731	**Arm Circumference** GA=15.89 (15.53 to 16.29) AA=16.03 (15.87 to 16.19) V=0.2%, P=0.524	**Waist Circumference** GA=49.59 (49.59 to 49.59) AA=49.59 (49.59 to 49.59) V=0%, P=0.997	**Hip Circumference** GA=52.71 (51.73 to 53.72) AA=52.84 (52.37 to 53.31) V=0%, P=0.818
Boys 3-4 yrs GG=1, GA=77,AA=323	**BMI** (GG=14.32 (14.32 to 14.32)) GG+GA=15.91 (15.58 to 16.26) AA=16.00 (15.84 to 16.16) V=0.1%, P=0.627 **Sum of Skinfolds** (GG=25.01 (25.01 to 25.01)) GG+GA=25.19 (24.85 to 25.56) AA=25.23 (25.05 to 25.42) V=0%, P=0.837	**Arm Circumference** (GG=14.49 (14.49 to 14.49)) GG+GA=16.28 (15.97 to 16.60) AA=16.34 (16.19 to 16.49) V=0%, P=0.708	**Waist Circumference** (GG=45.04 (45.04 to 45.04)) GG+GA=51.04 (50.28 to 51.90) AA=51.29 (50.90 to 51.70) V=0.1%, P=0.629	**Hip Circumference** (GG=52.99 (52.99 to 52.99)) GG+GA=55.10 (54.20 to 56.04) AA=54.83 (54.41 to 55.25) V=0.1%, P=0.575
Boys 4-6 yrs GG=6, GA=43,AA=243	**BMI** (GG=17.28 (15.39 to 19.87)) GG+GA=15.98 (15.61 to 16.37) AA=15.87 (15.68 to 16.06) V=0.1%, P=0.625 **Sum of Skinfolds** (GG=26.07 (24.20 to 29.66)) GG+GA=25.00 (24.60 to 25.46) AA=24.76 (24.59 to 24.95) V=0.4%, P=0.297	**Arm Circumference** (GG=17.44 (15.82 to 19.82)) GG+GA=16.74 (16.38 to 17.14) AA=16.72 (16.55 to 16.89) V=0%, P=0.890	**Waist Circumference** (GG=56.03 (50.36 to 64.20)) GG+GA=53.37 (52.18 to 54.64) AA=52.77 (52.29 to 53.27) V=0.3%, P=0.345	**Hip Circumference** (GG=58.71 (53.57 to 65.90)) GG+GA=58.08 (56.93 to 59.30) AA=57.11 (56.58 to 57.67) V=0.7%, P=0.163

Table 4 Associations of adiposity-related phenotypes with *ADRB1* C49 polymorphism in girls.

	BMI	Arm Circumference	Waist Circumference	Hip Circumference
Girls 1-2 yrs GG=0, AG=10,AA=77	GA=17.03 (16.22 to 17.96) AA=16.63 (16.31 to 16.97) V=0.7%, P=0.432 **Sum of Skinfolds** GA=24.86 (23.65 to 26.25) AA=25.29 (24.95 to 25.64) V=0.8%, P=0.430	GA=15.32 (14.33 to 16.26) AA=15.32 (15.04 to 16.60) V=0%, P=0.992	GA=46.54 (44.94 to 48.27) AA=46.03 (45.30 to 46.78) V=0.2%, P=0.646	GA=49.82 (48.14 to 51.53) AA=49.01 (48.08 to 49.94) V=0.4%, P=0.552
Girls 2-3 yrs GG=0, GA=30,AA=156	**BMI** GA=16.12 (15.60 to 16.67) AA=16.22 (15.99 to 16.45) V=0.1%, P=0.740 **Sum of Skinfolds** GA=25.72 (25.21 to 26.27) AA=25.69 (25.40 to 25.99) V=0%, P=0.929	**Arm Circumference** GA=15.73 (15.29 to 16.19) AA=16.11 (15.90 to 16.33) V=1.1%, P=0.147	**Waist Circumference** GA=48.88 (47.53 to 50.24) AA=49.65 (49.10 to 50.20) V=0.6%, P=0.274	**Hip Circumference** GA=52.28 (50.92 to 53.77) AA=53.06 (52.53 to 53.60) V=0.7%, P=0.263
Girls 3-4 yrs GG=1, GA=60, AA=289	**BMI** (GG=18.06 (18.06 to 18.06)) GG+GA=16.12 (15.75 to 16.51) AA=15.93 (15.77 to 16.10) V=0.2%, P=0.368 **Sum of Skinfolds** (GG=31.50 (31.50 to 31.50)) GG+GA=25.56 (25.12 to 26.04) AA=25.64 (25.44 to 25.85) V=0%, P=0.763	**Arm Circumference** (GG=17.93 (17.93 to 17.93)) GG+GA=16.34 (16.01 to 16.68) AA=16.39 (16.23 to 16.54) V=0%, P=0.798	**Waist Circumference** (GG=55.05 (55.05 to 55.05)) GG+GA=51.09 (50.16 to 52.05) AA=51.43 (50.96 to 51.90) V=0.1%, P=0.562	**Hip Circumference** (GG=58.95 (58.95 to 58.95)) GG+GA=55.21 (54.31 to 56.14) AA=55.65 (55.20 to 56.11) V=0.2%, P=0.417
Girls 4-6 yrs GG=5, GA=53,AA=252	**BMI** (GG=15.11 (14.46 to 15.84)) GA=15.72 (15.36 to 16.10) AA=15.95 (15.75 to 16.16) V=0.3%, P=0.319 **Sum of Skinfolds** (GG=26.11 (25.28 to 27.08)) GA=25.91 (25.46 to 26.40) AA=25.91 (25.66 to 26.18) V=0%, P=0.929	**Arm Circumference** (GG=16.49 (15.91 to 17.12)) GA=17.02 (16.63 to 17.42) AA=16.98 (16.79 to 17.18) V=0%, P=0.864	**Waist Circumference** (GG=52.35 (50.18 to 53.69)) GA=52.34 (51.38 to 53.36) AA=53.15 (52.64 to 53.69) V=0.6%, P=0.172	**Hip Circumference** (GG=55.88 (53.22 to 58.94)) GA=58.12 (56.95 to 59.37) AA=58.64 (58.07 to 59.22) V=0.2%, P=0.447

Table 5 Associations of adiposity-related phenotypes with *ADRB1* C389 polymorphism in boys.

	BMI	Arm Circumference	Waist Circumference	Hip Circumference
Boys 1-2 yrs CC=46,CG=28,GG=9	CC=16.84 (16.37 to 17.35) CG=16.61 (16.12 to 17.14) GG=17.06 (16.26 to 17.96) V=0.9%, P=0.707 **Sum of Skinfolds** CC=28.82 (27.02 to 30.79) CG=27.91 (26.85 to 29.03) GG=27.20 (26.18 to 28.27) V=3.2%, P=0.265	CC=15.68 (15.41 to 15.94) CG=15.13 (14.71 to 15.54) GG=15.58 (14.69 to 16.40) V=5.7%, P=0.094	CC=47.39 (46.62 to 48.67) CG=46.22 (45.07 to 47.38) GG=45.74 (44.09 to 47.42) V=5.3%, P=0.112	*CC=50.11 (49.30 to 50.89)* *CG=48.31 (47.00 to 49.57)* *GG=48.82 (47.24 to 50.31)* *V=7.7%, P=0.040*
Boys 2-3 yrs CC=96,CG=95,	**BMI** CC=16.51 (16.24 to 16.78) CG=16.40 (16.11 to 16.69) GG=16.58 (16.13 to 17.05) V=0.2%, P=0.794 **Sum of Skinfolds** CC=28.82 (27.02 to 30.79) CG=28.91 (26.85 to 29.03) GG=27.20 (26.18 to 28.27) V=1.2%, P=0.259	**Arm Circumference** CC=16.01 (15.80 to 16.23) CG=15.93 (15.70 to 16.18) GG=16.37 (15.98 to 16.80) V=1.2%, P=0.286	**Waist Circumference** CC=49.64 (49.05 to 50.24) CG=49.52 (48.88 to 50.18) GG=49.82 (48.76 to 50.95) V=0.1%, P=0.905	**Hip Circumference** CC=52.42 (51.77 to 53.08) CG=53.03 (52.33 to 53.75) GG=52.75 (51.40 to 54.17) V=0.7%, P=0.463
Boys 3-4 yrs CC=178,CG=160,GG=40	**BMI** CC=15.98 (15.77 to 16.21) CG=16.07 (15.84 to 16.30) GG=16.00 (15.54 to 16.50) V=0.1%, P=0.869 **Sum of Skinfolds** CC=25.35 (22.91 to 26.31) CG=25.51 (24.55 to 26.76) GG=25.97 (24.81 to 27.05) V=0.4%, P=0.436	**Arm Circumference** CC=16.26 (16.07 to 16.46) CG=16.42 (16.19 to 16.65) GG=16.47 (16.13 to 16.83) V=0.4%, P=0.500	**Waist Circumference** CC=50.92 (50.40 to 51.46) CG=51.68 (51.13 to 52.25) GG=51.53 (50.55 to 52.57) V=1.1%, P=0.134	**Hip Circumference** CC=54.72 (54.20 to 55.25) CG=55.21 (54.59 to 55.85) GG=54.98 (53.82 to 56.20) V=0.4%, P=0.500
Boys 4-6 yrs CC=136,CG=102,GG=29	**BMI** CC=16.00 (15.74 to 16.27) CG=15.83 (15.53 to 16.14) GG=15.55 (15.04 to 15.81) V=0.9%, P=0.323 **Sum of Skinfolds** CC=28.98 (26.85 to 29.03) CG=28.82 (27.02 to 30.79) GG=27.19 (26.18 to 28.27) V=1.1%, P=0.242	**Arm Circumference** CC=16.67 (16.43 to 16.92) CG=16.68 (16.43 to 16.95) GG=16.98 (16.61 to 17.17) V=0.5%, P=0.544	**Waist Circumference** CC=53.16 (52.47 to 53.87) CG=52.59 (51.79 to 53.43) GG=52.79 (51.50 to 53.45) V=0.4%, P=0.574	**Hip Circumference** CC=57.44 (56.68 to 58.22) CG=57.00 (56.19 to 57.85) GG=56.69 (55.25 to 57.43) V=0.4%, P=0.611

Table 6 Associations of adiposity-related phenotypes with *ADRB1* C389 polymorphism in girls.

Girls 1-2 yrs CC=37, CG=41,GG=7	**BMI** CC=16.71 (16.28 to 17.18) CG=16.90 (16.43 to 17.42) GG=15.70 (15.19 to 16.27) V=5.4%, P=0.102 **Sum of Skinfolds** CC=24.35 (22.91 to 26.31) CG=25.23 (24.55 to 26.76) GG=25.82 (24.81 to 27.05) V=2.1%, P=0.387	**Arm Circumference** CC=15.13 (14.77 to 15.48) CG=15.59 (15.14 to 16.04) GG=14.65 (14.14 to 15.15) V=5.2%, P=0.110	**Waist Circumference** CC=45.50 (44.56 to 46.49) CG=46.39 (45.37 to 47.47) GG=46.13 (44.23 to 48.22) V=1.8%, P=0.466	**Hip Circumference** CC=48.91 (47.69 to 50.15) CG=49.31 (47.96 to 50.68) GG=47.81 (46.63 to 49.02) V=1.1%, P=0.647
Girls 2-3 yrs CC=81, CG=86, GG=17	**BMI** CC=16.08 (15.79 to 16.38) CG=16.37 (16.03 to 16.72) GG=16.13 (15.64 to 16.63) V=0.9%, P=0.432 **Sum of Skinfolds** CC=25.19 (22.93 to 27.85) CG=25.59 (23.95 to 27.43) GG=24.37 (22.85 to 26.64) V=0.2%, P=0.783	**Arm Circumference** CC=15.90 (15.62 to 16.18) CG=16.28 (15.99 to 16.59) GG=15.68 (15.04 to 16.36) V=2.7%, P=0.088	**Waist Circumference** CC=49.40 (48.75 to 50.05) CG=49.93 (49.09 to 50.78) GG=49.27 (47.56 to 51.01) V=0.6%, P=0.566	**Hip Circumference** **CC=52.42 (51.75 to 53.12)** **CG=53.91 (53.13 to 54.73)** **GG=51.60 (50.13 to 53.21)** **V=5.8%, P=0.006**
Girls 3-4 yrs CC=151,CG=154,GG=45	**BMI** CC=15.96 (15.74 to 16.20) CG=15.94 (15.70 to 16.18) GG=16.26 (15.74 to 16.82) V=0.5%, P=0.456 **Sum of Skinfolds** CC=24.29 (22.95 to 26.24) CG=25.21 (24.55 to 26.75) GG=25.70 (24.81 to 27.00) V=0.5%, P=0.419	**Arm Circumference** CC=16.33 (16.12 to 16.55) CG=16.37 (16.15 to 16.59) GG=16.65 (16.21 to 17.10) V=0.5%, P=0.412	**Waist Circumference** CC=51.38 (50.75 to 52.01) CG=51.19 (50.54 to 51.85) GG=52.11 (50.86 to 53.41) V=0.5%, P=0.421	**Hip Circumference** CC=55.36 (54.74 to 56.00) CG=55.59 (54.96 to 56.24) GG=56.20 (55.10 to 57.37) V=0.4%, P=0.465
Girls 4-6 yrs CC=125,CG=138,GG=33	**BMI** CC=16.06 (15.77 to 16.36) CG=15.88 (15.62 to 16.16) GG=15.91 (15.43 to 16.44) V=0.3%, P=0.678 **Sum of Skinfolds** CC=25.24 (22.93 to 27.85) CG=25.62 (23.95 to 27.43) GG=24.66 (22.85 to 26.64) V=0.2%, P=0.688	**Arm Circumference** CC=17.21 (16.93 to 17.50) CG=16.90 (16.64 to 17.17) GG=16.74 (16.26 to 17.27) V=1.2%, P=0.170	**Waist Circumference** CC=53.39 (52.67 to 54.15) CG=52.85 (52.14 to 53.59) GG=53.03 (51.56 to 54.67) V=0.3%, P=0.604	**Hip Circumference** CC=59.05 (58.21 to 59.92) CG=58.04 (57.28 to 58.82) GG=58.35 (56.78 to 60.03) V=1.0%, P=0.223

Table 7 Associations of adiposity-related phenotypes with *ADRB2* C16 polymorphism in boys.

	BMI	Arm Circumference	Waist Circumference	Hip Circumference
Boys 1-2 yrs GG=40, GA=38,AA=9	GG=16.66 (16.26 to 17.08) GA=17.10 (16.57 to 17.68) AA=16.66(15.87 to 17.53) V=3.7%, P=0.202 **Sum of Skinfolds** GG=25.98 (24.85 to 26.03) GA=25.52 (26.02 to 26.79) AA=25.19 (24.18 to 26.27) V=4.8%, P=0.110	GG=15.31 (14.99 to 15.62) GA=15.70 (15.35 to 16.04) AA=15.66(15.09 to 16.21) V=3.3%, P=0.237	GG=46.38 (45.48 to 47.29) GA=47.11 (46.25 to 47.98) AA=47.37 (45.41 to 49.36) V=2.0%, P=0.430	GG=48.61 (49.58 to 49.58) GA=50.01 (50.87 to 50.87) AA=50.03 (47.71 to 52.19) V=5.5%, P=0.104
Boys 2-3 yrs GG=90,GA=105,AA=38	**BMI** GG=16.21 (15.93 to 16.48) GA=16.42 (16.15 to 16.69) AA=16.53 (16.09 to 16.98) V=0.8%, P=0.396 **Sum of Skinfolds** GG=24.66 (22.85 to 26.64) GA=25.62 (23.95 to 27.43) AA=25.24 (22.93 to 27.85) V=0.2%, P=0.625	**Arm Circumference** GG=15.95 (15.74 to 16.18) GA=15.96 (15.75 to 16.19) AA=16.03 (15.69 to 16.41) V=0%, P=0.933	**Waist Circumference** GG=49.39 (48.77 to 50.03) GA=49.61 (49.03 to 50.21) AA=49.65 (48.87 to 50.45) V=0.1%, P=0.844	**Hip Circumference** GG=52.39 (51.77 to 53.02) GA=52.92 (52.27 to 53.59) AA=52.92(51.89 to 53.99) V=0.6%, P=0.475
Boys 3-4 yrs GG=154,GA=202,AA=50	**BMI** **GG=15.93(15.17 to 18.29)** **GA=15.87 (14.54 to 17.57)** **AA=16.55(16.13 to 16.99)** **V=1.9%, P=0.020** **Sum of Skinfolds** GG=25.05 (24.79 to 25.33) GA=25.26 (25.05 to 25.49) AA=25.41 (24.93 to 25.94) V=0.3%, P=0.619	**Arm Circumference** GG=16.28 (16.06 to 16.49) GA=16.30 (16.11 to 16.50) AA=16.69 (16.34 to 17.06) V=0.9%, P=0.175	**Waist Circumference** GG=51.21 (50.68 to 51.76) GA=51.09 (50.58 to 51.61) AA=52.07(51.02 to 53.18) V=0.7%, P=0.243	**Hip Circumference** GG=54.77 (54.20 to 55.37) GA=54.76 (54.22 to 55.31) AA=56.11(55.05 to 57.23) V=1.2%, P=0.080
Boys 4-6 yrs GG=123,GA=140,AA=43	**BMI** GG=15.96 (15.69 to 16.23) GA=15.81 (15.57 to 16.06) AA=15.91(15.45 to 16.39) V=0.2%, P=0.721 **Sum of Skinfolds** GG=24.83 (24.59 to 25.10) GA=24.88 (24.65 to 25.13) AA=24.62 (24.23 to 25.07) V=0.4%, P=0.555	**Arm Circumference** GG=16.77 (16.54 to 17.02) GA=16.67 (16.46 to 16.88) AA=16.63(16.19 to 17.12) V=0.2%, P=0.756	**Waist Circumference** GG=53.12 (52.51 to 53.76) GA=52.87 (52.26 to 53.51) AA=51.93(50.47 to 55.53) V=1.0%, P=0.215	**Hip Circumference** GG=57.63 (56.88 to 58.41) GA=57.15 (56.48 to 57.86) AA=56.77 (55.45 to 58.19) V=0.5%, P=0.461

Table 8 Associations of adiposity-related phenotypes with *ADRB2* C16 polymorphism in girls.

Girls 1-2 yrs GG=37, GA=45,AA=6	**BMI** GG=16.68 (16.23 to 17.17) GA=16.72 (16.30 to 17.18) AA=16.19 (15.40 to 17.10) V=0.9%, P=0.687 **Sum of Skinfolds** GG=25.34 (23.90 to 26.71) GA=25.31 (24.87 to 25.77) AA=25.20 (23.90 to 26.71) V=0%, P=0.980	**Arm Circumference** GG=15.57 (15.18 to 15.95) GA=15.14 (14.76 to 15.51) AA=15.23 (13.73 to 16.63) V=2.6%, P=0.322	**Waist Circumference** GG=45.88 (44.78 to 47.05) GA=46.16 (45.27 to 47.08) AA=44.69 (42.95 to 46.62) V=1.3%, P=0.563	**Hip Circumference** GG=49.11 (47.72 to 50.53) GA=48.96 (47.82 to 50.13) AA=48.77 (46.97 to 50.61) V=0.1%, P=0.974
Girls 2-3 yrs GG=81, GA=88,AA=23	**BMI** GG=16.06 (15.75 to 16.36) GA=16.39 (16.07 to 16.71) AA=15.77 (15.36 to 16.20) V=2.3%, P=0.073 **Sum of Skinfolds** GG=25.56 (24.63 to 26.33) GA=25.81 (25.47 to 26.16) AA=25.43 (24.63 to 26.33) V=0.6%, P=0.383	**Arm Circumference** GG=16.07 (15.79 to 16.36) GA=16.07 (15.79 to 16.36) AA=15.64 (15.16 to 16.15) V=1.2%, P=0.236	**Waist Circumference** GG=49.21 (48.38 to 50.04) GA=49.99 (49.26 to 50.73) AA=48.68 (47.72 to 49.65) V=1.8%, P=0.132	**Hip Circumference** GG=52.53 (51.81 to 53.29) GA=53.52 (52.77 to 54.30) AA=51.87 (51.05 to 52.74) V=3.1%, P=0.051
Girls 3-4 yrs GG=185,GA=134,AA=50	**BMI** GG=15.99 (15.70 to 16.30) GA=16.03 (15.84 to 16.22) AA=15.95 (15.55 to 16.38) V=0.1%, P=0.945 **Sum of Skinfolds** GG=25.66 (25.35 to 25.98) GA=25.84 (25.56 to 26.12) AA=25.18 (24.77 to 25.62) V=1.5%, P=0.062	**Arm Circumference** GG=16.44 (16.20 to 16.71) GA=16.45 (16.25 to 16.64) AA=16.21 (15.89 to 16.56) V=0.3%, P=0.573	**Waist Circumference** GG=51.43 (50.63 to 52.54) GA=51.44 (50.90 to 52.00) AA=51.59 (50.67 to 52.54) V=0%, P=0.971	**Hip Circumference** GG=55.53 (54.84 to 56.25) GA=55.80 (55.25 to 56.36) AA=55.52 (54.42 to 56.66) V=0.1%, P=0.810
Girls 4-6 yrs GG=116,GA=162,AA=40	**BMI** GG=16.09 (15.79 to 16.40) GA=15.90 (15.66 to 16.14) AA=15.61 (15.07 to 16.20) V=0.9%, P=0.253 **Sum of Skinfolds** GG=26.24 (25.85 to 26.65) GA=25.76 (25.46 to 26.08) AA=25.85 (25.33 to 26.43) V=1.2%, P=0.142	**Arm Circumference** GG=17.25 (16.95 to 17.56) GA=16.94 (16.72 to 17.18) AA=16.87 (16.41 to 17.36) V=0.8 %, P=0.217	**Waist Circumference** GG=53.63 (52.88 to 54.42) GA=53.05 (52.44 to 53.69) AA=52.05 (50.62 to 53.62) V=1.4%, P=0.109	**Hip Circumference** GG=59.18 (58.33 to 60.06) GA=58.35 (57.65 to 59.06) AA=57.92 (56.50 to 59.43) V=1.0%, P=0.213

Table 9 Associations of adiposity-related phenotypes with *ADRB2* C27 polymorphism in boys.

Boys 1-2 yrs GG=16, GC=38,CC=34	**BMI** GG=16.33 (15.67 to 17.05) GC=16.86 (16.37 to 17.40) CC=16.88 (16.42 to 17.37) V=2.1%, P=0.414 **Sum of Skinfolds** GG=25.02 (24.41 to 25.73) GC= 25.75 (25.26 to 26.30) CC= 25.43 (24.93 to 25.99) V=3.0%, P=0.229	**Arm Circumference** *GG=15.03 (14.35 to 15.67)* *GC=15.81 (15.48 to 16.14)* *CC=15.48 (15.19 to 15.76)* *V=7.2%, P=0.042*	**Waist Circumference** GG=45.82 (44.52 to 47.12) GC=47.24 (46.36 to 48.13) CC=46.81 (45.84 to 47.78) V=3.3%, P=0.240	**Hip Circumference** *GG=47.60 (45.65 to 49.43)* *GC=49.78 (48.93 to 50.61)* *CC=49.80 (48.80 to 50.77)* *V=7.7%, P=0.039*
Boys 2-3 yrs GG=29, GC=115,CC=97	**BMI** GG=16.08 (15.69 to 16.49) GC=16.29 (16.03 to 16.55) CC=16.53 (16.26 to 16.80) V=1.2%, P=0.223 **Sum of Skinfolds** GG=25.76 (25.35 to 26.20) GC= 25.43 (25.14 to 25.73) CC=25.61 (25.26 to 25.97) V=0.5%, P=0.524	**Arm Circumference** GG=15.74 (15.43 to 16.08) GC=15.87 (15.67 to 16.08) CC=16.19 (15.98 to 16.41) V=2.5%, P=0.052	**Waist Circumference** *GG=48.84 (48.11 to 49.58)* *GC=49.19 (48.60 to 49.80)* *CC=50.08 (49.53 to 50.64)* *V=2.6%, P=0.043*	**Hip Circumference** **GG=50.94 (50.16 to 51.75)** **GC=52.47 (51.83 to 53.12)** **CC=53.46 (52.85 to 54.07)** **V=6.0%, P=0.001**
Boys 3-4 yrs GG=50, GC=206,CC=156	**BMI** GG=16.11 (15.66 to 16.61) GC=15.89 (15.70 to 16.08) CC=16.10 (15.87 to 16.34) V=0.5%, P=0.325 **Sum of Skinfolds** GG=25.06 (24.53 to 25.66) GC=25.28 (25.07 to 25.51) CC=25.21 (24.96 to 25.47) V=0.2%, P=0.652	**Arm Circumference** GG=16.24 (15.86 to 16.66) GC=16.39 (16.21 to 16.58) CC=16.32 (16.12 to 16.54) V=0.1%, P=0.759	**Waist Circumference** GG=50.94 (49.97 to 51.97) GC=51.09 (50.82 to 51.81) CC=51.34 (50.78 to 51.93) V=0.1%, P=0.777	**Hip Circumference** GG=54.66 (53.51 to 55.87) GC=54.82 (54.30 to 55.36) CC=55.20 (54.63 to 55.78) V=0.3%, P=0.564
Boys 4-6 yrs GG=43, GC=148,CC=118	**BMI** GG=15.98 (15.53 to 16.47) GC=15.83 (15.60 to 16.08) CC=15.92 (15.65 to 16.20) V=0.1%, P=0.814 **Sum of Skinfolds** GG=24.83 (24.48 to 25.23) GC=25.03 (24.79 to 25.28) CC=24.62 (24.37 to 24.90) V=1.7%, P=0.064	**Arm Circumference** GG=16.82 (16.46 to 17.20) GC=16.73 (16.52 to 16.95) CC=16.66 (16.42 to 16.92) V=0.1%, P=0.800	**Waist Circumference** GG=53.23 (52.34 to 54.16) GC=52.95 (52.32 to 53.60) CC=52.66 (51.91 to 53.44) V=0.2%, P=0.693	**Hip Circumference** GG=58.05 (56.88 to 59.29) GC=57.14 (56.44 to 57.88) CC=57.27 (56.54 to 58.02) V=0.5%, P=0.483

Table 10 Associations of adiposity-related phenotypes with *ADRB2* C27 polymorphism in girls.

	BMI	Arm Circumference	Waist Circumference	Hip Circumference
Girls 1-2 yrs GG=16,GC=37,CC=36	GG=16.73 (15.98 to 17.58) GC=16.78 (16.38 to 17.20) CC=16.56 (16.07 to 17.10) V=0.5%, P=0.809	GG=15.76 (15.28 to 16.23) GC=15.25 (14.85 to 15.64) CC=15.23 (14.75 to 15.69) V=2.5%, P=0.333	GG=46.63 (44.95 to 48.48) GC=46.36 (45.49 to 47.27) CC=45.35 (44.24 to 46.54) V=2.8%, P=0.290	GG=48.91 (47.15 to 50.71) GC=49.57 (48.39 to 50.76) CC=48.58 (47.14 to 50.05) V=1.3%, P=0.571
	Sum of Skinfolds GG=25.10 (24.15 to 26.16) GC=25.26 (24.86 to 25.67) CC=25.35 (24.79 to 25.95) V=0.3%, P=0.856			
Girls 2-3 yrs GG=28, GC=93,CC=74	**BMI** GG=16.02 (15.48 to 16.58) GC=16.15 (15.85 to 16.47) CC=16.25 (15.93 to 16.58) V=0.3%, P=0.639	**Arm Circumference** GG=15.89 (15.32 to 16.49) GC=16.16 (15.90 to 16.43) CC=15.92 (15.62 to 16.24) V=0.8%, P=0.394	**Waist Circumference** GG=49.39 (48.04 to 50.76) GC=49.78 (49.04 to 50.50) CC=49.39 (48.58 to 50.21) V=0.7%, P=0.392	**Hip Circumference** GG=52.76 (51.39 to 54.26) GC=53.09 (52.41 to 53.83) CC=52.71 (51.94 to 53.52) V=0.3%, P=0.738
	Sum of Skinfolds GG=25.70 (24.94 to 26.56) GC= 25.74 (25.38 to 26.11) CC=25.60 (25.21 to 26.00) V=0.1%, P=0.832			
Girls 3-4 yrs GG=46, GC=185,CC=145	**BMI** GG=16.06 (15.53 to 16.64) GC=15.91 (15.71 to 16.13) CC=16.07 (15.84 to 16.30) V=0.3%, P=0.616	**Arm Circumference** GG=16.61 (16.15 to 17.08) GC=16.43 (16.24 to 16.63) CC=16.34 (16.13 to 16.57) V=0.3%, P=0.534	**Waist Circumference** GG=51.30 (49.87 to 52.78) GC=51.51 (50.91 to 52.12) CC=51.44 (50.83 to 52.05) V=0%, P=0.950	**Hip Circumference** GG=56.01 (54.82 to 57.26) GC=55.69 (55.12 to 56.28) CC=55.46 (54.84 to 56.10) V=0.2%, P=0.700
	Sum of Skinfolds GG=25.80 (25.21 to 26.45) GC=25.80 (25.53 to 26.07) CC=25.55 (25.28 to 25.85) V=0.4%, P=0.439			
Girls 4-6 yrs GG=38, GC=155,CC=124	**BMI** GG=16.06 (15.53 to 16.64) GC=15.98 (15.72 to 16.25) CC=15.84 (15.58 to 16.12) V=0.2%, P=0.691	**Arm Circumference** GG=17.23 (16.67 to 17.82) GC=17.03 (16.78 to 17.29) CC=17.05 (16.81 to 17.31) V=0.1%, P=0.792	**Waist Circumference** GG=53.39 (52.13 to 54.77) GC=53.44 (52.78 to 54.13) CC=52.72 (52.00 to 53.48) V=0.7%, P=0.345	**Hip Circumference** GG=58.65 (57.28 to 60.12) GC=58.89 (58.15 to 59.66) CC=58.18 (57.29 to 59.10) V=0.5%, P=0.438
	Sum of Skinfolds GG=26.17 (25.56 to 26.87) GC=25.92 (25.61 to 26.26) CC=25.93 (25.57 to 26.31) V=0.1%, P=0.782			

Table 11 Associations of adiposity-related phenotypes with *ADRB3* C64 polymorphism in boys.

Boys 1-2 yrs CC=1, CT=8,TT=79	**BMI** (CC=17.71 (17.71 to 17.71)) CT+CC=17.31 (16.19 to 18.61) TT=16.70 (16.38 to 17.02 V=1.5%, P=0.262 **Sum of Skinfolds** (CC=24.00 (24.00 to 24.00)) CT+CC=25.16 (24.25 to 26.27) TT=25.52 (25.14 to 26.20) V=0.5%, P=0.482	**Arm Circumference** (CC=15.49 (15.49 to 15.49)) CT+CC=15.54 (14.82 to 16.21) TT=15.49 (15.26 to 15.72) V=0%, P=0.904	**Waist Circumference** (CC=44.50 (44.50 to 44.50)) CT+CC=46.38 (44.56 to 48.21) TT=46.75 (46.13 to 47.37) V=0.2%, P=0.710	**Hip Circumference** (CC=52.98 (52.98 to 52.98)) CT+CC=50.49 (48.38 to 52.46) TT=49.17 (48.49 to 49.83) V=1.8%, P=0.216
Boys 2-3 yrs CC=0, CT=19,TT=217	**BMI** CT=16.13 (15.65 to 16.62) TT=16.39 (16.21 to 16.58) V=0.3%, P=0.421 **Sum of Skinfolds** CT=25.87 (25.17 to 26.67) TT=25.47 (25.26 to 25.69) V=0.4%, P=0.329	**Arm Circumference** *CT=15.45 (15.01 to 15.98)* *TT=16.02 (15.88 to 16.17)* *V=2.1%, P=0.027*	**Waist Circumference** CT=49.03 (48.01 to 50.09) TT=49.57 (49.17 to 49.99) V=0.2%, P=0.448	**Hip Circumference** CT=51.97 (50.47 to 53.55) TT=52.73 (52.29 to 53.17) V=0.4%, P=0.331
Boys 3-4 yrs CC=1, CT=47,TT=372	**BMI** (CC=14.79 (14.79 to 14.79)) CT+CC=15.93 (15.58 to 16.30) TT=15.98 (15.98 to 15.98) V=0%, P=0.829 **Sum of Skinfolds** (CC=24.01 (24.01 to 24.01)) CT+CC=25.19 (24.74 to 25.70) TT=25.21 (25.05 to 25.38) V=0%, P=0.982	**Arm Circumference** (CC=15.49 (15.49 to 15.49)) CT+CC=16.46 (16.12 to 16.82) TT=16.32 (16.18 to 16.46) V=0.1%, P=0.511	**Waist Circumference** (CC=49.04 (49.04 to 49.04)) CT+CC=50.97 (49.93 to 52.08) TT=51.28 (50.91 to 51.65) V=0.1%, P=0.585	**Hip Circumference** (CC=51.99 (51.99 to 51.99)) CT+CC=54.82 (53.76 to 55.89) TT=54.93 (54.53 to 55.33) V=0%, P=0.827
Boys 4-6 yrs CC=1, CT=25,TT=281	**BMI** (CC=16.27 (16.27 to 16.27)) CT+CC=16.46 (15.83 to 17.16) TT=15.85 (15.68 to 16.03) V=1.2%, P=0.059 **Sum of Skinfolds** (CC=24.00 (24.00 to 24.00)) CT+CC=24.83 (24.23 to 25.57) TT=24.85 (24.68 to 25.02) V=0%, P=0.958	**Arm Circumference** (CC=17.01 (17.01 to 17.01)) CT+CC=16.92 (16.30 to 17.63) TT=16.72 (16.57 to 16.87) V=0.2%, P=0.472	**Waist Circumference** *(CC=53.05 (53.05 to 53.05))* *CT+CC=54.88 (53.30 to 56.62)* TT=52.74 (52.29 to 53.21) *V=2.0%, P=0.013*	**Hip Circumference** *(CC=59.97 (59.97 to 59.97)* *CT+CC=59.38 (57.56 to 61.40)* TT=57.19 (56.70 to 57.69) *V=1.7%, P=0.022*

Table 12 Associations of adiposity-related phenotypes with *ADRB3* C64 polymorphism in girls.

	BMI	Arm Circumference	Waist Circumference	Hip Circumference
Girls 1-2 yrs CC=0, CT=7, TT=80	CT=16.64 (16.19 to 17.12) TT=16.68 (16.36 to 17.02) V=0%, P=0.946 **Sum of Skinfolds** CT=24.61 (23.74 to 25.57) TT=25.40 (25.05 to 25.75) V=2.0%, P=0.178	CT=14.58 (13.95 to 15.20) TT=15.42 (15.14 to 15.70) V=3.2%, P=0.101	*CT=43.54 (41.38 to 45.99)* *TT=46.20 (45.51 to 46.90)* *V=6.0%, P=0.026*	*CT=46.14 (44.26 to 48.07)* *TT=49.32 (48.45 to 50.21)* *V=5.1%, P=0.039*
Girls 2-3 yrs CC=0, CT=17, TT=177	**BMI** CT=16.57 (15.75 to 17.43) TT=16.14 (15.93 to 16.35) V=0.7%, P=0.263 **Sum of Skinfolds** CT=26.22 (25.17 to 27.43) TT=25.64 (25.38 to 25.91) V=0.7%, P=0.287	**Arm Circumference** CT=16.44 (15.79 to 17.12) TT=15.99 (15.79 to 16.19) V=0.8%, P=0.209	**Waist Circumference** CT=50.83 (49.03 to 52.66) TT=49.38 (48.86 to 49.90) V=1.3%, P=0.117	**Hip Circumference** CT=53.64 (51.98 to 55.47) TT=52.84 (52.33 to 53.35) V=0.4%, P=0.387
Girls 3-4 yrs CC=1, CT=32, TT=341	**BMI** (CC=16.47 (16.47 to 16.47)) CT+CC=15.70 (15.67 to 15.68) TT=16.02 (15.86 to 16.18) V=0.4%, P=0.244 **Sum of Skinfolds** (CC=28.00 (28.00 to 28.00)) CT+CC=25.46 (24.80 to 26.21) TT=25.70 (25.51 to 25.89)) V=0.1%, P=0.453	**Arm Circumference** (CC=17.93 (17.93 to 17.93)) CT+CC=16.14 (15.76 to 16.54) TT=16.45 (16.30 to 16.60) V=0.4%, P=0.228	**Waist Circumference** (CC=53.05 (53.05 to 53.05)) CT+CC=50.56 (49.40 to 51.76) (TT=51.52 (51.07 to 51.97) V=0.4%, P=0.203	**Hip Circumference** (CC=51.95 (51.95 to 51.95)) CT+CC=55.22 (54.07 to 56.42) TT=55.63 (55.27 to 56.14) V=0.1%, P=0.450
Girls 4-6 yrs CC=3, CT=30, TT=293	**BMI** (CC=16.71 (15.34 to 18.43)) CT+CC=15.84 (15.31 to 16.42) TT=15.93 (15.75 to 16.12) V=0%, P=0.753 **Sum of Skinfolds** (CC=26.65 (24.48 to 30.04)) CT+CC=25.77 (25.16 to 26.46) TT=25.96 (25.73 to 26.20) V=0.1%, P=0.638	**Arm Circumference** (CC=18.30 (17.04 to 19.78)) CT+CC=17.28 (16.77 to 17.83) TT=17.01 (16.83 to 17.19) V=0.3%, P=0.346	**Waist Circumference** (CC=58.89 (53.10 to 67.71)) CT+CC=54.03 (52.65 to 55.55) TT=53.00 (52.53 to 53.49) V=0.5%, P=0.187	**Hip Circumference** (CC=65.08 (62.67 to 67.76)) CT+CC=59.46 (58.00 to 61.03) TT=58.43 (57.90 to 58.97) V=0.4%, P=0.259

Table 13 Associations of adiposity-related phenotypes with *PPARγ* Pro12Ala polymorphism in boys.

Boys 1-2 yrs CC=77,CG=7,GG=0	**BMI** CC=16.69 (16.37 to 17.03) CG=17.65 (16.64 to 18.77) V=2.9%, P=0.123 **Sum of Skinfolds** CC=25.47 (25.13 to 25.83) CG=26.55 (25.44 to 27.89) V=3.1%, P=0.109	**Arm Circumference** CC=15.43 (15.19 to 15.67) CG=15.97 (15.31 to 16.61) V=2.0%, P=0.194	**Waist Circumference** CC=46.47 (45.85 to 47.09) CG=48.02 (46.29 to 49.77) V=2.5%, P=0.154	**Hip Circumference** CC=49.08 (48.39 to 49.76) CG=49.97 (47.85 to 52.00) V=0.7%, P=0.458
Boys 2-3 yrs CC=184, CG=35, GG=2	**BMI** CC=16.45(16.24 to 16.65) CG+GG=16.17 (15.76 to 16.60) (GG=16.04 (13.59 to 18.94)) V=0.5%, P=0.273 **Sum of Skinfolds** CC=25.53 (25.30 to 25.78) CG+GG=25.21 (24.82 to 25.62) (GG=24.00 (24.00 to 24.00)) V=0.6%, P=0.235	**Arm Circumference** CC=16.00 (15.84 to 16.16) CG+GG=15.81 (15.47 to 16.19) (GG=15.47 (14.58 to 16.56)) V=0.8%, P=0.319	**Waist Circumference** CC=49.40 (48.96 to 49.85) CG+GG=49.37 (48.43 to 50.34) (GG=46.94 (46.94 to 46.94)) V=0%, P=0.954	**Hip Circumference** CC=52.90 (52.45 to 53.36) CG+GG=52.44 (51.32 to 53.61) (GG=52.99 (51.09 to 55.01)) V=0.3%, P=0.425
Boys 3-4 yrs CC=334,CG=54,GG=1	**BMI** CC=15.99 (15.83 to 16.16) CG+GG=16.04 (15.70 to 16.40) (GG=18.16 to 18.16) V=0%, P=0.829 **Sum of Skinfolds** CC=25.23 (25.06 to 25.42) CG+GG=25.17 (24.78 to 25.60) (GG=27.01 to 27.01) V=0%, P=0.786	**Arm Circumference** CC=16.36 (16.21 to 16.51) CG+GG=16.35 (15.99 to 16.73) (GG=17.49 to 17.49) V=0%, P=0.954	**Waist Circumference** CC=51.24 (50.84 to 51.65) CG+GG=51.09 (50.27 to 51.95) (GG=54.46 to 54.46) V=0%, P=0.779	**Hip Circumference** CC=54.95 (54.54 to 55.38) CG+GG=54.97 (53.97 to 56.03) (GG=59.07 to 59.07) V=0%, P=0.974
Boys 4-6 yrs CC=245, CG=42,GG=0	**BMI** CC=15.93 (15.75 to 16.11) CG=15.80 (15.31 to 16.32) V=0.1%, P=0.583 **Sum of Skinfolds** CC=24.85 (24.68 to 25.04) CG=24.92 (24.54 to 25.35) V=0%, P=0.737	**Arm Circumference** CC=16.70 (16.53 to 16.87) CG=16.84 (16.46 to 17.25) V=0.1%, P=0.549	**Waist Circumference** CC=52.83 (52.33 to 53.34) CG=52.74 (51.71 to 53.82) V=0%, P=0.887	**Hip Circumference** CC=57.34 (56.80 to 57.89) CG=57.32 (56.04 to 58.70) V=0%, P=0.986

Table 14 Associations of adiposity-related phenotypes with *PPARγ* Pro12Ala polymorphism in girls.

Girls 1-2 yrs CC=68,CG=11,GG=0	**BMI** CC=16.67 (16.31 to 17.05) CG=16.80 (16.11 to 17.55) V=0.1%, P=0.805 **Sum of Skinfolds** CC=25.26 (24.88 to 25.66) CG=25.12 (24.08 toc26.26) V=0.1%, P=0.734	**Arm Circumference** CC=15.35 (15.03 to 15.67) CG=15.60 (15.13 to 16.06) V=0.4%, P=0.557	**Waist Circumference** CC=46.00 (45.23 to 46.80) CG=45.91 (43.81 to 48.24) V=0%, P=0.937	**Hip Circumference** CC=48.77 (47.80 to 49.77) CG=49.44 (47.47 to 51.53) V=0.3%, P=0.616
Girls 2-3 yrs CC=160, CG=19, GG=0	**BMI** CC=16.11 (15.90 to 16.34) CG=16.38 (15.74 to 17.04) V=0.3%, P=0.454 **Sum of Skinfolds** CC=25.68 (25.40 to 25.96) CG=25.70 (24.92 to 26.56) V=0%, P=0.962	**Arm Circumference** CC=16.00 (15.81 to 16.20) CG=15.92 (15.22 to 16.66) V=0%, P=0.783	**Waist Circumference** CC=49.39 (48.83 to 49.95) CG=50.21 (48.64 to 51.81) V=0.5%, P=0.350	**Hip Circumference** CC=52.78 (52.26 to 53.31) CG=53.71 (51.98 to 55.63) V=0.8%, P=0.278
Girls 3-4 yrs CC=300,CG=38,GG=0	**BMI** CC=15.88 (15.72 to 16.04) CG=16.25 (15.74 to 16.80) V=0.6%, P=0.150 **Sum of Skinfolds** **CC=25.57 (25.38 to 25.76)** **CG=26.24 (25.67 to 26.97)** **V=1.6%, P=0.011**	**Arm Circumference** **CC=16.30 (16.15 to 16.45)** **CG=16.91 (16.50 to 17.34)** **V=2.0%, P=0.010**	**Waist Circumference** *CC=50.92 (50.48 to 51.37)* *CG=52.50 (51.26 to 53.79)* *V=1.5%, P=0.022*	**Hip Circumference** **CC=55.28 (54.86 to 55.70)** **CG=57.12 (55.98 to 58.32)** **V=3.7%, P=0.005**
Girls 4-6 yrs CC=260, CG=49, GG=2	**BMI** CC=15.90 (15.71 to 16.11) CG+GG=15.84 (15.44 to 16.27) (GG=14.48 (12.77 to 18.40)) V=0%, P=0.805 **Sum of Skinfolds** CC=25.99 (25.74 to 26.24) CG+GG=25.77 (25.25 to 26.35) (GG=25.46 (24.58 to 27.06)) V=0.1%, P=0.439	**Arm Circumference** CC=17.04 (16.85 to 17.24) CG+GG=16.91 (16.54 to 17.29) (GG=16.47 (15.55 to 17.99)) V=0.1%, P=0.557	**Waist Circumference** CC=53.06 (52.55 to 53.59) CG+GG=52.94 (51.86 to 54.07) (GG=50.24 (46.06 to 59.25)) V=0%, P=0.841	**Hip Circumference** CC=58.69 (58.12 to 59.28) CG+GG=57.70 (56.62 to 58.83) (GG=56.23 (51.85 to 64.42)) V=0.7%, P=0.184

Table 15 Associations of adiposity-related phenotypes with *PPARγ* C1431T polymorphism in boys.

Boys 1-2 yrs CC=76,CT=12,TT=0	**BMI** CC=16.76 (16.43 to 17.10) CT=16.91 (16.09 to 17.82) V=0.1%, P=0.747 **Sum of Skinfolds** CC=25.39 (25.05 to 25.75) CT=26.15 (25.40 to 26.99) V=2.5%, P=0.129	**Arm Circumference** CC=15.49 (15.24 to 15.73) CT=15.57 (15.14 to 16.00) V=0.1%, P=0.791	**Waist Circumference** CC=46.75 (46.09 to 47.41) CT=46.99 (45.73 to 48.26) V=0.1%, P=0.784	**Hip Circumference** CC=49.29 (48.58 to 49.99) CT=50.02 (48.63 to 51.37). V=0.7%, P=0.440
Boys 2-3 yrs CC=189,CT=46,TT=1	**BMI** CC=16.34 (16.15 to 16.54) CT+TT=16.41 (15.99 to 16.83) (TT=14.74 to 14.74) V=0%, P=0.775 **Sum of Skinfolds** CC=25.47 (25.25 to 25.70) CT+TT=25.61 (25.11 to 26.15) (TT=24.00 to 24.00) V=0.1%, P=0.590	**Arm Circumference** CC=16.00 (15.85 to 16.15) CT+TT=15.86 (15.54 to 16.20) (TT=14.99 to 14.99) V=0.2%, P=0.501	**Waist Circumference** CC=49.45 (49.04 to 49.87) CT+TT=49.06 (48.11 to 50.06) (TT=46.94 to 46.94) V=0.3%, P=0.433	**Hip Circumference** *CC=52.92 (52.48 to 53.37)* *CT+TT=51.82 (50.81 to 52.87)* *(TT=52.00 to 52.00)* *V=1.9%, P=0.036*
Boys 3-4 yrs CC=330,CT=65,TT=4	**BMI** CC=15.98 (15.82 to 16.14) CT+TT=16.17 (15.81 to 16.54) (TT=15.64 (14.42 to 17.16)) V=0.2%, P=0.334 **Sum of Skinfolds** CC=25.19 (25.02 to 25.37) CT+TT=25.39 (24.99 to 25.83) (TT=24.61 (23.27 to 26.69)) V=0.2%, P=0.299	**Arm Circumference** CC=16.33 (16.19 to 16.48) CT+TT=16.63 (16.33 to 16.94) (TT=16.64 (15.71 to 17.71)) V=0.7%, P=0.105	**Waist Circumference** CC=51.25 (50.86 to 51.65) CT+TT=51.23 (50.42 to 52.06) (TT=49.80 (49.57 to 50.03)) V=0%, P=0.964	**Hip Circumference** CC=54.89 (54.50 to 55.29) CT+TT=55.43 (54.44 to 56.47) (TT=55.66 (53.01 to 58.75)) V=0.3%, P=0.267
Boys 4-6 yrs CC=244,CT=51,TT=1	**BMI** CC=15.91 (15.74 to 16.08) CT+TT=15.81 (15.29 to 16.39) (TT=14.20 to 14.20) V=0.1%, P=0.686 **Sum of Skinfolds** *CC=24.75 (24.59 to2 4.93)* *CT+TT=25.22 (24.78 to 25.74)* *(TT=24.00 to2 4.00)* *V=1.4%, P=0.038*	**Arm Circumference** CC=16.69 (16.53 to 16.86) CT+TT=16.93 (16.52 to 17.37) (TT=14.99 to 14.99) V=0.4%, P=0.266	**Waist Circumference** CC=52.80 (52.32 to 53.30) CT+TT=53.33 (52.18 to 54.56) (TT=46.98 to 46.98) V=0.2%, P=0.408	**Hip Circumference** CC=57.25 (56.73 to 57.80) CT+TT=57.90 (56.68 to 59.22) (TT=51.97 to 51.97) V=0.2%, P=0.398

Table 16 Associations of adiposity-related phenotypes with *PPARγ* C1431T polymorphism in girls.

Girls 1-2 yrs CC=71,CT=15,TT=0	**BMI** CC=16.80 (16.44 to 17.17) CT=16.45 (15.97 to 16.97) V=0.8%, P=0.411 **Sum of Skinfolds** CC=25.19 (24.81 to 25.57) CT=25.59 (24.77 to 26.47) V=0.9%, P=0.339	**Arm Circumference** CC=15.35 (15.06 to 15.63) CT=15.60 (14.75 to 16.17) V=0.1%, P=0.754	**Waist Circumference** CC=46.24 (45.45 to 47.06) CT=45.13 (43.91 to 46.42) V=1.7%, P=0.233	**Hip Circumference** CC=49.10 (48.18 to 50.05) CT=49.04 (47.62 to 50.51) V=0%, P=0.953
Girls 2-3 yrs CC=154,CT=30,TT=1	**BMI** CC=16.11 (15.89 to 16.34) CT+TT=16.27 (15.85 to 16.69) (TT=14.13 to 14.13) V=0.2%, P=0.577 **Sum of Skinfolds** CC=25.69 (25.41 to 25.98) CT+TT=25.38 (24.82 to 25.99) (TT=25.90 to 25.90) V=0.2%, P=0.359	**Arm Circumference** CC=16.01 (15.80 to 16.21) CT+TT=16.09 (15.66 to 16.53) (TT=14.08 to 14.08) V=0.1%, P=0.748	**Waist Circumference** CC=49.49 (48.93 to 50.05) CT+TT=49.23 (48.09 to 50.39) (TT=46.24 to 46.24) V=0.1%, P=0.703	**Hip Circumference** CC=52.84 (52.30 to 53.39) CT+TT=52.86 (51.78 to 53.99) (TT=51.63 to 51.63) V=0%, P=0.929
Girls 3-4 yrs CC=295,CT=62,TT=1	**BMI** CC=15.91 (15.74 to 16.08) CT+TT=16.26 (15.87 to 16.67) (TT=19.59 to 19.59) V=0.7%, P=0.102 **Sum of Skinfolds** CC=25.56 (25.36 to 25.76) CT+TT=25.94 (25.49 to 26.43) (TT=26.00 to 26.00) V=0.7%, P=0.110	**Arm Circumference** CC=16.33 (16.18 to 16.49) CT+TT=16.66 (16.33 to 17.01) (TT=17.93 to 17.93) V=0.8%, P=0.090	**Waist Circumference** **CC=51.08 (50.63 to 51.53)** **CT+TT=52.42 (51.40 to 53.47)** **(TT=59.91 to 59.91)** **V=1.6%, P=0.018**	**Hip Circumference** CC=55.40 (54.98 to 55.84) CT+TT=56.18 (55.18 to 57.23) (TT=60.98 to 60.98) V=0.6%, P=0.135
Girls 4-6 yrs CC=257,CT=01,TT=1	**BMI** CC=15.99 (15.79 to 16.20) CT+TT=15.58 (15.21 to 15.98) (TT=15.82 to 15.82) V=0.9%, P=0.090 **Sum of Skinfolds** CC=26.05 (25.80 to 26.31) CT+TT=25.60 (25.12 to 26.14) (TT=23.99 to 23.99) V=0.7%, P=0.128	**Arm Circumference** CC=17.07 (16.87 to 17.27) CT+TT=16.91 (16.55 to 17.30) (TT=16.98 to 16.98) V=0.1%, P=0.525	**Waist Circumference** CC=53.13 (52.62 to 53.66) CT+TT=52.83 (51.64 to 54.12) (TT=57.44 to 57.44) V=0.1%, P=0.642	**Hip Circumference** CC=58.70 (58.12 to 59.30) CT+TT=57.70 (56.58 to 58.89) (TT=59.04 to 59.04) V=0.8%, P=0.127

Appendix 2: ANOVA analysis for associations between adiposity-reiated phenotypes and *ADRB2* and *PPARγ* diplotypes in different age groups for boys and girls.

Mean values and 95% CIs are given for the measured parameters for each *ADRB2* and *PPARγ* diplotype at different ages in boys and girls. Associations between the diplotypes and the adiposity-related phenotypes in different age groups were analysed by ANOVA. Data have been normalised, then analysed, then back-transformed to values appropriate for each age group. For each test, the percentage of variance explained by ANOVA (V) and the probability (P) are given. P values are given to three significant figures. Tests were considered significant at the level of $P<0.05$ and they are indicated in bold. N values indicate the size of each age group.

Table 1 Associations of adiposity-related phenotypes with *ADRB2* diplotypes in boys

ADRB2		BMI	Arm Circum	Waist Circum	Hip Circum
		P=0.572,V=4.6%	P=0.228,V=8.0%	P=0.612,V=4.2%	P=0.173,V=8.9%
Boys 1-2 yrs N=87	AC/AC	16.50 (15.35-17.85)	15.66 (14.78-16.49)	47.37 (44.37-50.42)	50.03 (46.40-53.29)
	AC/GC	17.08 (15.84-18.56)	15.48 (14.54-16.35)	46.75 (43.76-49.78)	50.14 (47.00-53.00)
	AC/GG	17.14 (15.30-19.57)	15.89 (14.64-17.04)	47.44 (45.00-49.91)	49.89 (47.16-52.39)
	GC/GC	16.86 (15.23-18.94)	15.24 (14.48-15.96)	46.23 (43.52-48.97)	48.61 (46.62-50.48)
	GG/GC	16.60 (15.68-17.64)	15.60 (14.86-16.31)	46.97 (43.72-50.27)	49.53 (46.60-52.20)
	GG/GG	16.33 (15.05-17.88)	15.03 (13.61-16.30)	45.82 (43.18-48.49)	47.60 (43.47-51.23)
		P=0.660,V=1.4%	P=0.167,V=3.9%	P=0.171,V=3.3%	**P=0.011,V=6.3%**
Boys 2-3 yrs N=232	AC/AC	16.53 (15.18-18.01)	16.03 (15.01-17.35)	49.62 (47.23-52.29)	**52.92 (49.77-56.48)**
	AC/GC	16.52 (15.22-17.93)	16.29 (15.28-17.57)	50.38 (47.59-53.57)	**53.94 (51.06-57.15)**
	AC/GG	16.34 (14.95-17.86)	15.79 (14.78-17.09)	49.08 (46.23-52.35)	**52.21 (48.90-55.97)**
	GC/GC	16.51 (15.23-17.98)	16.37 (15.56-17.34)	50.49 (47.90-53.41)	**53.41 (51.09-55.93)**
	GG/GC	16.20 (14.83-17.69)	15.97 (14.96-17.27)	49.27 (46.03-53.08)	**52.77 (49.52-56.44)**
	GG/GG	16.07 (14.98-17.24)	15.73 (14.88-16.78)	49.03 (47.10-51.14)	**51.15 (49.13-53.33)**
		P=0.145,V=2.0%	P=0.341,V=1.4%	P=0.501,V=1.1%	P=0.395,V=1.3%
Boys 3-4 yrs N=400	AC/AC	16.55 (15.17-18.29)	16.69 (15.50-18.15)	52.08 (48.57-56.49)	56.26 (52.63-60.64)
	AC/GC	15.85 (14.46-17.65)	16.15 (14.93-17.66)	50.83 (47.57-54.87)	54.82 (51.50-58.78)
	AC/GG	15.90 (14.61-17.54)	16.42 (15.14-18.02)	51.26 (47.86-55.51)	54.82 (51.08-59.40)
	GC/GC	15.96 (15.05-17.01)	16.17 (14.99-17.61)	51.19 (48.20-54.82)	54.54 (51.66-57.90)
	GG/GC	15.91 (14.69-17.43)	16.39 (15.17-17.92)	51.55 (48.50-55.28)	55.10 (51.76-59.09)
	GG/GG	16.11 (14.61-18.10)	16.24 (14.95-17.87)	50.97 (47.70-55.03)	54.54 (50.71-59.26)
		P=0.950,V=0.4%	P=0.912,V=0.9%	P=0.402,V=1.7%	P=0.644,V=1.1%
Boys 4-5 yr N=306	AC/AC	15.91 (14.50-17.70)	16.63 (15.28-18.50)	51.93 (47.47-57.94)	56.76 (52.72-62.02)
	AC/GC	15.90 (14.47-17.74)	16.73 (15.54-18.31)	53.34 (50.05-57.39)	57.67 (54.10-62.14)
	AC/GG	15.74 (14.48-17.31)	16.62 (15.58-17.96)	52.54 (49.06-56.89)	56.76 (52.96-61.61)
	GC/GC	16.03 (15.05-17.18)	16.52 (15.51-17.80)	52.31 (49.56-55.58)	57.21 (54.21-60.82)
	GG/GC	15.92 (14.47-17.78)	16.82 (15.50-18.62)	53.26 (49.67-57.78)	57.44 (53.23-62.96)
	GG/GG	15.98 (14.59-17.76)	16.82 (15.72-18.24)	53.26 (50.45-56.60)	58.14 (54.50-62.71)

Table 2 Associations of adiposity-related phenotypes with *ADRB2* diplotypes in girls

ADRB2		BMI	Arm Circum	Waist Circum	Hip Circum
Girls 1-2 yrs N=88		P=0.949,V=1.4%	P=0.731,V=3.3%	P=0.579,V=4.3%	P=0.947,V=1.4%
	AC/AC	16.19 (15.22-17.35)	15.23 (13.34-16.97)	44.71 (42.55-47.14)	48.77 (46.53-51.07)
	AC/GC	16.71 (15.18-18.75)	15.11 (13.69-16.46)	45.92 (42.51-50.04)	48.48 (44.55-52.64)
	AC/GG	16.74 (15.56-18.18)	15.16 (13.93-16.33)	46.40 (44.16-48.92)	49.43 (45.66-53.40)
	GC/GC	16.44 (15.04-18.27)	15.54 (14.05-16.93)	44.38 (40.94-48.58)	48.70 (42.21-55.83)
	GG/GC	16.86 (15.70-18.27)	15.39 (14.03-16.68)	46.28 (43.15-49.99)	49.79 (46.39-53.36)
	GG/GG	16.65 (15.21-18.51)	15.74 (14.72-16.72)	46.34 (43.15-50.14)	48.71 (45.14-52.46)
Girls 2-3 yrs N=192		P=0.280,V=3.3%	P=0.373,V=2.8%	P=0.476,V=2.4%	P=0.285,V=3.2%
	AC/AC	15.77 (14.78-16.84)	15.64 (14.51-16.93)	48.68 (46.35-51.06)	51.87 (49.92-54.07)
	AC/GC	16.51 (15.02-18.16)	15.98 (14.59-17.62)	49.94 (46.16-53.86)	53.25 (49.68-57.78)
	AC/GG	16.32 (14.88-17.91)	16.12 (14.90-17.52)	50.02 (46.72-53.43)	53.62 (50.44-57.52)
	GC/GC	16.40 (14.90-18.07)	16.17 (14.90-17.64)	49.32 (45.07-53.75)	52.78 (49.20-57.32)
	GG/GC	16.01 (14.71-17.43)	16.28 (15.17-17.54)	49.57 (45.88-53.38)	52.55 (49.71-55.96)
	GG/GG	15.87 (14.59-17.28)	15.71 (14.42-17.22)	48.63 (45.11-52.27)	52.43 (49.25-56.36)
Girls 3-4 yrs N=366		P=0.645,V=0.9%	P=0.452,V=1.3%	P=0.977,V=2.2%	P=0.278,V=1.6%
	AC/AC	15.95 (14.61-17.64)	16.22 (15.06-17.48)	51.59 (48.37-55.12)	55.48 (51.79-59.91)
	AC/GC	16.03 (14.85-17.47)	16.30 (14.98-17.75)	51.24 (47.67-55.18)	55.08 (51.71-59.06)
	AC/GG	16.02 (14.82-17.49)	16.53 (15.27-17.91)	51.58 (47.92-55.62)	56.16 (52.58-60.41)
	GC/GC	16.39 (14.98-18.17)	16.71 (15.23-18.35)	51.69 (47.40-56.51)	56.16 (52.41-60.66)
	GG/GC	15.76 (14.28-17.69)	16.24 (14.86-17.77)	51.39 (46.90-56.47)	54.95 (51.18-59.49)
	GG/GG	15.99 (14.33-18.20)	16.54 (15.02-18.23)	51.08 (46.38-56.43)	55.89 (52.05-60.55)
Girls 4-5 yrs N=313		P=0.450,V=1.5%	P=0.392,V=1.7%	P=0.123,V=2.8%	P=0.430,V=1.6%
	AC/AC	15.58 (13.99-17.71)	16.85 (15.45-18.57)	52.03 (47.84-57.86)	57.80 (53.53-63.14)
	AC/GC	15.78 (14.52-17.35)	16.92 (15.69-18.39)	52.40 (49.16-56.54)	57.80 (54.13-62.22)
	AC/GG	15.98 (14.49-17.90)	17.01 (15.55-18.82)	53.54 (49.72-58.62)	58.78 (54.30-64.43)
	GC/GC	16.42 (15.29-17.79)	17.66 (16.54-18.97)	54.72 (51.19-59.28)	59.46 (55.48-64.47)
	GG/GC	15.95 (14.41-17.97)	17.05 (15.50-19.00)	53.34 (49.56-58.34)	59.12 (55.04-64.13)
	GG/GG	16.09 (14.54-18.12)	17.28 (15.65-19.33)	53.42 (49.72-58.29)	58.78 (54.05-64.82)

Table 3 Associations of adiposity-related phenotypes with *PPARγ* diplotypes in girls.

PPARγ		BMI	Arm Circum	Waist Circum	Hip Circum
Boys 1-2 yrs N=88	Pro-C	16.74 (16.39-17.10)	15.45 (15.20-15.70)	46.52 (45.86-47.18)	49.07 (48.35-49.78)
	Pro-T	16.11 (14.98-17.40)	15.26 (14.62-15.89)	46.10 (44.45-47.76)	50.19 (48.11-52.18)
	Ala-C	18.02 (18.02-18.02)	16.99 (16.99-16.99)	49.00 (49.00-49.00)	54.05 (54.05-54.05)
	Ala-T	17.59 (16.43-18.91)	15.79 (15.12-16.44)	47.86 (45.85-49.89)	49.26 (47.37-51.07)
		P=0.356,V=4.0%	P=0.396,V=3.7%	P=0.534,V=2.7%	P=0.317,V=4.3%
Boys 2-3 yrs N=192	Pro-C	16.47 (16.25-16.69)	16.05 (15.88-16.22)	49.46 (49.00-49.93)	53.01 (52.54-53.49)
	Pro-T	16.31 (15.66-16.98)	15.81 (15.33-16.35)	48.76 (49.61-52.26)	51.89 (50.40-53.46)
	Ala-C	15.69 (15.12-16.28)	15.64 (15.13-16.21)	49.40 (49.68-52.69)	53.05 (52.00-54.13)
	Ala-T	16.43 (15.87-17.02)	15.86 (15.40-16.37)	49.29 (50.51-52.54)	51.82 (50.27-53.46)
		P=0.266,V=1.8%	P=0.483,V=0.8%	P=0.806,V=0.5%	P=0.195,V=2.2%
Boys 3-4 yrs N=366	Pro-C	16.00 (15.83-16.18)	16.35 (16.19-16.51)	51.33 (50.90-51.76)	54.97 (54.55-55.41)
	Pro-T	16.14 (15.58-16.75)	16.60 (16.17-17.06)	50.89 (49.61-52.26)	55.24 (53.63-57.01)
	Ala-C	15.96 (15.44-16.53)	16.13 (15.53-16.79)	51.12 (49.68-52.69)	54.53 (52.95-56.25)
	Ala-T	16.25 (15.79-16.73)	16.66 (16.25-17.10)	51.50 (50.51-52.54)	55.70 (54.52-56.97)
		P=0.809,V=0.3%	P=0.389,V=0.8%	P=0.898,V=0.2%	P=0.698,V=0.4%
Boys 4-5 yrs N=313	Pro-C	15.92 (15.74-16.11)	16.71 (16.53-16.89)	52.77 (52.24-53.32)	57.21 (56.63-57.81)
	Pro-T	16.02 (15.35-16.77)	16.87 (16.29-17.53)	53.64 (51.92-55.53)	58.38 (56.62-60.34)
	Ala-C	15.97 (15.37-16.64)	16.87 (16.37-17.43)	52.92 (51.38-54.59)	57.44 (55.63-59.45)
	Ala-T	15.62 (14.82-16.55)	16.87 (16.30-17.52)	52.59 (51.22-54.07)	57.44 (55.60-59.49)
		P=0.825,V=0.3%	P=0.752,V=0.4%	P=0.746,V=0.4%	P=0.676,V=0.6%

Table 4 Associations of adiposity-related phenotypes with *PPARγ* diplotypes in girls.

PPARγ		BMI	Arm Circum	Waist Circum	Hip Circum
Girls 1-2 yrs N=88	Pro-C	16.72 (16.32-17.14)	15.40 (15.08-15.73)	46.08 (45.21-46.99)	48.87 (47.84-49.94)
	Pro-T	16.49 (15.69-17.38)	15.26 (14.03-16.48)	45.63 (44.23-47.13)	49.30 (47.20-51.53)
	Ala-C	17.60 (16.33-19.11)	15.49 (15.05-15.92)	47.74 (44.46-51.58)	50.26 (46.80-54.07)
	Ala-T	16.18 (15.83-16.56)	15.69 (14.88-16.49)	44.50 (42.13-47.17)	48.78 (46.46-51.25)
		P=0.482,V=3.3%	P=0.937,V=0.5%	P=0.456,V=3.5%	P=0.894,V=0.8%
Girls 2-3 yrs N=192	Pro-C	16.12 (15.88-16.36)	16.00 (15.79-16.22)	49.50 (48.90-50.11)	52.86 (52.29-53.45)
	Pro-T	16.37 (15.85-16.92)	16.14 (15.61-16.69)	48.81 (47.34-50.30)	52.23 (50.95-53.62)
	Ala-C	16.20 (15.29-17.18)	16.23 (15.35-17.19)	50.25 (47.89-52.67)	53.51 (50.98-56.45)
	Ala-T	16.28 (15.42-17.20)	15.96 (14.98-17.06)	50.54 (48.20-52.93)	54.70 (52.42-57.31)
		P=0.891,V=0.4%	P=0.937,V=0.2%	P=0.599,V=1.1%	P=0.354,V=1.9%
Girls 3-4 yrs N=326	Pro-C	15.86 (15.68-16.04)	16.28 (16.11-16.44)	**50.87 (50.41-51.34)**	**55.24 (54.79-55.69)**
	Pro-T	16.09 (15.67-16.54)	16.43 (16.00-16.87)	**52.04 (50.70-53.44)**	**55.32 (54.00-56.74)**
	Ala-C	15.88 (15.41-16.40)	16.76 (16.28-17.25)	**51.40 (49.71-53.17)**	**56.40 (55.24-57.62)**
	Ala-T	16.40 (15.72-17.18)	16.98 (16.42-17.55)	**52.96 (51.37-54.64)**	**57.44 (55.91-59.09)**
		P=0.298,V=1.1%	P=0.061,V=2.3%	**P=0.035,V=2.6%**	**P=0.038,V=3.8%**
Girls 4-5 yrs N=313	Pro-C	15.99 (15.78-16.22)	17.11 (16.90-17.33)	53.15 (52.59-53.72)	58.81 (58.19-59.46)
	Pro-T	15.48 (14.89-16.15)	17.00 (16.42-17.63)	53.04 (51.22-55.10)	58.36 (56.32-60.60)
	Ala-C	16.09 (15.41-16.84)	17.03 (16.44-17.67)	53.33 (51.92-54.88)	58.59 (56.69-60.65)
	Ala-T	15.65 (15.18-16.16)	16.79 (16.32-17.30)	52.52 (50.92-54.31)	56.85 (55.70-58.06)
		P=0.351,V=1.1%	P=0.787,V=0.4%	P=0.897,V=0.2%	P=0.244,V=1.6%

REFERENCES

1. Akey,J., Jin,L., and Xiong,M. (2001). Haplotypes vs single marker linkage disequilibrium tests: what do we gain? *Eur. J. Hum. Genet.,* **9,** 291-300.

2. Altshuler,D., Hirschhorn,J.N., Klannemark,M., Lindgren,C.M., Vohl,M.C., Nemesh,J., Lane,C.R., Schaffner,S.F., Bolk,S., Brewer,C., Tuomi,T., Gaudet,D., Hudson,T.J., Daly,M., Groop,L., and Lander,E.S. (2000). The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. *Nat. Genet.,* **26,** 76-80.

3. Ardlie,K.G., Kruglyak,L., and Seielstad,M. (2002). Patterns of linkage disequilibrium in the human genome. *Nat. Rev. Genet.,* **3,** 299-309.

4. Arner,P. (2005). Human fat cell lipolysis: biochemistry, regulation and clinical role. *Best. Pract. Res. Clin. Endocrinol. Metab,* **19,** 471-482.

5. Auwerx,J., Cock,T.A., and Knouff,C. (2003). PPAR-gamma: a thrifty transcription factor. *Nucl. Recept. Signal.,* **1,** e006.

6. Bachman,E.S., Dhillon,H., Zhang,C.Y., Cinti,S., Bianco,A.C., Kobilka,B.K., and Lowell,B.B. (2002). betaAR signaling required for diet-induced thermogenesis and obesity resistance. *Science,* **297,** 843-845.

7. Barak,Y., Nelson,M.C., Ong,E.S., Jones,Y.Z., Ruiz-Lozano,P., Chien,K.R., Koder,A., and Evans,R.M. (1999). PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol. Cell,* **4,** 585-595.

8. Beamer,B.A., Yen,C.J., Andersen,R.E., Muller,D., Elahi,D., Cheskin,L.J., Andres,R., Roth,J., and Shuldiner,A.R. (1998). Association of the Pro12Ala variant in the peroxisome proliferator-activated receptor-gamma2 gene with obesity in two Caucasian populations. *Diabetes,* **47,** 1806-1808.

9. Bernstein,K.E., Martin,B.M., Edwards,A.S., and Bernstein,E.A. (1989). Mouse angiotensin-converting enzyme is a protein composed of two homologous domains. *J. Biol. Chem.,* **264,** 11945-11951.

10. Bhatti,P., Church,D.M., Rutter,J.L., Struewing,J.P., and Sigurdson,A.J. (2006). Candidate single nucleotide polymorphism selection using publicly available tools: a guide for epidemiologists. *Am. J. Epidemiol.,* **164,** 794-804.

11. Bouatia-Naji,N., Meyre,D., Lobbens,S., Seron,K., Fumeron,F., Balkau,B., Heude,B., Jouret,B., Scherer,P.E., Dina,C., Weill,J., and Froguel,P. (2006). ACDC/adiponectin polymorphisms are associated with severe childhood and adult obesity. *Diabetes,* **55,** 545-550.

12. Bouchard,C. (1995). The genetics of obesity: from genetic epidemiology to molecular markers. *Mol. Med. Today,* **1,** 45-50.

13. Bouchard,C. (1997). Genetics of human obesity: recent results from linkage studies. *J. Nutr.,* **127,** 1887S-1890S.

14. Bouchard,C. and Perusse,L. (1988). Heredity and body fat. *Annu. Rev. Nutr.*, **8,** 259-277.

15. Brookes,A.J. (1999). The essence of SNPs. *Gene,* **234,** 177-186.

16. Candelore,M.R., Deng,L., Tota,L.M., Kelly,L.J., Cascieri,M.A., and Strader,C.D. (1996). Pharmacological characterization of a recently described human beta 3-adrenergic receptor mutant. *Endocrinology,* **137,** 2638-2641.

17. Cardon,L.R. and Bell,J.I. (2001). Association study designs for complex diseases. *Nat. Rev. Genet.*, **2,** 91-99.

18. Cardon,L.R. and Palmer,L.J. (2003). Population stratification and spurious allelic association. *Lancet,* **361,** 598-604.

19. Carlson,C.S., Eberle,M.A., Kruglyak,L., and Nickerson,D.A. (2004). Mapping complex disease loci in whole-genome association studies. *Nature,* **429,** 446-452.

20. Cassis,L.A., English,V.L., Bharadwaj,K., and Boustany,C.M. (2004). Differential effects of local versus systemic angiotensin II in the regulation of leptin release from adipocytes. *Endocrinology,* **145,** 169-174.

21. Cecil,J.E., Watt,P., Palmer,C.N., and Hetherington,M. (2006). Energy balance and food intake: the role of PPARgamma gene polymorphisms. *Physiol Behav.*, **88,** 227-233.

22. Chinn,S. (2006). Definitions of childhood obesity: current practice. *Eur. J. Clin. Nutr.*, **60,** 1189-1194.

23. Clayton,D. and McKeigue,P.M. (2001). Epidemiological methods for studying genes and environmental factors in complex diseases. *Lancet,* **358,** 1356-1360.

24. Clement,K. (2006). Genetics of human obesity. *C. R. Biol.*, **329,** 608-622.

25. Clement,K., Vaisse,C., Manning,B.S., Basdevant,A., Guy-Grand,B., Ruiz,J., Silver,K.D., Shuldiner,A.R., Froguel,P., and Strosberg,A.D. (1995). Genetic variation in the beta 3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *N. Engl. J. Med.*, **333,** 352-354.

26. Cole,T.J., Bellizzi,M.C., Flegal,K.M., and Dietz,W.H. (2000). Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ,* **320,** 1240-1243.

27. Cole,T.J., Freeman,J.V., and Preece,M.A. (1995). Body mass index reference curves for the UK, 1990. *Arch. Dis. Child,* **73,** 25-29.

28. Collins,S., Cao,W., Daniel,K.W., Dixon,T.M., Medvedev,A.V., Onuma,H., and Surwit,R. (2001). Adrenoceptors, uncoupling proteins, and energy expenditure. *Exp. Biol. Med. (Maywood.),* **226,** 982-990.

29. Collins,S., Cao,W., and Robidoux,J. (2004). Learning new tricks from old dogs: beta-adrenergic receptors teach new lessons on firing up adipose tissue metabolism. *Mol. Endocrinol.*, **18,** 2123-2131.

30. Cooper,R., McFarlane-Anderson,N., Bennett,F.I., Wilks,R., Puras,A., Tewksbury,D., Ward,R., and Forrester,T. (1997). ACE, angiotensinogen and obesity: a potential pathway leading to hypertension. *J. Hum. Hypertens.*, **11,** 107-111.

31. Corella,D., Guillen,M., Portoles,O., Sorli,J.V., Alonso,V., Folch,J., and Saiz,C. (2001). Gender specific associations of the Trp64Arg mutation in the beta3-adrenergic receptor gene with obesity-related phenotypes in a Mediterranean population: interaction with a common lipoprotein lipase gene variation. *J. Intern. Med.*, **250,** 348-360.

32. Cox,R., Bouzekri,N., Martin,S., Southam,L., Hugill,A., Golamaully,M., Cooper,R., Adeyemo,A., Soubrier,F., Ward,R., Lathrop,G.M., Matsuda,F., and Farrall,M. (2002). Angiotensin-1-converting enzyme (ACE) plasma concentration is influenced by multiple ACE-linked quantitative trait nucleotides. *Hum. Mol. Genet.*, **11,** 2969-2977.

33. Crisan,D. and Carr,J. (2000). Angiotensin I-converting enzyme: genotype and disease associations. *J. Mol. Diagn.*, **2,** 105-115.

34. Danser,A.H. (2003). Local renin-angiotensin systems: the unanswered questions. *Int. J. Biochem. Cell Biol.*, **35,** 759-768.

35. Dedoussis,G.V., Theodoraki,E.V., Manios,Y., Yiannakouris,N., Panagiotakos,D., Papoutsakis,C., Skenderi,K., and Zampelas,A. (2007). The Pro12Ala polymorphism in PPARgamma2 gene affects lipid parameters in Greek primary school children: A case of gene-to-gender interaction. *Am. J. Med. Sci.*, **333,** 10-15.

36. Deeb,S.S., Fajas,L., Nemoto,M., Pihlajamaki,J., Mykkanen,L., Kuusisto,J., Laakso,M., Fujimoto,W., and Auwerx,J. (1998). A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. *Nat. Genet.*, **20,** 284-287.

37. Dehghan,M., Akhtar-Danesh,N., and Merchant,A.T. (2005). Childhood obesity, prevalence and prevention. *Nutr. J.*, **4,** 24.

38. Dietz,W.H. (1997). Periods of risk in childhood for the development of adult obesity--what do we need to learn? *J. Nutr.*, **127,** 1884S-1886S.

39. Dietz,W.H. (1998). Health consequences of obesity in youth: childhood predictors of adult disease. *Pediatrics*, **101,** 518-525.

40. Dionne,I.J., Garant,M.J., Nolan,A.A., Pollin,T.I., Lewis,D.G., Shuldiner,A.R., and Poehlman,E.T. (2002). Association between obesity and a polymorphism in the beta(1)-adrenoceptor gene (Gly389Arg ADRB1) in Caucasian women. *Int. J. Obes. Relat Metab Disord.*, **26,** 633-639.

41. Doney,A., Fischer,B., Frew,D., Cumming,A., Flavell,D.M., World,M., Montgomery,H.E., Boyle,D., Morris,A., and Palmer,C.N. (2002). Haplotype analysis of the PPARgamma Pro12Ala and C1431T variants reveals opposing associations with body weight. *BMC. Genet.,* **3,** 21.

42. Dorosty,A.R., Emmett,P.M., Cowin,S., and Reilly,J.J. (2000). Factors associated with early adiposity rebound. ALSPAC Study Team. *Pediatrics,* **105,** 1115-1118.

43. Drysdale,C.M., McGraw,D.W., Stack,C.B., Stephens,J.C., Judson,R.S., Nandabalan,K., Arnold,K., Ruano,G., and Liggett,S.B. (2000). Complex promoter and coding region beta 2-adrenergic receptor haplotypes alter receptor expression and predict in vivo responsiveness. *Proc. Natl. Acad. Sci. U. S. A,* **97,** 10483-10488.

44. Ebbeling,C.B., Pawlak,D.B., and Ludwig,D.S. (2002). Childhood obesity: public-health crisis, common sense cure. *Lancet,* **360,** 473-482.

45. Ek,J., Urhammer,S.A., Sorensen,T.I., Andersen,T., Auwerx,J., and Pedersen,O. (1999). Homozygosity of the Pro12Ala variant of the peroxisome proliferation-activated receptor-gamma2 (PPAR-gamma2): divergent modulating effects on body mass index in obese and lean Caucasian men. *Diabetologia,* **42,** 892-895.

46. Ellsworth,D.L., Coady,S.A., Chen,W., Srinivasan,S.R., Boerwinkle,E., and Berenson,G.S. (2005). Interactive effects between polymorphisms in the beta-adrenergic receptors and longitudinal changes in obesity. *Obes. Res.,* **13,** 519-526.

47. Ellsworth,D.L., Coady,S.A., Chen,W., Srinivasan,S.R., Elkasabany,A., Gustat,J., Boerwinkle,E., and Berenson,G.S. (2002). Influence of the beta2-adrenergic receptor Arg16Gly polymorphism on longitudinal changes in obesity from childhood through young adulthood in a biracial cohort: the Bogalusa Heart Study. *Int. J. Obes. Relat Metab Disord.,* **26,** 928-937.

48. Engeli,S., Gorzelniak,K., Kreutz,R., Runkel,N., Distler,A., and Sharma,A.M. (1999). Co-expression of renin-angiotensin system genes in human adipose tissue. *J. Hypertens.,* **17,** 555-560.

49. Evans,R.M., Barish,G.D., and Wang,Y.X. (2004). PPARs and the complex journey to obesity. *Nat. Med.,* **10,** 355-361.

50. Fajas,L., Auboeuf,D., Raspe,E., Schoonjans,K., Lefebvre,A.M., Saladin,R., Najib,J., Laville,M., Fruchart,J.C., Deeb,S., Vidal-Puig,A., Flier,J., Briggs,M.R., Staels,B., Vidal,H., and Auwerx,J. (1997). The organization, promoter analysis, and expression of the human PPARgamma gene. *J. Biol. Chem.,* **272,** 18779-18789.

51. Flegal,K.M., Ogden,C.L., Wei,R., Kuczmarski,R.L., and Johnson,C.L. (2001). Prevalence of overweight in US children: comparison of US growth charts

from the Centers for Disease Control and Prevention with other reference values for body mass index. *Am. J. Clin. Nutr.*, **73,** 1086-1093.

52. Fomon,S.J., Haschke,F., Ziegler,E.E., and Nelson,S.E. (1982). Body composition of reference children from birth to age 10 years. *Am. J. Clin. Nutr.*, **35,** 1169-1175.

53. Frederich,R.C., Jr., Kahn,B.B., Peach,M.J., and Flier,J.S. (1992). Tissue-specific nutritional regulation of angiotensinogen in adipose tissue. *Hypertension*, **19,** 339-344.

54. Fredriks,A.M., van Buuren,S., Fekkes,M., Verloove-Vanhorick,S.P., and Wit,J.M. (2005). Are age references for waist circumference, hip circumference and waist-hip ratio in Dutch children useful in clinical practice? *Eur. J. Pediatr.*, **164,** 216-222.

55. Freedman,D.S., Kettel,K.L., Serdula,M.K., Srinivasan,S.R., and Berenson,G.S. (2001). BMI rebound, childhood height and obesity among adults: the Bogalusa Heart Study. *Int. J. Obes. Relat Metab Disord.*, **25,** 543-549.

56. Garrow,J.S. and Webster,J. (1985). Quetelet's index (W/H2) as a measure of fatness. *Int. J. Obes.*, **9,** 147-153.

57. Georgiadis,G. and Nassis,G.P. (2007). Prevalence of overweight and obesity in a national representative sample of Greek children and adolescents. *Eur. J. Clin. Nutr.*.

58. Ghoussaini,M., Meyre,D., Lobbens,S., Charpentier,G., Clement,K., Charles,M.A., Tauber,M., Weill,J., and Froguel,P. (2005). Implication of the Pro12Ala polymorphism of the PPAR-gamma 2 gene in type 2 diabetes and obesity in the French population. *BMC. Med. Genet.*, **6,** 11.

59. Giacchetti,G., Faloia,E., Mariniello,B., Sardu,C., Gatti,C., Camilloni,M.A., Guerrieri,M., and Mantero,F. (2002). Overexpression of the renin-angiotensin system in human visceral adipose tissue in normal and overweight subjects. *Am. J. Hypertens.*, **15,** 381-388.

60. Goldstein,D.B. (2001). Islands of linkage disequilibrium. *Nat. Genet.*, **29,** 109-111.

61. Gonzalez Sanchez,J.L., Proenza,A.M., Martinez Larrad,M.T., Ramis,J.M., Fernandez,P.C., Palou,A., and Serrano,R.M. (2003). The glutamine 27 glutamic acid polymorphism of the beta2-adrenoceptor gene is associated with abdominal obesity and greater risk of impaired glucose tolerance in men but not in women: a population-based study in Spain. *Clin. Endocrinol. (Oxf)*, **59,** 476-481.

62. Goossens,G.H., Blaak,E.E., Saris,W.H., and van Baak,M.A. (2004). Angiotensin II-induced effects on adipose and skeletal muscle tissue blood flow and lipolysis in normal-weight and obese subjects. *J. Clin. Endocrinol. Metab*, **89,** 2690-2696.

63. Goossens,G.H., Blaak,E.E., and van Baak,M.A. (2003). Possible involvement of the adipose tissue renin-angiotensin system in the pathophysiology of obesity and obesity-related disorders. *Obes. Rev.*, **4,** 43-55.

64. Goran,M.I. (2001). Metabolic precursors and effects of obesity in children: a decade of progress, 1990-1999. *Am. J. Clin. Nutr.*, **73,** 158-171.

65. Gray,I.C., Campbell,D.A., and Spurr,N.K. (2000). Single nucleotide polymorphisms as tools in human genetics. *Hum. Mol. Genet.*, **9,** 2403-2408.

66. Green,S.A., Cole,G., Jacinto,M., Innis,M., and Liggett,S.B. (1993). A polymorphism of the human beta 2-adrenergic receptor within the fourth transmembrane domain alters ligand binding and functional properties of the receptor. *J. Biol. Chem.*, **268,** 23116-23121.

67. Green,S.A., Turki,J., Innis,M., and Liggett,S.B. (1994). Amino-terminal polymorphisms of the human beta 2-adrenergic receptor impart distinct agonist-promoted regulatory properties. *Biochemistry,* **33,** 9414-9419.

68. Gurnell,M. (2005). Peroxisome proliferator-activated receptor gamma and the regulation of adipocyte function: lessons from human genetic studies. *Best. Pract. Res. Clin. Endocrinol. Metab,* **19,** 501-523.

69. Hall,D.M. and Cole,T.J. (2006). What use is the BMI? *Arch. Dis. Child,* **91,** 283-286.

70. Hamann,A., Munzberg,H., Buttron,P., Busing,B., Hinney,A., Mayer,H., Siegfried,W., Hebebrand,J., and Greten,H. (1999). Missense variants in the human peroxisome proliferator-activated receptor-gamma2 gene in lean and obese subjects. *Eur. J. Endocrinol.*, **141,** 90-92.

71. Haschke,F. and van't Hof,M.A. (2000). Euro-Growth references for length, weight, and body circumferences. Euro-Growth Study Group. *J. Pediatr. Gastroenterol. Nutr.*, **31 Suppl 1,** S14-S38.

72. Hattersley,A.T. and McCarthy,M.I. (2005). What makes a good genetic association study? *Lancet,* **366,** 1315-1323.

73. He,W., Barak,Y., Hevener,A., Olson,P., Liao,D., Le,J., Nelson,M., Ong,E., Olefsky,J.M., and Evans,R.M. (2003). Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. *Proc. Natl. Acad. Sci. U. S. A,* **100,** 15712-15717.

74. Hellstrom,L., Large,V., Reynisdottir,S., Wahrenberg,H., and Arner,P. (1999). The different effects of a Gln27Glu beta 2-adrenoceptor gene polymorphism on obesity in males and in females. *J. Intern. Med.*, **245,** 253-259.

75. Hill,J.O., Wyatt,H.R., Reed,G.W., and Peters,J.C. (2003). Obesity and the environment: where do we go from here? *Science,* **299,** 853-855.

76. Hooper,N.M. and Turner,A.J. (2003). An ACE structure. *Nat. Struct. Biol.*, **10,** 155-157.

77. Hsueh,W.C., Cole,S.A., Shuldiner,A.R., Beamer,B.A., Blangero,J., Hixson,J.E., MacCluer,J.W., and Mitchell,B.D. (2001). Interactions between variants in the beta3-adrenergic receptor and peroxisome proliferator-activated receptor-gamma2 genes and obesity. *Diabetes Care,* **24,** 672-677.

78. Ishiyama-Shigemoto,S., Yamada,K., Yuan,X., Ichikawa,F., and Nonaka,K. (1999). Association of polymorphisms in the beta2-adrenergic receptor gene with obesity, hypertriglyceridaemia, and diabetes mellitus. *Diabetologia,* **42,** 98-101.

79. Jackson,D.M., Reilly,J.J., Kelly,L.A., Montgomery,C., Grant,S., and Paton,J.Y. (2003). Objectively measured physical activity in a representative sample of 3- to 4-year-old children. *Obes. Res.,* **11,** 420-425.

80. Jebb,S.A., Rennie,K.L., and Cole,T.J. (2004). Prevalence of overweight and obesity among young people in Great Britain. *Public Health Nutr.,* **7,** 461-465.

81. Jones,B.H., Standridge,M.K., and Moustaid,N. (1997). Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology,* **138,** 1512-1519.

82. Karayiannis,D., Yannakoulia,M., Terzidou,M., Sidossis,L.S., and Kokkevi,A. (2003). Prevalence of overweight and obesity in Greek school-aged children and adolescents. *Eur. J. Clin. Nutr.,* **57,** 1189-1192.

83. Kawamura,T., Egusa,G., Fujikawa,R., and Okubo,M. (2001). Beta(3)-adrenergic receptor gene variant is associated with upper body obesity only in obese Japanese-American men but not in women. *Diabetes Res. Clin. Pract.,* **54,** 49-55.

84. Kim-Motoyama,H., Yasuda,K., Yamaguchi,T., Yamada,N., Katakura,T., Shuldiner,A.R., Akanuma,Y., Ohashi,Y., Yazaki,Y., and Kadowaki,T. (1997). A mutation of the beta 3-adrenergic receptor is associated with visceral obesity but decreased serum triglyceride. *Diabetologia,* **40,** 469-472.

85. Knoblauch,H., Busjahn,A., Muller-Myhsok,B., Faulhaber,H.D., Schuster,H., Uhlmann,R., and Luft,F.C. (1999). Peroxisome proliferator-activated receptor gamma gene locus is related to body mass index and lipid values in healthy nonobese subjects. *Arterioscler. Thromb. Vasc. Biol.,* **19,** 2940-2944.

86. Koivisto Hursti,U.K. (1999). Factors influencing children's food choice. *Ann. Med.,* **31 Suppl 1,** 26-32.

87. Krassas,G.E., Tzotzas,T., Tsametis,C., and Konstantinidis,T. (2001). Prevalence and trends in overweight and obesity among children and adolescents in Thessaloniki, Greece. *J. Pediatr. Endocrinol. Metab,* **14 Suppl 5,** 1319-1326.

88. Kuczmarski,R.J., Ogden,C.L., Grummer-Strawn,L.M., Flegal,K.M., Guo,S.S., Wei,R., Mei,Z., Curtin,L.R., Roche,A.F., and Johnson,C.L. (2000). CDC growth charts: United States. *Adv. Data,* 1-27.

89. Lafontan,M. and Berlan,M. (1993). Fat cell adrenergic receptors and the control of white and brown fat cell function. *J. Lipid Res.,* **34,** 1057-1091.

90. Large,V., Hellstrom,L., Reynisdottir,S., Lonnqvist,F., Eriksson,P., Lannfelt,L., and Arner,P. (1997). Human beta-2 adrenoceptor gene polymorphisms are highly frequent in obesity and associate with altered adipocyte beta-2 adrenoceptor function. *J. Clin. Invest,* **100,** 3005-3013.

91. Lavoie,J.L. and Sigmund,C.D. (2003). Minireview: overview of the renin-angiotensin system--an endocrine and paracrine system. *Endocrinology,* **144,** 2179-2183.

92. Lehrke,M. and Lazar,M.A. (2005). The many faces of PPARgamma. *Cell,* **123,** 993-999.

93. Leineweber,K. and Brodde,O.E. (2004). Beta2-adrenoceptor polymorphisms: relation between in vitro and in vivo phenotypes. *Life Sci.,* **74,** 2803-2814.

94. Leineweber,K., Buscher,R., Bruck,H., and Brodde,O.E. (2004). Beta-adrenoceptor polymorphisms. *Naunyn Schmiedebergs Arch. Pharmacol.,* **369,** 1-22.

95. Linne,Y., Dahlman,I., and Hoffstedt,J. (2005). beta1-Adrenoceptor gene polymorphism predicts long-term changes in body weight. *Int. J. Obes. (Lond),* **29,** 458-462.

96. Lissau,I. (2004). Overweight and obesity epidemic among children. Answer from European countries. *Int. J. Obes. Relat Metab Disord.,* **28 Suppl 3,** S10-S15.

97. Lissau,I., Overpeck,M.D., Ruan,W.J., Due,P., Holstein,B.E., and Hediger,M.L. (2004). Body mass index and overweight in adolescents in 13 European countries, Israel, and the United States. *Arch. Pediatr. Adolesc. Med.,* **158,** 27-33.

98. Lowell,B.B. and Bachman,E.S. (2003). Beta-Adrenergic receptors, diet-induced thermogenesis, and obesity. *J. Biol. Chem.,* **278,** 29385-29388.

99. Luan,J., Browne,P.O., Harding,A.H., Halsall,D.J., O'Rahilly,S., Chatterjee,V.K., and Wareham,N.J. (2001). Evidence for gene-nutrient interaction at the PPARgamma locus. *Diabetes,* **50,** 686-689.

100. Lundberg,U. (1983). Sex differences in behaviour pattern and catecholamine and cortisol excretion in 3-6 year old day-care children. *Biol. Psychol.,* **16,** 109-117.

101. Macho-Azcarate,T., Marti,A., Gonzalez,A., Martinez,J.A., and Ibanez,J. (2002). Gln27Glu polymorphism in the beta2 adrenergic receptor gene and lipid metabolism during exercise in obese women. *Int. J. Obes. Relat Metab Disord.,* **26,** 1434-1441.

102. Maes,H.H., Neale,M.C., and Eaves,L.J. (1997). Genetic and environmental factors in relative body weight and human adiposity. *Behav. Genet.*, **27,** 325-351.

103. Magarey,A.M., Daniels,L.A., Boulton,T.J., and Cockington,R.A. (2003). Predicting obesity in early adulthood from childhood and parental obesity. *Int. J. Obes. Relat Metab Disord.*, **27,** 505-513.

104. Magkos,F., Manios,Y., Christakis,G., and Kafatos,A.G. (2005). Secular trends in cardiovascular risk factors among school-aged boys from Crete, Greece, 1982-2002. *Eur. J. Clin. Nutr.*, **59,** 1-7.

105. Mamalakis,G., Kafatos,A., Manios,Y., Anagnostopoulou,T., and Apostolaki,I. (2000). Obesity indices in a cohort of primary school children in Crete: a six year prospective study. *Int. J. Obes. Relat Metab Disord.*, **24,** 765-771.

106. Manios,Y. (2006). Design and descriptive results of the "Growth, Exercise and Nutrition Epidemiological Study In preSchoolers": the GENESIS study. *BMC. Public Health,* **6,** 32.

107. Maqbool,A., Hall,A.S., Ball,S.G., and Balmforth,A.J. (1999). Common polymorphisms of beta1-adrenoceptor: identification and rapid screening assay. *Lancet,* **353,** 897.

108. Marti,A., Moreno-Aliaga,M.J., Hebebrand,J., and Martinez,J.A. (2004). Genes, lifestyles and obesity. *Int. J. Obes. Relat Metab Disord.*, **28 Suppl 3,** S29-S36.

109. Martin,G., Schoonjans,K., Staels,B., and Auwerx,J. (1998). PPARgamma activators improve glucose homeostasis by stimulating fatty acid uptake in the adipocytes. *Atherosclerosis,* **137 Suppl,** S75-S80.

110. Mason,D.A., Moore,J.D., Green,S.A., and Liggett,S.B. (1999). A gain-of-function polymorphism in a G-protein coupling domain of the human beta1-adrenergic receptor. *J. Biol. Chem.*, **274,** 12670-12674.

111. Masud,S. and Ye,S. (2003). Effect of the peroxisome proliferator activated receptor-gamma gene Pro12Ala variant on body mass index: a meta-analysis. *J. Med. Genet.*, **40,** 773-780.

112. Masugi,J., Tamori,Y., Mori,H., Koike,T., and Kasuga,M. (2000). Inhibitory effect of a proline-to-alanine substitution at codon 12 of peroxisome proliferator-activated receptor-gamma 2 on thiazolidinedione-induced adipogenesis. *Biochem. Biophys. Res. Commun.*, **268,** 178-182.

113. Mayeux,R. (2005). Mapping the new frontier: complex genetic disorders. *J. Clin. Invest,* **115,** 1404-1407.

114. McCarthy,H.D., Jarrett,K.V., Emmett,P.M., and Rogers,I. (2005). Trends in waist circumferences in young British children: a comparative study. *Int. J. Obes. (Lond),* **29,** 157-162.

115. Meirhaeghe,A. and Amouyel,P. (2004). Impact of genetic variation of PPARgamma in humans. *Mol. Genet. Metab,* **83,** 93-102.

116. Meirhaeghe,A., Cottel,D., Amouyel,P., and Dallongeville,J. (2005a). Association between peroxisome proliferator-activated receptor gamma haplotypes and the metabolic syndrome in French men and women. *Diabetes,* **54,** 3043-3048.

117. Meirhaeghe,A., Fajas,L., Helbecque,N., Cottel,D., Auwerx,J., Deeb,S.S., and Amouyel,P. (2000a). Impact of the Peroxisome Proliferator Activated Receptor gamma2 Pro12Ala polymorphism on adiposity, lipids and non-insulin-dependent diabetes mellitus. *Int. J. Obes. Relat Metab Disord.,* **24,** 195-199.

118. Meirhaeghe,A., Fajas,L., Helbecque,N., Cottel,D., Lebel,P., Dallongeville,J., Deeb,S., Auwerx,J., and Amouyel,P. (1998). A genetic polymorphism of the peroxisome proliferator-activated receptor gamma gene influences plasma leptin levels in obese humans. *Hum. Mol. Genet.,* **7,** 435-440.

119. Meirhaeghe,A., Helbecque,N., Cottel,D., and Amouyel,P. (1999). Beta2-adrenoceptor gene polymorphism, body weight, and physical activity. *Lancet,* **353,** 896.

120. Meirhaeghe,A., Helbecque,N., Cottel,D., and Amouyel,P. (2000b). Impact of polymorphisms of the human beta2-adrenoceptor gene on obesity in a French population. *Int. J. Obes. Relat Metab Disord.,* **24,** 382-387.

121. Meirhaeghe,A., Tanck,M.W., Fajas,L., Janot,C., Helbecque,N., Cottel,D., Auwerx,J., Amouyel,P., and Dallongeville,J. (2005b). Study of a new PPARgamma2 promoter polymorphism and haplotype analysis in a French population. *Mol. Genet. Metab,* **85,** 140-148.

122. Meyre,D., Bouatia-Naji,N., Tounian,A., Samson,C., Lecoeur,C., Vatin,V., Ghoussaini,M., Wachter,C., Hercberg,S., Charpentier,G., Patsch,W., Pattou,F., Charles,M.A., Tounian,P., Clement,K., Jouret,B., Weill,J., Maddux,B.A., Goldfine,I.D., Walley,A., Boutin,P., Dina,C., and Froguel,P. (2005). Variants of ENPP1 are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes. *Nat. Genet.,* **37,** 863-867.

123. Mi,J., Cheng,H., Hou,D.Q., Duan,J.L., Teng,H.H., and Wang,Y.F. (2006). [Prevalence of overweight and obesity among children and adolescents in Beijing in 2004]. *Zhonghua Liu Xing. Bing. Xue. Za Zhi.,* **27,** 469-474.

124. Miller,J., Rosenbloom,A., and Silverstein,J. (2004). Childhood obesity. *J. Clin. Endocrinol. Metab,* **89,** 4211-4218.

125. Moffett,S.P., Feingold,E., Barmada,M.M., Damcott,C.M., Marshall,J.A., Hamman,R.F., and Ferrell,R.E. (2005). The C161-->T polymorphism in peroxisome proliferator-activated receptor gamma, but not P12A, is associated with insulin resistance in Hispanic and non-Hispanic white women: evidence

for another functional variant in peroxisome proliferator-activated receptor gamma. *Metabolism,* **54,** 1552-1556.

126. Moran,C.N., Vassilopoulos,C., Tsiokanos,A., Jamurtas,A.Z., Bailey,M.E., Wilson,R.H., and Pitsiladis,Y.P. (2005). Effects of interaction between angiotensin I-converting enzyme polymorphisms and lifestyle on adiposity in adolescent Greeks. *Obes. Res.,* **13,** 1499-1504.

127. Moreno,L.A., Rodriguez,G., Guillen,J., Rabanaque,M.J., Leon,J.F., and Arino,A. (2002). Anthropometric measurements in both sides of the body in the assessment of nutritional status in prepubertal children. *Eur. J. Clin. Nutr.,* **56,** 1208-1215.

128. Mori,Y., Kim-Motoyama,H., Katakura,T., Yasuda,K., Kadowaki,H., Beamer,B.A., Shuldiner,A.R., Akanuma,Y., Yazaki,Y., and Kadowaki,T. (1998). Effect of the Pro12Ala variant of the human peroxisome proliferator-activated receptor gamma 2 gene on adiposity, fat distribution, and insulin sensitivity in Japanese men. *Biochem. Biophys. Res. Commun.,* **251,** 195-198.

129. Newton-Cheh,C. and Hirschhorn,J.N. (2005). Genetic association studies of complex traits: design and analysis issues. *Mutat. Res.,* **573,** 54-69.

130. Nicklas,T.A., Baranowski,T., Cullen,K.W., and Berenson,G. (2001). Eating patterns, dietary quality and obesity. *J. Am. Coll. Nutr.,* **20,** 599-608.

131. O'Donnell,C.J., Lindpaintner,K., Larson,M.G., Rao,V.S., Ordovas,J.M., Schaefer,E.J., Myers,R.H., and Levy,D. (1998). Evidence for association and genetic linkage of the angiotensin-converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham Heart Study. *Circulation,* **97,** 1766-1772.

132. O'Neill,J.L., McCarthy,S.N., Burke,S.J., Hannon,E.M., Kiely,M., Flynn,A., Flynn,M.A., and Gibney,M.J. (2007). Prevalence of overweight and obesity in Irish school children, using four different definitions. *Eur. J. Clin. Nutr.,* **61,** 743-751.

133. Ochoa,M.C., Marti,A., Azcona,C., Chueca,M., Oyarzabal,M., Pelach,R., Patino,A., Moreno-Aliaga,M.J., Martinez-Gonzalez,M.A., and Martinez,J.A. (2004). Gene-gene interaction between PPAR gamma 2 and ADR beta 3 increases obesity risk in children and adolescents. *Int. J. Obes. Relat Metab Disord.,* **28 Suppl 3,** S37-S41.

134. Ogden,C.L., Carroll,M.D., Curtin,L.R., McDowell,M.A., Tabak,C.J., and Flegal,K.M. (2006). Prevalence of overweight and obesity in the United States, 1999-2004. *JAMA,* **295,** 1549-1555.

135. Perusse,L., Rankinen,T., Zuberi,A., Chagnon,Y.C., Weisnagel,S.J., Argyropoulos,G., Walts,B., Snyder,E.E., and Bouchard,C. (2005). The human obesity gene map: the 2004 update. *Obes. Res.,* **13,** 381-490.

136. Pietri-Rouxel,F., St John,M.B., Gros,J., and Strosberg,A.D. (1997). The biochemical effect of the naturally occurring Trp64-->Arg mutation on human beta3-adrenoceptor activity. *Eur. J. Biochem.*, **247,** 1174-1179.

137. Pietrobelli,A., Peroni,D.G., and Faith,M.S. (2003). Pediatric body composition in clinical studies: which methods in which situations? *Acta Diabetol.*, **40 Suppl 1,** S270-S273.

138. Pietrobelli,A. and Tato,L. (2005). Body composition measurements: from the past to the future. *Acta Paediatr. Suppl,* **94,** 8-13.

139. Poirier,O., Nicaud,V., Cambien,F., and Tiret,L. (2000). The Pro12Ala polymorphism in the peroxisome proliferator-activated receptor gamma2 gene is not associated with postprandial responses to glucose or fat tolerance tests in young healthy subjects: the European Atherosclerosis Research Study II. *J. Mol. Med.*, **78,** 346-351.

140. Poulsen,P., Andersen,G., Fenger,M., Hansen,T., Echwald,S.M., Volund,A., Beck-Nielsen,H., Pedersen,O., and Vaag,A. (2003). Impact of two common polymorphisms in the PPARgamma gene on glucose tolerance and plasma insulin profiles in monozygotic and dizygotic twins: thrifty genotype, thrifty phenotype, or both? *Diabetes,* **52,** 194-198.

141. Prentice,A.M. and Jebb,S.A. (1995). Obesity in Britain: gluttony or sloth? *BMJ,* **311,** 437-439.

142. Rankinen,T., Zuberi,A., Chagnon,Y.C., Weisnagel,S.J., Argyropoulos,G., Walts,B., Perusse,L., and Bouchard,C. (2006). The human obesity gene map: the 2005 update. *Obesity. (Silver. Spring),* **14,** 529-644.

143. Reilly,J.J. (2002). Assessment of childhood obesity: national reference data or international approach? *Obes. Res.*, **10,** 838-840.

144. Reilly,J.J. (2005). Descriptive epidemiology and health consequences of childhood obesity. *Best. Pract. Res. Clin. Endocrinol. Metab,* **19,** 327-341.

145. Reilly,J.J. (2006). Diagnostic accuracy of the BMI for age in paediatrics. *Int. J. Obes. (Lond),* **30,** 595-597.

146. Reilly,J.J., Kelly,L., Montgomery,C., Williamson,A., Fisher,A., McColl,J.H., Lo,C.R., Paton,J.Y., and Grant,S. (2006). Physical activity to prevent obesity in young children: cluster randomised controlled trial. *BMJ,* **333,** 1041.

147. Rieder,M.J., Taylor,S.L., Clark,A.G., and Nickerson,D.A. (1999). Sequence variation in the human angiotensin converting enzyme. *Nat. Genet.*, **22,** 59-62.

148. Riera-Fortuny,C., Real,J.T., Chaves,F.J., Morales-Suarez-Varela,M., Martinez-Triguero,M.L., Morillas-Arino,C., and Hernandez-Mijares,A. (2005). The relation between obesity, abdominal fat deposit and the angiotensin-converting enzyme gene I/D polymorphism and its association with coronary heart disease. *Int. J. Obes. (Lond),* **29,** 78-84.

149. Rigat,B., Hubert,C., Alhenc-Gelas,F., Cambien,F., Corvol,P., and Soubrier,F. (1990). An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J. Clin. Invest,* **86,** 1343-1346.

150. Rio-Navarro,B.E., Velazquez-Monroy,O., Sanchez-Castillo,C.P., Lara-Esqueda,A., Berber,A., Fanghanel,G., Violante,R., Tapia-Conyer,R., and James,W.P. (2004). The high prevalence of overweight and obesity in Mexican children. *Obes. Res.,* **12,** 215-223.

151. Risch,N.J. (2000). Searching for genetic determinants in the new millennium. *Nature,* **405,** 847-856.

152. Rolland-Cachera,M.F., Deheeger,M., Bellisle,F., Sempe,M., Guilloud-Bataille,M., and Patois,E. (1984). Adiposity rebound in children: a simple indicator for predicting obesity. *Am. J. Clin. Nutr.,* **39,** 129-135.

153. Rosen,E.D., Sarraf,P., Troy,A.E., Bradwin,G., Moore,K., Milstone,D.S., Spiegelman,B.M., and Mortensen,R.M. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol. Cell,* **4,** 611-617.

154. Rosen,E.D. and Spiegelman,B.M. (2001). PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. *J. Biol. Chem.,* **276,** 37731-37734.

155. Ryden,M., Hoffstedt,J., Eriksson,P., Bringman,S., and Arner,P. (2001). The Arg 389 Gly beta1-adrenergic receptor gene polymorphism and human fat cell lipolysis. *Int. J. Obes. Relat Metab Disord.,* **25,** 1599-1603.

156. Salmon,J., Campbell,K.J., and Crawford,D.A. (2006). Television viewing habits associated with obesity risk factors: a survey of Melbourne schoolchildren. *Med. J. Aust.,* **184,** 64-67.

157. Savage,D.B., Tan,G.D., Acerini,C.L., Jebb,S.A., Agostini,M., Gurnell,M., Williams,R.L., Umpleby,A.M., Thomas,E.L., Bell,J.D., Dixon,A.K., Dunne,F., Boiani,R., Cinti,S., Vidal-Puig,A., Karpe,F., Chatterjee,V.K., and O'Rahilly,S. (2003). Human metabolic syndrome resulting from dominant-negative mutations in the nuclear receptor peroxisome proliferator-activated receptor-gamma. *Diabetes,* **52,** 910-917.

158. Savva,S.C., Tornaritis,M., Chadjigeorgiou,C., Kourides,Y.A., Savva,M.E., Panagi,A., Chrictodoulou,E., and Kafatos,A. (2005). Prevalence and socio-demographic associations of undernutrition and obesity among preschool children in Cyprus. *Eur. J. Clin. Nutr.,* **59,** 1259-1265.

159. Seals,D.R. and Esler,M.D. (2000). Human ageing and the sympathoadrenal system. *J. Physiol,* **528,** 407-417.

160. Small,K.M., McGraw,D.W., and Liggett,S.B. (2003). Pharmacology and physiology of human adrenergic receptor polymorphisms. *Annu. Rev. Pharmacol. Toxicol.,* **43,** 381-411.

161. Soeder,K.J., Snedden,S.K., Cao,W., Della Rocca,G.J., Daniel,K.W., Luttrell,L.M., and Collins,S. (1999). The beta3-adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a Gi-dependent mechanism. *J. Biol. Chem.,* **274,** 12017-12022.

162. Soubrier,F., Martin,S., Alonso,A., Visvikis,S., Tiret,L., Matsuda,F., Lathrop,G.M., and Farrall,M. (2002). High-resolution genetic mapping of the ACE-linked QTL influencing circulating ACE activity. *Eur. J. Hum. Genet.,* **10,** 553-561.

163. Stenhouse,E., Wright,D.E., Hattersley,A.T., and Millward,B.A. (2004). Weight differences in Plymouth toddlers compared to the British Growth Reference Population. *Arch. Dis. Child,* **89,** 843-844.

164. Stephens,J.C., Schneider,J.A., Tanguay,D.A., Choi,J., Acharya,T., Stanley,S.E., Jiang,R., Messer,C.J., Chew,A., Han,J.H., Duan,J., Carr,J.L., Lee,M.S., Koshy,B., Kumar,A.M., Zhang,G., Newell,W.R., Windemuth,A., Xu,C., Kalbfleisch,T.S., Shaner,S.L., Arnold,K., Schulz,V., Drysdale,C.M., Nandabalan,K., Judson,R.S., Ruano,G., and Vovis,G.F. (2001). Haplotype variation and linkage disequilibrium in 313 human genes. *Science,* **293,** 489-493.

165. Strazzullo,P. and Galletti,F. (2004). Impact of the renin-angiotensin system on lipid and carbohydrate metabolism. *Curr. Opin. Nephrol. Hypertens.,* **13,** 325-332.

166. Strazzullo,P., Iacone,R., Iacoviello,L., Russo,O., Barba,G., Russo,P., D'Orazio,A., Barbato,A., Cappuccio,F.P., Farinaro,E., and Siani,A. (2003). Genetic variation in the renin-angiotensin system and abdominal adiposity in men: the Olivetti Prospective Heart Study. *Ann. Intern. Med.,* **138,** 17-23.

167. Strobel,G., Friedmann,B., Siebold,R., and Bartsch,P. (1999). Effect of severe exercise on plasma catecholamines in differently trained athletes. *Med. Sci. Sports Exerc.,* **31,** 560-565.

168. Strosberg,A.D. (1997). Structure and function of the beta 3-adrenergic receptor. *Annu. Rev. Pharmacol. Toxicol.,* **37,** 421-450.

169. Stumvoll,M. and Haring,H. (2002). The peroxisome proliferator-activated receptor-gamma2 Pro12Ala polymorphism. *Diabetes,* **51,** 2341-2347.

170. Suh,Y. and Vijg,J. (2005). SNP discovery in associating genetic variation with human disease phenotypes. *Mutat. Res.,* **573,** 41-53.

171. Swarbrick,M.M., Chapman,C.M., McQuillan,B.M., Hung,J., Thompson,P.L., and Beilby,J.P. (2001). A Pro12Ala polymorphism in the human peroxisome proliferator-activated receptor-gamma 2 is associated with combined hyperlipidaemia in obesity. *Eur. J. Endocrinol.,* **144,** 277-282.

172. Tafel,J., Branscheid,I., Skwarna,B., Schlimme,M., Morcos,M., Algenstaedt,P., Hinney,A., Hebebrand,J., Nawroth,P., and Hamann,A. (2004). Variants in the human beta 1-, beta 2-, and beta 3-adrenergic receptor genes are not associated

with morbid obesity in children and adolescents. *Diabetes Obes. Metab,* **6,** 452-455.

173. Tai,E.S., Corella,D., Deurenberg-Yap,M., Adiconis,X., Chew,S.K., Tan,C.E., and Ordovas,J.M. (2004). Differential effects of the C1431T and Pro12Ala PPARgamma gene variants on plasma lipids and diabetes risk in an Asian population. *J. Lipid Res.,* **45,** 674-685.

174. Tiret,L., Poirier,O., Nicaud,V., Barbaux,S., Herrmann,S.M., Perret,C., Raoux,S., Francomme,C., Lebard,G., Tregouet,D., and Cambien,F. (2002). Heterogeneity of linkage disequilibrium in human genes has implications for association studies of common diseases. *Hum. Mol. Genet.,* **11,** 419-429.

175. Tiret,L., Rigat,B., Visvikis,S., Breda,C., Corvol,P., Cambien,F., and Soubrier,F. (1992). Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am. J. Hum. Genet.,* **51,** 197-205.

176. Tontonoz,P., Hu,E., and Spiegelman,B.M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell,* **79,** 1147-1156.

177. Townsend,R.R. (2001). The effects of angiotensin-II on lipolysis in humans. *Metabolism,* **50,** 468-472.

178. Trost,S.G., Sirard,J.R., Dowda,M., Pfeiffer,K.A., and Pate,R.R. (2003). Physical activity in overweight and nonoverweight preschool children. *Int. J. Obes. Relat Metab Disord.,* **27,** 834-839.

179. Twyman,R.M. and Primrose,S.B. (2003). Techniques patents for SNP genotyping. *Pharmacogenomics.,* **4,** 67-79.

180. Uhl,G.R., Gold,L.H., and Risch,N. (1997). Genetic analyses of complex behavioral disorders. *Proc. Natl. Acad. Sci. U. S. A,* **94,** 2785-2786.

181. Ukkola,O., Rankinen,T., Weisnagel,S.J., Sun,G., Perusse,L., Chagnon,Y.C., Despres,J.P., and Bouchard,C. (2000). Interactions among the alpha2-, beta2-, and beta3-adrenergic receptor genes and obesity-related phenotypes in the Quebec Family Study. *Metabolism,* **49,** 1063-1070.

182. Ukkola,O., Tremblay,A., and Bouchard,C. (2001). Beta-2 adrenergic receptor variants are associated with subcutaneous fat accumulation in response to long-term overfeeding. *Int. J. Obes. Relat Metab Disord.,* **25,** 1604-1608.

183. Um,J.Y., Moon,K.S., Lee,K.M., Cho,K.H., Heo,Y., Moon,B.S., and Kim,H.M. (2003). Polymorphism of angiotensin-converting enzyme, angiotensinogen, and apolipoprotein E genes in Korean patients with cerebral infarction. *J. Mol. Neurosci.,* **21,** 23-28.

184. Urhammer,S.A., Fridberg,M., Sorensen,T.I., Echwald,S.M., Andersen,T., Tybjaerg-Hansen,A., Clausen,J.O., and Pedersen,O. (1997). Studies of genetic

variability of the uncoupling protein 1 gene in Caucasian subjects with juvenile-onset obesity. *J. Clin. Endocrinol. Metab,* **82,** 4069-4074.

185. Valli-Jaakola,K., Lipsanen-Nyman,M., Oksanen,L., Hollenberg,A.N., Kontula,K., Bjorbaek,C., and Schalin-Jantti,C. (2004). Identification and characterization of melanocortin-4 receptor gene mutations in morbidly obese finnish children and adults. *J. Clin. Endocrinol. Metab,* **89,** 940-945.

186. Valve,R., Sivenius,K., Miettinen,R., Pihlajamaki,J., Rissanen,A., Deeb,S.S., Auwerx,J., Uusitupa,M., and Laakso,M. (1999). Two polymorphisms in the peroxisome proliferator-activated receptor-gamma gene are associated with severe overweight among obese women. *J. Clin. Endocrinol. Metab,* **84,** 3708-3712.

187. Viswanathan,M., Selby,D.M., and Ray,P.E. (2000). Expression of renal and vascular angiotensin II receptor subtypes in children. *Pediatr. Nephrol.,* **14,** 1030-1036.

188. Wake,M., Hardy,P., Canterford,L., Sawyer,M., and Carlin,J.B. (2007). Overweight, obesity and girth of Australian preschoolers: prevalence and socio-economic correlates. *Int. J. Obes. (Lond),* **31,** 1044-1051.

189. Walston,J., Silver,K., Bogardus,C., Knowler,W.C., Celi,F.S., Austin,S., Manning,B., Strosberg,A.D., Stern,M.P., Raben,N., and . (1995). Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the beta 3-adrenergic-receptor gene. *N. Engl. J. Med.,* **333,** 343-347.

190. Weiss,K.M. and Clark,A.G. (2002). Linkage disequilibrium and the mapping of complex human traits. *Trends Genet.,* **18,** 19-24.

191. Wells,J.C. and Fewtrell,M.S. (2006). Measuring body composition. *Arch. Dis. Child,* **91,** 612-617.

192. Whitaker,R.C., Wright,J.A., Pepe,M.S., Seidel,K.D., and Dietz,W.H. (1997). Predicting obesity in young adulthood from childhood and parental obesity. *N. Engl. J. Med.,* **337,** 869-873.

193. Widen,E., Lehto,M., Kanninen,T., Walston,J., Shuldiner,A.R., and Groop,L.C. (1995). Association of a polymorphism in the beta 3-adrenergic-receptor gene with features of the insulin resistance syndrome in Finns. *N. Engl. J. Med.,* **333,** 348-351.

194. Wright,H.M., Clish,C.B., Mikami,T., Hauser,S., Yanagi,K., Hiramatsu,R., Serhan,C.N., and Spiegelman,B.M. (2000). A synthetic antagonist for the peroxisome proliferator-activated receptor gamma inhibits adipocyte differentiation. *J. Biol. Chem.,* **275,** 1873-1877.

195. Yen,C.J., Beamer,B.A., Negri,C., Silver,K., Brown,K.A., Yarnall,D.P., Burns,D.K., Roth,J., and Shuldiner,A.R. (1997). Molecular scanning of the human peroxisome proliferator activated receptor gamma (hPPAR gamma) gene in diabetic Caucasians: identification of a Pro12Ala PPAR gamma 2 missense mutation. *Biochem. Biophys. Res. Commun.,* **241,** 270-274.

196. Zaccai,J.H. (2004). How to assess epidemiological studies. *Postgrad. Med. J.*, **80,** 140-147.

197. Zhu,X., McKenzie,C.A., Forrester,T., Nickerson,D.A., Broeckel,U., Schunkert,H., Doering,A., Jacob,H.J., Cooper,R.S., and Rieder,M.J. (2000). Localization of a small genomic region associated with elevated ACE. *Am. J. Hum. Genet.*, **67,** 1144-1153.

198. Zondervan,K.T. and Cardon,L.R. (2004). The complex interplay among factors that influence allelic association. *Nat. Rev. Genet.*, **5,** 89-100.