EFFECTS OF INTERACTION BETWEEN VARIANTS OF SELECTED CANDIDATE GENES AND LIFESTYLE ON HEALTH AND PERFORMANCE-RELATED PHYSICAL FITNESS

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SUBMITTED FOR THE DEGREE OF
DOCTORATE OF PHILOSOPHY
IN THE FACULTY OF SCIENCE,
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PhD

APRIL 2005

In loving memory of my mother...

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 peer-reviewed journals as follows:

1. Colin N. Moran[1], Christos Vassilopoulos[1], Athanasios Tsiokanos[2], Athanasios Z. Jamurtas[2], Mark E.S. Bailey[1], Richard H. Wilson[1], Yannis P. Pitsiladis[1] (2005) Effects of interaction between ACE polymorphisms and lifestyle on obesity-related phenotypes in adolescent Greeks. Obes Res (submitted).

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

1. Tsiokanos A, E Georgiades, P Tsimeas, C Vassilopoulos, G Rennie, RH Wilson, YP Pitsiladis. Body composition and aerobic capacity of Greek school-aged children. European College of Sports Science meeting, Athens, July 2002.

2. Vassilopoulos C, RH Wilson, W Goodwin, A Tsiokanos, AZ Jamurtas, YP Pitsiladis. Maximal oxygen uptake is associated with ACE genotype in school-aged boys. European College of Sports Science meeting, Athens, July 2002.

3. Wilson RH, C Vassilopoulos, W Goodwin, GP Rontoyiannis, A Tsiokanos, AZ Jamurtas, YP Pitsiladis. International Conference on Genetic Variation and Physical Activity. The IUNS Committee on Genetics, Nutrition and Chronic

Diseases and The Directors of the Centres on Genetics, Nutrition, Exercise and Health. Cagliari, Italy, May 2003.

4. Vassilopoulos C, E Georgiades, A Tsiokanos, AZ Jamurtas, P Tsimeas, RH Wilson, YP Pitsiladis. The ACE I/D polymorphism is associated with strength related performance in school-aged girls. VIIth IOC Olympic World Congress on Sport Sciences. "Physical, Nutritional and Psychological care of the Athlete in the 21st century", Athens, October, 2003.

5. Wilson RH, CN Moran, C Vassilopoulos, A Tsiokanos, A Jamurtas, YP Pitsiladis. Components of Exercise-Related Phenotypes and their Responses to Genetic and Environmental Variation. American College of Sports Medicine, Annual Meeting, Indianapolis, Indiana, USA, June, 2004.

6. Lagou V, Y Manios, E Grammatikaki, E Oikonomou, E Ioannou, G Moschonis, G Dedoussis, C Vassilopoulos, MES Bailey, RH Wilson, CN Moran, YP Pitsiladis. Impact of ACE polymorphisms on obesity-related phenotypes in Greek children aged 0-6 years. 14th European Congress on Obesity, Athens, Greece, June, 2005.

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Acknowledgements

I would like to thank first and foremost Dr. Y.P. Pitsiladis for his faith and unyielding commitment to see me successfully through my studies despite all the difficulties, personal and academic. It has certainly been a long journey. I will always consider it a privilege to have had as my supervisor a man of his courage, ethos and integrity.

I thank Dr. R.H. Wilson for the many stimulating discussions and ceaseless advice.

I thank Dr. Colin Moran for his expert advice, his excellent technical assistance and his friendship.

I thank Professor A. Tsiokanos and Mr. P. Tsimeas for making this study possible and their catalytic intervention at critical moments.

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

I thank the members of the Academic staff of the Division of Neuroscience & Biomedical Systems and the Graduate School for their understanding of my situation and their continuing support through a most difficult period for me and my family.

I thank all my colleagues from the Anderson College 5th floor for having me in their facilities and their technical assistance.

I thank my parents for their love, courage and patience during all the years of my studies which have kept me away from our family. Any man should feel blessed to be given so much and so freely with so little asked in return.

I thank Despoina for her love, support and encouragement throughout the many difficult moments I experienced during the course of this PhD.

I thank my closest friends, Fivo, Giorgo, Lysandro, Vangeli, and Yanni, for their support and for being there in my hour of need.

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 primary objective of these experiments was to investigate the impact of the interaction between variants of selected candidate genes, gender and lifestyle on health and physical fitness-related phenotypes in a large, representative population of adolescent children from Greece using both genotype and haplotype approaches. 1198 subjects, all at school in Greece, filled out questionnaires giving details of age and amount of organised physical activity regularly undertaken. Subjects provided a buccal swab that was used to extract DNA while measurements were taken to describe a series of physiological, physical and skill-related phenotypes.

The aim of the first experimental investigation (Chapter 3) was to assess the general health and physical fitness of the present population of adolescent children from Greece. This was achieved by comparing the prevalence of overweight and obesity in the present cohort with findings from the published literature. The physiological, physical and skill-related physical fitness of Greek adolescents was further assessed relative to normative data from Australian children of similar age. In the present population of Greek adolescents overweight and obesity rates were high with 23.6% of children being overweight and 6.9% being obese. Relative to previous findings for overweight rates, the participants of the present cohort ranked second highest among 12 countries, significantly including Australia. Compared to normative data from Australian adolescents, Greek children, irrespective of gender, had a higher mean body mass, and were, on average, outperformed in upper-body strength, agility and skill-related trials. In addition, the aerobic fitness of Greek adolescents was lower than Australian and European norms.

The aim of the second experimental investigation (Chapter 4) was to assess the potential influence of individual genotypes and haplotypes of the ACE gene, when controlled for lifestyle and gender differences, on obesity-related phenotypes in the cohort of Greek adolescents. Significant, albeit modest, associations were observed between several of the ACE polymorphisms and the obesity-related phenotypes in total and inactive females. The ACE I-allele, in an additive fashion, was associated with lower obesity-related scores. In contrast, the ACE D-allele was, on average, associated with higher scores for the obesity-related measures.

The third experimental investigation (Chapter 5) aimed to assess potential associations between variants of the ACE gene and physical, physiological and skill parameters in Greek adolescents. In young females, polymorphic variants within the ACE gene were significantly associated with several of the measured phenotypes. In particular, the ACE I-allele, and the corresponding H6 haplotype were associated with improved physical, physiological and skill parameter scores. The observed associations of ACE variants with physical, physiological and skill-related phenotypes were dependant on gender and participation in physical activity. These findings were consistent with a broader, albeit modest, influence of the ACE gene on human physical performance than previously described.

The aim of the fourth experimental investigation (Chapter 6) was to assess the influence of polymorphic variants in the ADRB genes on obesity-related phenotypes in the Greek adolescents. Variants of the ADRB2 gene associated with subtle influences on obesity-related phenotypes in young males when controlled for activity status. In particular, the ADRB2 Gly16 allele and corresponding Gly16Glu27/Gly16Glu27 and Gly16Gln27

/Gly16Glu27 diplotypes associated with increased adiposity. In contrast, the ADRB2 Arg16 allele and corresponding Arg16Gln27/Gly16Glu27 and Arg16Gln27/Arg16Gln27 diplotypes, associated with low and moderate adiposity. The data for young males were consistent with a previously unreported, protective effect of allelic combinations of the Arg16Gln27 haplotype against the obese phenotype that was dominantly mediated by the Arg16 variant.

The fifth and final experimental investigation (Chapter 7) aimed to assess the impact of variants in the ADRB genes on physical, physiological and skill-related parameters in the Greek adolescents. In young males, polymorphisms within the ADRB1 and ADRB2 genes associated with physiological, physical and skill-related phenotypes. These effects were modest and influenced by activity status. The ADRB1 Ser49 and the Gly389 alleles as well as the corresponding Ser49Gly389 haplotype associated with enhanced performance-related parameter scores. For the ADRB2 gene, the Gly16 allele and the corresponding Gly16Gln27 haplotype were associated with enhanced hand-grip strength. The findings for the ADRB2 Gly16 allele suggested pleiotropic genetic effects of the Gly16Arg variant mediating receptor function in young males.

The present population, considered to be representative of Greek adolescents, is the sole cohort of its type in Greece that includes such a wide variety of health and performance-related physical fitness parameters. Relative to the Australian normative data, Greek adolescents had a higher mean body mass and lower than average aerobic fitness that, most likely, accounted for their lower performance-related physical fitness. The results of the genetic analyses suggested that polymorphisms in the ACE, ADRB1 and ADRB2 genes influenced obesity and performance-related phenotypes in a gender-specific manner. They

also indicated that these effects could be altered by participation in organised physical activity. As such, these findings suggested that genes simply predispose individuals to particular traits in a manner that can be overridden by lifestyle choices. Haplotype-based methods offer greater analytic ability relative to individual SNPs but their analytical power is dependant on sample size and the strength of the genetic effect under investigation. Thorough discussion of experimental results based on their strength, consistency and biological plausibility in addition to adjustments for multiple testing, where these are practical, may be the best approach to avoid reporting of biased results.

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ACE	Angiotensin Converting Enzyme
ADRA2	α_2-Adrenergic Receptor
ADRB	β-Adrenergic Receptor
ADBR1	β_1-Adrenergic Receptor
ADBR2	β_2-Adrenergic Receptor
ADRB3	β_3-Adrenergic Receptor
ApoE	Apolipoprotein E gene
ANOVA	one way Analysis Of Variance
BMI	Body Mass Index
bp	base pair(s)
cAMP	cyclic-Adenosine Monophosphate
CI	Confidence Interval
CKMM	muscle-specific Creatine Kinase M chain
CLD	Chronic Lung Disease
cm	centimetre(s)
CVD	Cardio-Vascular Disease
DNA	Deoxyribonucleic Acid
FWER	Family-Wise Error Rate
GPCR	G-protein coupled receptor
HWE	Hardy-Weinberg Equilibrium
IOTF	International Obesity Task Force
G_I	adenylyl cyclase inhibitory G protein
G_S	adenylyl cyclase stimulatory G protein
kg	kilogram(s)
LD	Linkage Disequilibrium
LEPR	Leptin Receptor
log10	base10 logarithm
LPL	Lipoprotein Lipase
MAP	Mitogen Activated Protein

µl	microlitre(s)
min	minute(s)
ml	millilitre(s)
mlkg-1min-1	millilitre per kilogram per minute
mRNA	messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
OR	Odds Ratio
P	Probability
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
pmol	picomole(s)
QTL	Quantitative Trait Locus (or Loci)
RAS	Renin-Angiotensin System
RFLP	Restriction Fragment Length Polymorphism
r^2	Disequilibrium coefficient
rs	reference SNP identification number
s	second(s)
SD	Standard Deviation
SISA	Simple Interactive Statistical Analysis website
SNP	Single Nucleotide Polymorphism
U	Unit of enzymic activity
UTR	Untranslated Region
V_A	additive genetic variance
V_D	dominant genetic variance
V_E	environmental variance
V_G	genetic variance
V_{GE}	gene-environment interaction variance
V_I	interaction genetic variance
$\dot{V}o_{2\ max}$	maximal oxygen uptake
V_P	total phenotypic variance

CHAPTER 1

GENERAL INTRODUCTION

1.1 Health and physical fitness

It is well established that daily physical activity and participation in sport are important in defining daily energy expenditure (Ravussin and Bogardus 1989),(Bouchard and Tremblay 1997). Currently, sedentary lifestyles are common in developed societies (James 1995),(Jeffery and Utter 2003); physical activity levels during childhood are low (Yamada et al. 1999) and decline further in adolescent years (Aaron et al. 2002). In addition, modern eating habits favour an over-consumption of high energy-density products such as fast-foods that, in addition to a high saturated-fat content, tend to have low levels of important, for health, dietary components such as fiber and antioxidants (Slavin et al. 1999),(French et al. 2001). The health consequences of such behaviours early in life are now clearly emerging (Hill et al. 2003). Recent reports indicate an explosive increase in the prevalence of overweight and obesity in developed and developing countries (for a review (Ebbeling et al. 2002)) (for example table 3.6). As indicated by epidemiological investigations, the increase in overweight and obesity rates is detectable in both adult and adolescent populations (Chinn and Rona 2001),(Wang et al. 2002) and people of all socioeconomic status (James et al. 1997). In developed countries such as the USA, Britain and Australia the prevalence of childhood obesity has increased 2.8-fold over twenty-five years in the USA (Ebbeling et al. 2002), 2.4-fold over ten years in Britain (Chinn and Rona 2001) and between 2 and 4-fold over forty years in Australia (Magarey et al. 2001). Similar observations have been made in developing countries; obesity rates have increased 3.9-fold in Egypt over nineteen years, 3.5-fold in Brazil over twenty-three years (de Onis and Blossner 2000) and 1.1 to 1.4-fold in China over a period of six years (Wang et al. 2002).

A consistent positive correlation between child and adult overweight and obesity has been seen (Bouchard 1995),(Bouchard 1997). Overweight and obese children are more likely to

become overweight and obese adults and to experience chronic health problems associated with adult obesity (Borecki et al. 1991),(Moll et al. 1991),(Goodman et al. 2002). A study in monozygotic and dizygotic pairs of twins by (Selby et al. 1989) has reported heritability estimates of 70% for BMI and 73% for total skinfolds. On average, family and twin studies suggest that body fat and body fat distribution have heritability estimates ranging from 10% to 80% (for reviews (Bouchard and Perusse 1988),(Bouchard 1997)). These findings suggest that obesity has a strong genetic component (for reviews (Bouchard and Perusse 1988),(Bouchard 1997)) that is likely to implicate synergistic interactions between polymorphic variants in a number of minor genes involved in the process of adipogenesis and adipose tissue metabolism (Arner 2001). Nevertheless, overweight and obesity rates have risen in a relatively constant gene pool hence against a constant metabolic background (Prentice and Jebb 1995). Considering changes in the dietary intake in Britain over the last 50 years would indicate that the proportion of dietary energy originating from fat has increased by 50% (Prentice and Jebb 1995). Numerous dietary studies have previously suggested a link between body fatness and the increased consumption of fat (for review (Lissner and Heitmann 1995)) although such a relation has been questioned by an inability to consistently associate dietary fat and adiposity in children (Atkin and Davies 2000) and adults (Ludwig et al. 1999). On the other hand, data from British adults over a forty year period suggest that the increase of physical inactivity, as indicated by proxy measures like the number of cars per household and hours of television viewing per week, matched more closely increases in the rate of obesity than energy and fat intake (Prentice and Jebb 1995) (figure 1.1). Furthermore, as studies have indicated (Meirhaeghe et al. 1999), the contribution of genetic determinants in obesity may change in response to lifestyle modifications such as when individuals partake physical activity (figure 1.2). Taken together, these findings would suggest that obesity is caused by the synergistic

interaction of underlying genetic susceptibility with environmental factors such as poor diet and physical inactivity (Prentice and Jebb 2003) and that the obese phenotype is progressively affected more and more by environmental and behavioural changes (Ebbeling et al. 2002). However, as longitudinal twin studies have shown (Maes et al. 1997), genetics play a larger role than behaviour or environment in determining relative body mass and adiposity of younger age groups as the environment has had less time to take effect; hence, youngsters may be a better subject population, relative to adults, to investigate genetic influences on obesity despite growth and sexual maturation that occur naturally at this age.

The rise of the obesity epidemic has been accompanied by a worldwide reduction in physical fitness (Tomkinson et al. 2003b),(Tomkinson et al. 2003a). Body fat is inversely associated with $\dot{V}o_{2\,max}$ (Johnson et al. 2000),(Janz et al. 2002) and most performance-related physical fitness phenotypes such as running speed and vertical jump (for example (Minck et al. 2000)); consequently, the increases in adiposity associated with obesity will have a negative impact on $\dot{V}o_{2\,max}$ and physical fitness. Obesity causes a clustering of cardiovascular disease factors known as the insulin resistance syndrome that has been identified in children as young as 5 years of age (Young-Hyman et al. 2001). In a British cohort (Gunnell et al. 1998), children that were overweight had a two-fold higher risk of death from ischemic heart disease in adulthood over 57 years. The paediatric obesity rise is also partly responsible for the sharp increase of type-II diabetes (Ludwig and Ebbeling 2001) while glucose intolerance and insulin resistance are highly prevalent amongst obese children independent of ethnic group (Sinha et al. 2002). Obesity in children is also associated with pulmonary complications such as asthma (Figueroa-Munoz et al. 2001) while other serious systemic and psychosocial consequences have also been recognised.

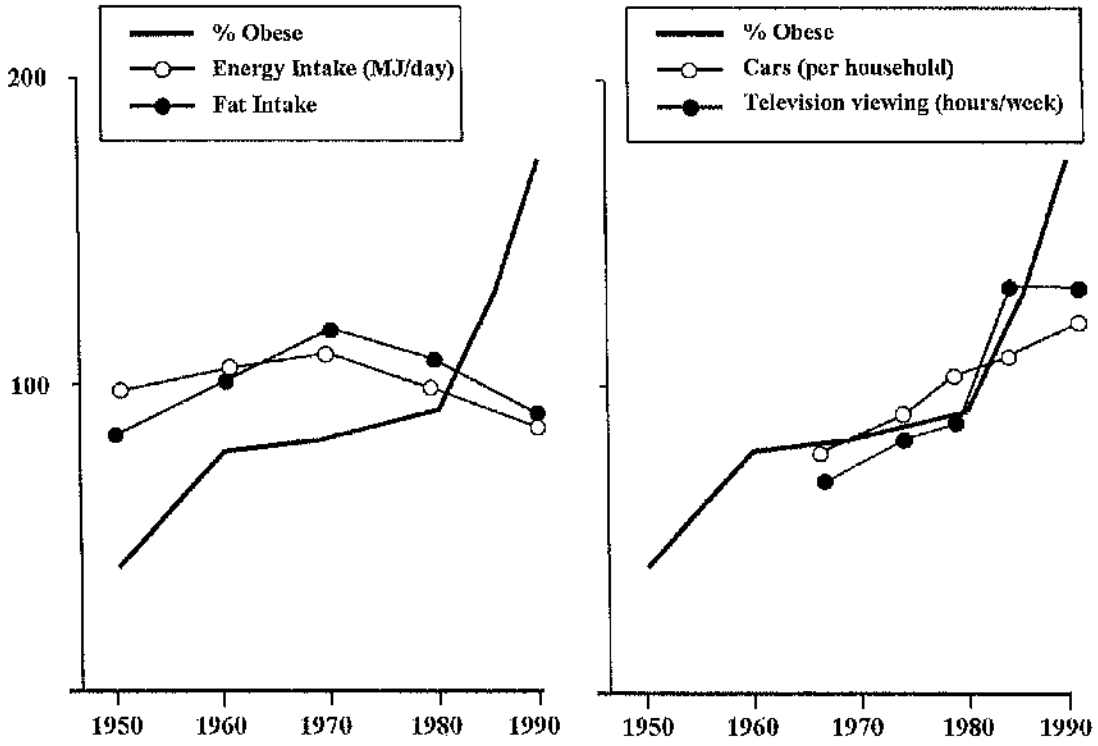


Figure 1.1: Changes in the % of obese adult individuals from Britain over a 40-year period relative to changes in energy and fat intake (left) and proxy measurements of physical inactivity (right). Data indicate a closer tracking of changes in % of obese individuals with increases of car ownership and television viewing relative to changes in energy and fat intake. These findings are suggestive of a stronger influence of physical inactivity in the manifestation of obesity relative to dietary intake. **(from Prentice and Jebb 1995)**

For example, in a study of adolescent patients (Adelman et al. 2001) indicated that severe obesity was associated with proteinuria and abnormal renal histological features such as glomerular hypertrophy while (Strauss 2000), in a study of 1520 children aged 9-10, indicated that obesity associated with significantly lower levels of self-esteem.

Like obesity, performance-related physical fitness is a multi-factorial trait that, as evidence from family and twin studies have indicated, has a strong genetic component (for review (Rankinen et al. 2004)). Early work in monozygotic and dizygotic twins by (Klissouras 1971) has suggested heritability estimates for $\dot{V}o_{2\,max}$ as high as 93.4%; however, as later work indicated (for example (Bouchard et al. 1986)), such high heritability estimates were strongly influenced by environmental factors. Considering age, gender and body mass-adjusted results from twin and familial studies (for examples (Bouchard et al. 1986),(Bouchard et al. 1998)) would indicate that $\dot{V}o_{2\,max}$ has a significant genetic component with heritability estimates ranging from 40%-50%. In addition, genetic predisposition affects $\dot{V}o_{2\,max}$ in both the untrained and the post-training states (Bouchard et al. 1999). Twin studies (Lauderdale et al. 1997),(Thomis et al. 1998) have suggested that human body morphology and composition as well as training responses may be heavily influenced by genetic factors. (Maes et al. 1996), reported that over three quarters of the variance in explosive strength in pre-pubertal females may be explained by genetic parameters while (Thomis et al. 1998) suggested that 20% of the variation in post-training isometric strength gains was explained by training-specific genetic factors. In a large number of twin pairs and their parents, Maes et al 1996 attempted to quantify genetic and environmental sources of variation in physical fitness components; the findings from this study indicated that genetic factors had a dominant role, relative to behaviour or environment, in determining fitness of younger age groups. These findings suggest that,

like for obesity, younger individuals may be a better subject population, relative to adults, to study the genetic influences on performance-related physical fitness parameters.

1.2 Variation in multi-factorial traits

Variation of multifactorial traits such as obesity and performance-related physical fitness can be described in terms of phenotypic and genetic heterogeneity that is measured by the total phenotypic variance, V_P. As seen in figure 1.3, V_P is the sum of inter-individual genetic differences V_G, non-genetic sources of variation V_E and gene-environment interactions V_{GE} (Russel et al. 1994). Considering obesity as an example, V_G would reflect the number of genetic variants thought to influence the obese phenotype (Arner 2001), V_E would represent variation from sources such as diet and levels of physical activity (Prentice and Jebb 2003) and V_{GE} would account for the interaction between V_G and V_E such as the impact of lifestyle modifications in the effect of genotype (Meirhaeghe et al. 1999). V_G may be further subdivided by the presence of additive, dominance and epistatic genetic effects on phenotype, giving rise to V_A, V_D and V_I genetic variance, respectively (figure 1.2) (Russel et al. 1994). V_G is generated through the high degree of polymorphic variation spaced throughout the coding and non-coding regions of DNA sequences (Halushka et al. 1999),(Stephens et al. 2001) while gene diversity is dependent upon the population considered; the polymorphic variability of the transcribed sequences is greater in populations of African origin relative to Europeans (Cargill et al. 1999),(Halushka et al. 1999),(Stephens et al. 2001) or Asians (Ohnishi et al. 2000). Considering the ACE gene as an example, a study of the sequence variation by (Rieder et al. 1999) has reported a higher sequence diversity in individuals of African-American descent in all coding and non-coding regions relative to European-Americans. Nevertheless, it must be noted that, in European populations at least, the polymorphic variability of transcribed sequences does

not differ significantly between genes according to their chromosomal location (Tiret et al. 2002).

Sequence heterogeneity and trait variation may be generated by the occurrence of single-base differences called SNPs. In contrast to mutations that are rare differences occurring in less than 1% of the population, SNPs have been defined as the least common allele occurring in 1% or greater of the population (Marez et al. 1997). They are present throughout the human genome with an average frequency of approximately 1 per 1,000-2,600bp pending on the population origin (Brookes 1999),(Tiret et al. 2002). SNPs, although frequently not having functional relevance (Brookes 1999), can result in drastic alterations in protein function through changes in amino acid and regulatory elements sequence or splicing. For example, (Clement et al. 1998) in their work on three morbidly obese sisters have described a G to A transition at the +1 position of intron 16 of the LEPR that resulted in a truncated LEPR lacking both the transmembrane and the intracellular domains and resulted in early-onset morbid obesity. SNP-derived sequence diversity may be best described in terms of multilocus variation or else haplotypes (Akey et al. 2001) that are defined as sets of genetic variation at multiple loci that tend to be co-inherited and are largely unaffected by recombination events. In a study of the ADRB2 gene SNP variation, (Drysdale et al. 2000) described 13 SNPs; however out of the possible 8,192 SNP combinations only 12 haplotypes were identified. These findings suggest that although the number of theoretically possible haplotypes depends on the number of polymorphic loci and variants at each locus, natural selection differentially restricts the frequency of observable haplotypes in genes (Drysdale et al. 2000) and populations (Moriarty et al. 1997).

In human populations, non-random patterns of SNP co-inheritance between different loci are the result of LD (Goldstein 2001),(Tiret et al. 2002). Unlike linkage that measures co-segregation at a single locus, LD measures co-segregation of SNPs in a population at a higher frequency than would be predicted by random chance alone. LD resulted from much earlier, ancestral recombination events and has been perpetuated by demographic events such as natural selection and genetic drift (Wilson and Goldstein 2000),(Reich et al. 2001). Co-inheritance of SNPs due to LD must have persisted through multiple generations to become a property of the population (Ardlie et al. 2002). Although clustered in specific genomic regions appreciable LD may occur over considerable genomic distances (Zavattari et al. 2000),(Service et al. 2001),(Abecasis et al. 2001). Fine-mapping studies of trait loci in the ACE gene performed in a large European family cohort by (Soubrier et al. 2002) have suggested the presence of very strong LD; in contrast, findings from another study of the ACE polymorphic variation in Nigerian families by (Cox et al. 2002) indicated that in people of African descent the levels of LD in the ACE gene were much lower. These findings suggest that the extent of LD is not universally the same but differs across populations (for reviews (Reich et al. 2001),(Service et al. 2001),(Kehoe et al. 2003)).

The varying patterns of LD between populations may be particularly important in investigations of gene-environment interactions as they may help to distinguish the nature of association between marker loci and phenotypic traits and between marker loci themselves (Goldstein 2001),(McKenzie et al. 2001). As (Drysdale et al. 2000) have indicated for the ADRB2 gene, close pairs of SNPs may have lower levels of LD relative to more distant SNPs; Tiret et al. 2002 (Tiret et al. 2002) in an investigation of the characteristics of LD in 50 candidate genes have confirmed the findings by (Drysdale et al. 2000). Therefore, the variation of LD with distance between sites (Eaves et al. 2000) is highly important as it could affect the design and interpretation of the results from studies

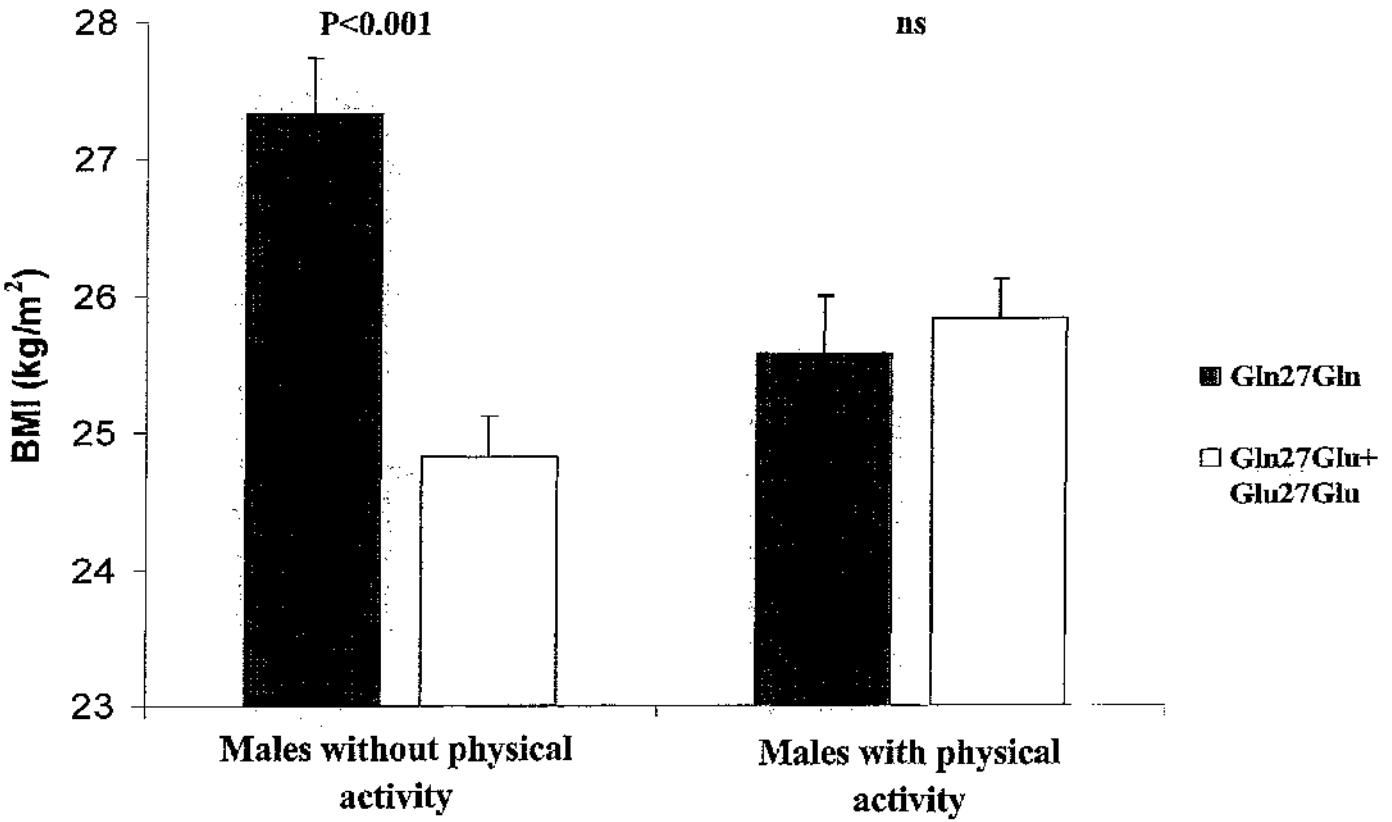


Figure 1.2: Changes in the influence of genotype on phenotype in response to physical activity participation. Homozygous ADRB2 Gln27Gln adult males that do not partake in physical activity have significantly higher mean BMI (P<0.001) when compared to their ADRB2 Gln27Glu and ADRB2 Glu27Glu counterparts. In contrast, ADRB2 Gln27Gln homozygotes that partake in physical activity have no significant difference in mean BMI when compared to their ADRB2 Gln27Glu and ADRB2 Glu27Glu counterparts. **(from Meirhaeghe et al. 1999)**

attempting to analyse the relationship of polymorphic variants and multi-factorial phenotypic traits (Goldstein 2001),(Stephens et al. 2001). Nevertheless, pending on sequence variation, LD may provide evidence of association as well as the proximity of marker loci and causal variants (Moriarty et al. 1997),(Clark et al. 1998),(Goldstein 2001). Gene localisation can also significantly influence the variation of LD (Stephens et al. 2001),(Tiret et al. 2002); for example, LD has been reported to be stronger in GC-poor sequences due to a lower recombination frequency in these regions (Eisenbarth et al. 2001).

1.3 Methods for the genetic analysis of multi-factorial traits

As already described, a large number of twin (for example (Maes et al. 1996)), familial (for example (Bouchard et al. 1998)), commingling (for example (Borecki et al. 1991)) and segregation analysis studies (for example (Rice et al. 1994), collectively known as unmeasured genotype approaches, have been performed to measure the heritability of obesity and performance-related physical fitness phenotypes (figure 1.4). Nevertheless, identifying and locating specific genetic variables that influence complex traits involves methods based, primarily, on measured genotype approaches; currently the most commonly used measured genotype approaches are linkage and association studies (Risch 2000) (figure 1.4). Linkage analysis attempts to calculate the likelihood of obtaining the parent-to-offspring transmission patterns between a trait and a marker locus taking advantage of the linkage between genetic loci in close proximity (for a review (Risch 2001)). The most widely-used linkage analysis method is the sib-pair method (Haseman and Elston 1972). In a linkage analysis of 277 full sib-pairs, (Rivera et al. 1999), based on the principal that in linkage the squared sib-paired trait difference decreases with an increase in the alleles shared identically by the sib-pair at the marker locus, have indicated that a CKMM-NcoI

RLFP in the 3' UTR of the gene contributes to individual differences in $\dot{V}o_{2\,max}$ responses to endurance training. As demonstrated by a low false-positive rate history, linkage analysis is especially robust when applied to Mendelian traits (Rao et al. 1978). However, when genes do not have singly large effects or strong genotype-phenotype correlations it is the general consensus that linkage analysis may be less likely to detect susceptibility genes (Risch 2001). Nevertheless, linkage studies can be very successful in identifying specific genetic variables that influence complex traits in the presence of very strong or complete LD between traits and genetic markers (Risch 2001); as (Rivera et al. 1999) have suggested, an alternative explanation of their findings would involve the presence of another variant in close LD with the CKMM gene. The reason for such results is that in LD, the association between alleles at linked loci is preserved at the population level (Ardlie et al. 2002) unlike linkage where, under random mating, repeated recombination events will cause the trait and the marker loci to segregate independently.

Considering genes with modest influences on phenotype, like most genes influencing complex traits, and if the candidate variant is at hand (candidate gene), detection of gene effects by direct association has greater power than linkage analysis (Risch 2000). Generally, association studies are either family-based or population-based. Family-based association studies are best suited for studies of very rare conditions (Spielman and Ewens 1996). Population-based association studies can be either case-control or cohort-based designs; case-control studies investigate relatively rare conditions but not so rare that only family-based designs could realistically assess potential associations (for a review (Zondervan and Cardon 2004)). Cohort-based association studies, at large, attempt to associate polymorphic variants in candidate genes with influences on the phenotype under investigation (Pritchard and Przeworski 2001). Despite their greater demands on sample

size (Clayton and McKeigue 2001), unlike case-control designs, cohort-based studies can provide information for both the relative and absolute risk (penetrance) of a trait in a population (Clayton and McKeigue 2001).

With respect to the genetics of obesity and performance-related physical fitness, a significant portion of the studies published in the literature are cohort-based association studies (for reviews (Rankinen et al. 2004) (Snyder et al. 2004)). Many investigators have remarked on the, as yet, limited success of association studies to dissect the genetic basis of complex traits (Weiss and Terwilliger 2000). For example, the ACE I/D polymorphism has been previously suggested to be a potent risk factor for myocardial infarction (Cambien et al. 1992) a finding later shown to have been subjected to publication bias (Keavney et al. 2000). However, the application of association studies in the investigation of multi-factorial traits has led to the conclusive identification of genetic risk factors in complex disorders such as type-II diabetes (Altshuler et al. 2000), breast cancer (Meijers-Heijboer et al. 2000) and Crohn's disease (Hugot et al. 2001). Hence, despite certain restrictions, association studies are in fact a practical and powerful approach for the investigation of multi-factorial phenotypes (Salo et al. 1995),(Cardon and Palmer 2003). Nevertheless, the shortcomings of many investigations (Weiss and Terwilliger 2000) indicate that gene association studies have limitations (Clayton and McKeigue 2001),(Pritchard and Przeworski 2001) that must carefully considered.

1.4 Gene association studies: problems and limitations

Based on large samples of unrelated individuals, association studies attempt to directly link population traits with alleles at marker loci by taking advantage of the LD in the human genome (for example (Cox et al. 2002),(Tiret et al. 2002)). Variations of LD with distance

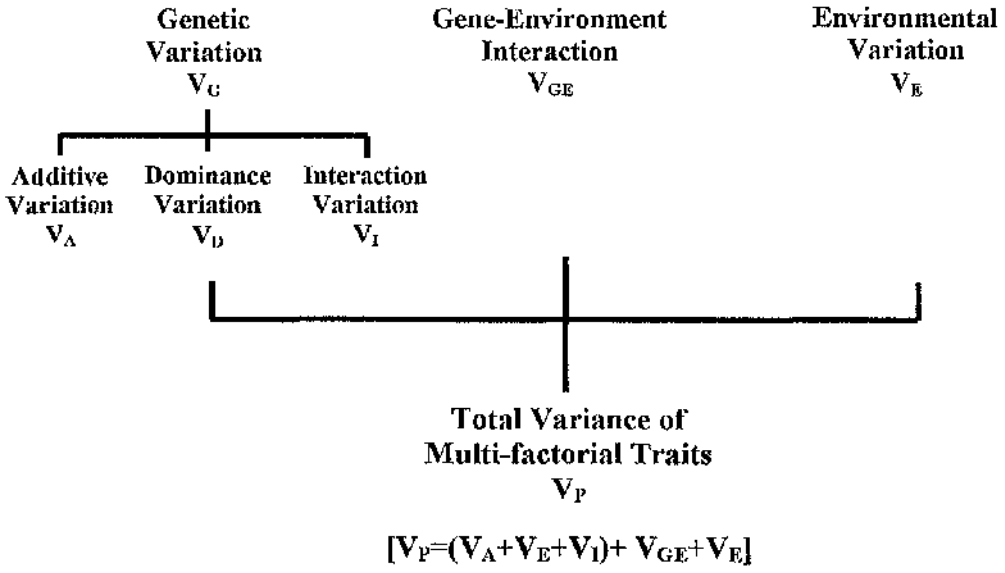


Figure 1.3: The total phenotypic variance in multi-factorial traits V_P is composed by differences arising from genetic variation (V_G), environmental variation (V_E) and genetic-environmental variation (V_{GE}). The genetic variance can be further sub-divided into components arising from different types of interaction between genes; additive effects generate additive variation (V_A), dominant effects generate dominance variance (V_D) while epistatic effects add interaction variance (V_I).

(Eaves et al. 2000) and population origin (Reich et al. 2001),(Kehoe et al. 2003) can significantly affect the design and the interpretation of the results of association studies. Furthermore, the power of association studies to detect effects is, by design, proportional to the levels of LD present in the genomic area under investigation; decreases in LD would reduce the power of an association study to identify functional associations. Diminishing LD also has an impact on sample size in order for studies to maintain sufficient investigative power, the relationship being approximately $1/r^2$; in cases where LD is very low, association studies may have sample requirements that are unrealistically high (Pritchard and Przeworski 2001). In a study of the functional relevance of ADRB2 gene haplotypes in asthmatics, (Drysdale et al. 2000) indicated that the mean bronchodilator responses to β-agonists significantly associated with the haplotype pair (diplotype) considered but not with individual variants suggesting that haplotypes have a greater analytical ability when compared to individual SNPs. Consequently, the aforementioned findings have pointed out the critical need for an understanding of the LD in the genomic area and the population under investigation and the use of haplotypes to maximise the genetic variation tested for association with a given trait (Akey et al. 2001),(Weiss and Clark 2002),(Kehoe et al. 2003). Failing to acknowledge these issues may serve to explain, in part, the shortcomings of previous studies.

Localisation of genetic determinants by association studies crucially depends on the adequate modelling of the population trait(s) under investigation (for a review (Strauch et al. 2003)). The classification of human phenotypic variance is troublesome and dominantly influenced by the selection of the parameters used to quantify phenotypes (for reviews (Weiss and Terwilliger 2000),(Strauch et al. 2003)). Accurately measuring certain phenotypes such as $\dot{V}o_{2\,max}$ (as described in (Bouchard et al. 1998)) requires the tightly

controlled environment of a laboratory and complex equipment impractical to use in the field with the large numbers of subjects required for such studies. To maximise the amount of phenotypic information obtained and counter these problems association studies resort to batteries of simpler, descriptive tests (for example (Australian Sports Commission 1993)); the combination of large numbers of tests with large numbers of subjects is considered to adequately summarise phenotypic variance. Nevertheless, this approach has weaknesses that can significantly alter the outcome of a study (Weiss and Terwilliger 2000),(Cardon and Bell 2001). Descriptive measurements are likely to be representative of variance attributed to external factors as well as that of the phenotype they assess and are further subjected to correlations between the measured parameters themselves; for example, distribution variations in arm span correlate strongly with variations in height (Cheng et al. 1998) while skeletal growth patterns are influenced by gender, age and environment (Gasser et al. 2000). These issues suggest that caution must be exercised when assessing similar traits.

Certain genes may differentially influence traits according to gender resulting in confounding or else selection bias; for example, the ADRB3 Trp64Arg variant has been associated with upper body obesity in obese men but not in women (Kawamura et al. 2001). One approach to counterbalance selection bias is to match the population under investigation for potential confounding factors such as gender and age. However, accounting for the presence of gender-specific influences of certain genetic determinants on phenotype is, in most cases, problematic and speculative. Considering the ACE gene, it has been previously shown that circulating ACE activity is responsive to oestrogen levels (Proudler et al. 1995). It is possible therefore that the circulating levels of hormones may modify or render the effects of certain genetic variants redundant in one of the two genders. In a cross-sectional study in an adult Spanish population, (Corella et al. 2001)

reported an association of the ADRB3 Arg64 allele with BMI in males that was absent in females; however, when the interaction with the HindIII-LPL polymorphism was taken into account the ADRB3 Arg64 allele was associated with higher BMI in females as well. These findings suggest that the complimentary actions of separate genes influencing the same trait on multi-factorial phenotypes could also help explain a number of gender-specific differences. From the genetics perspective, perhaps the most serious potential confounding factor is population stratification that can lead to biased results (Cardon and Palmer 2003). To counter problems of confounding by ethnicity (population stratification) population-based association studies focus on homogeneous and randomly mating populations (Cardon and Palmer 2003) although populations where only random mating occurs may be more of a theoretical ideal than reality (Risch 2001).

The large numbers of descriptive measurements considered by association studies result in multiple comparisons during statistical analysis (Sokal and Rohlf 2001). The liberal interpretation of multiple comparisons in the past was responsible, at least in part, for publication biased associations (Goodman 1998). In order to lower the probability of type I errors several techniques have been developed (Sokal and Rohlf 2001); examples include the Bonferroni, the Holm and the Šidak methods (Holm 1979),(Sokal and Rohlf 2001). Currently, despite the large number of available alternatives (Goodman 1998), (Bender and Lange 1999) there is no widely accepted correction for multiple testing that is routinely used in association studies. Although considered as good practice by some (Bender and Lange 1999),(Bland and Altman 1995) these methods have been severely criticised by others as very conservative and, as such, add to the problems associated with multiple testing (Perneger TV 1998),(Sabatti et al. 2003). Nevertheless, and irrespective of other

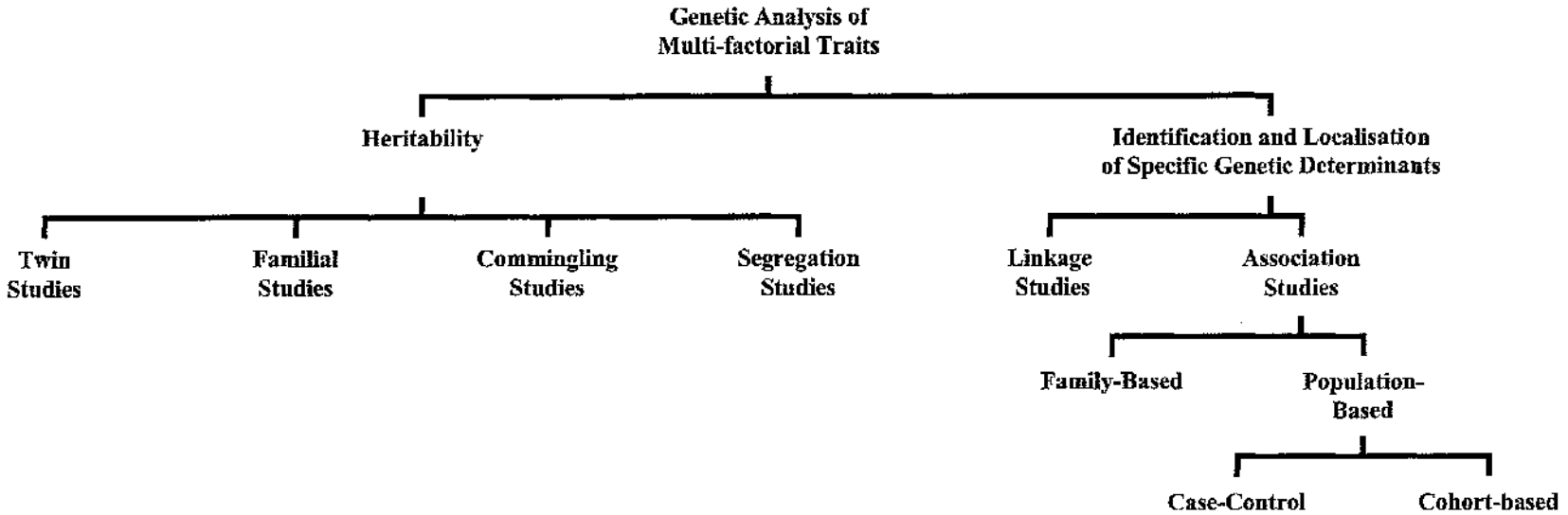


Figure 1.4: Tools for the genetic analysis of multi-factorial traits. Questions with regards to estimates of the heritability of multi-factorial traits may be best answered by unmeasured genotype approaches, significantly including twin, familial, commingling and segregation analysis studies. However the identification and localisation of specific genetic determinants influencing complex traits may be best addressed by measured genotype approaches that exploit linkage (linkage studies) or LD (association studies) to assess genetic influences on particular phenotypes.

considerations, it is the general consensus that the findings of prospective association studies must be carefully interpreted to avoid reporting of biased results.

1.5 Selecting candidate genes: the ACE gene and the ADRB gene family

Candidate genes for cohort-based association studies may be identified based on a number of approaches. A gene may be selected on the basis of its biological function and be subsequently tested for association with given phenotypes; however, this approach may be unsatisfactory as, presently, little is known of the complete functional spectrum of most gene products and even less about the detailed molecular biology of most multi-factorial traits (Gray et al. 2000). Alternatively, evidence from linkage studies may be used to narrow the genomic regions that harbour prospective candidate genes; an example of this strategy is the discovery of the association of the ApoE gene with late onset Alzheimer's disease after evidence of linkage between the disease phenotype with genomic region 19q13.2 that harbours the ApoE gene (Pericak-Vance et al. 1991),(Strittmatter et al. 1993). Nevertheless, this approach has limitations since, as previously indicated, linkage analysis is unlikely to detect small genetic effects i.e. effects from individual variants that account for less than 5-10% of the variance of a trait in the population (Uhl et al. 1997),(Risch 2001). As each of the aforementioned methods has its limitations, a more practical approach to evaluate the plausibility of candidate genes may rely on combining evidence from functional studies and linkage analyses as well as other sources such as previous case-control and cohort-based designs or model organisms (Zondervan and Cardon 2004). Based on the aforementioned approaches, a number of candidate genetic loci have been associated with subtle differences in obesity-related phenotypes and performance-related physical fitness; overall, more than 530 genes, markers, and chromosomal regions have been associated or linked with differences in human obesity and fitness phenotypes (for

reviews (Rankinen et al. 2004),(Snyder et al. 2004)) giving a measure of the genetic heterogeneity of these traits. Much of this research has concentrated on selected adult populations and, for fitness phenotypes in particular, on assessing the extent of association between elite athletic status and particular performance genes (for reviews (Rankinen et al. 2004),(Snyder et al. 2004)). Few studies have assessed genetic influences on obesity and fitness-related phenotypes in unselected populations; even fewer, if any, studies have investigated the genetics of obesity and performance-related physical fitness in younger populations despite evidence suggesting that genetic factors are more important than the environment or behaviour in determining health and fitness in younger age-groups (Maes et al. 1996),(Maes et al. 1997). At present, the extent to which obesity and fitness-related performance associate with particular genes is questioned (Weiss and Terwilliger 2000), due to a number of contrasting results produced by relevant studies; as discussed in the previous section, a number of reasons may explain these discrepancies. Among the most widely studied putative genes influencing complex human phenotypes are the ACE gene and the ADRB gene family.

1.5.1 The ACE gene

The ACE peptidase plays a key role in the endocrine Renin-Angiotensin System (RAS). ACE is responsible for the conversion of the inactive decapeptide Angiotensin I to the physiologically active octapeptide Angiotensin II; Angiotensin II is a potent vasopressor that mediates a variety of physiological responses (Crisan and Carr 2000) and the degradation of vasodilator kinins and other active oligopeptides (Skidgel and Erdos 2004) (figure 1.5). In humans, Angiotensin II effects are mediated predominantly through two specific receptors, namely AT1 and AT2 (Danser 2003). In addition to the endocrine RAS, local RAS systems have been described in several tissues in humans (Karlsson et al. 1998)

and animal models significantly including the adipose, the cardiac and skeletal muscle tissues (Engeli et al. 1999),(Schling et al. 1999). Although the biological processes involved have not been conclusively determined, previous studies have suggested that Angiotensin II (an inducer of cell proliferation) is involved, among others, in the growth, differentiation (Negrel et al. 1989) and metabolism of the adipose (Hennes et al. 1996),(Cooper et al. 1997),(Casselman et al. 2004), cardiac and skeletal muscle tissues (for reviews (Danser 2003),(Jones and Woods 2003)). These findings suggest that RAS has a significant paracrine/autocrine function (Lavoie and Sigmund 2003). Nevertheless, it is unknown whether all the components of RAS (rennin, angiotensinogen and ACE) are produced locally, get uptaken from the circulation through diffusion/local receptors (Danser 2003) or there is a combination of local de novo synthesis and uptake (Jones and Woods 2003). Current evidence suggest that, most likely, the production or uptake of RAS components may vary in a tissue specific manner (Jones and Woods 2003),(Lavoie and Sigmund 2003).

Many polymorphisms have been reported in the ACE gene: over 100 were listed in SNP databases at the date of writing. Historically work has centred on an insertion/deletion (I/D respectively) polymorphism found in the 16th intron of the gene, which is characterised by the presence (I-allele) or absence (D-allele) of a 287 base-pair (bp) *Alu* sequence (Rigat et al. 1990). In Caucasians, the I-allele is associated with low circulating ACE activity levels (Rigat et al. 1990),(Tiret et al. 1992) and the D-allele with high circulating ACE activity levels. Nevertheless, no similarly strong association is found between the I/D polymorphism and ACE activity in people of African origin (Bloem et al. 1996). The I allele has also been associated with lower levels of ACE expression in patients with

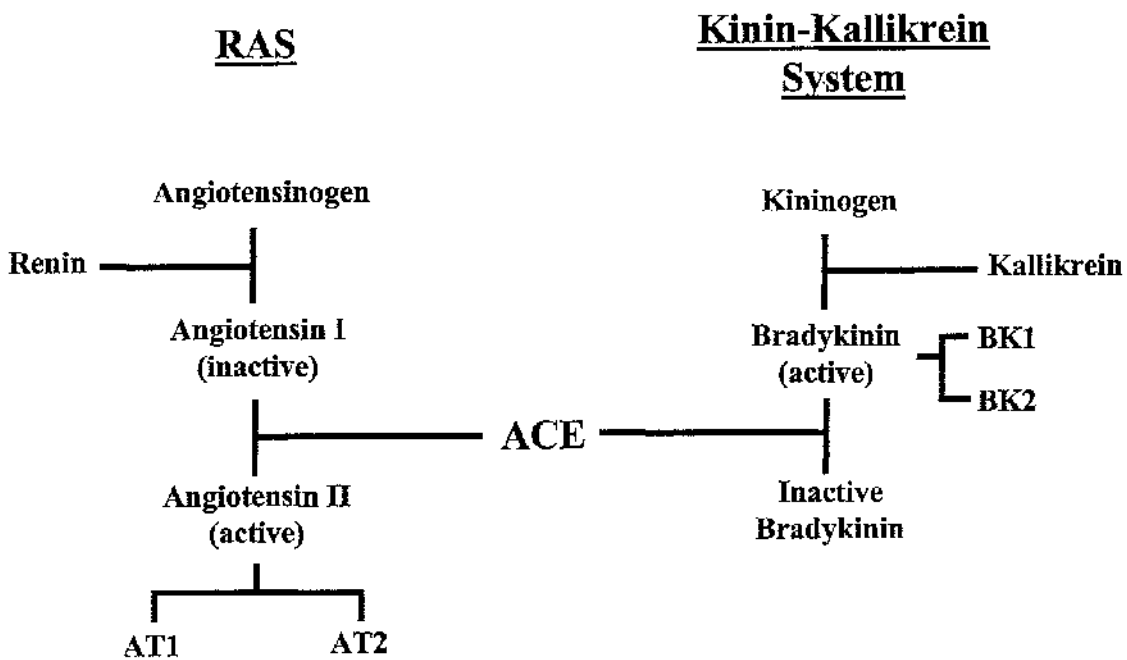


Figure 1.5: The role of the ACE peptidase in the RAS and Kinin-Kallikrein systems. **(adapted from (Crisan and Carr 2000))**

ischaemic heart disease (Davis et al. 2000) and healthy individuals (Mizuiri et al. 2001) and with increased endothelial cell survival (Hamdi and Castellon 2003). Some studies have suggested the presence of more than one QTL influencing circulating ACE activity levels (Villard et al. 1996),(Cox et al. 2002). Therefore, describing how multiple variants influence circulating ACE activity levels possibly involves identifying haplotypes and understanding how these combinations of variants work together and influence each other. (Rieder et al 1999) defined 13 ACE haplotypes (H1-H13) in 11 individuals, 6 European and 5 African-Americans. Only four haplotypes, H1, H6, H7 and H9, were found in the European-Americans; haplotype H6 is the only type of I-allele and haplotypes H1, H7 and H9 being different types of D-allele (Rieder et al. 1999). 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. Such a hypothesis would explain the weaker association between ACE activity levels and the I/D polymorphism in Africans (Cox et al. 2002) and would be highlighted by haplotype analysis.

Many studies have sought to associate the I/D polymorphism with disease phenotypes, such as myocardial infarction (Schelleman et al. 2004), Alzheimer's disease (Elkins et al. 2004) and renal disorders (Marre et al. 1994). The ACE I/D polymorphism has been associated with sporting phenotypes amongst elite athletes, and the response to structured exercise regimes in adults. The I–allele has been shown to be associated with elite endurance performance (Montgomery et al. 1998),(Myerson et al. 1999) with greater $\dot{V}o_{2\,max}$ in postmenopausal women (Hagberg et al. 1998) and with improved responses to physical training (Montgomery et al. 1998),(Montgomery et al. 1999),(Woods et al. 2001). Correspondingly, the D-allele has been associated with elite power-oriented performance

(Nazarov et al. 2001),(Woods et al. 2001) quadriceps strength in patients with chronic lung disease (Hopkinson et al. 2004) and with responses to physical training in male subjects (Folland et al. 2000). However, other studies have failed to demonstrate such associations (for examples (Rankinen et al. 2000),(Sonna et al. 2001)) a disparity perhaps partly due to the use of population samples of mixed age, sex and race (Woods et al. 2001),(Montgomery and Dhamrait 2002). A recent cross-sectional longitudinal study (Strazzulo et al. 2001) of Italian adult men reported a significant interaction of the ACE DD genotype with increased BMI, waist circumference and diastolic blood pressure in relation to age. Again, however, other studies have failed to consistently associate the ACE I/D polymorphism with BMI (for example (Cooper et al. 1997).

1.5.2 The ADRB gene family

Among the candidate genes for obesity and health and performance-related physical fitness are the three members of the ADRB family, namely the ADRB1, ADRB2 and ADRB 3 genes. All three genes encode for 7-transmembrane GPCRs that are differentially expressed throughout the body; ADRB1 is expressed mainly in cardiac muscle, ADRB2 is expressed in a variety of tissues significantly including the adipose tissue and ADRB3 is expressed mainly in the adipose tissue (for reviews (Small et al. 2003),(Leineweber et al. 2004),(Collins and Surwit 2001)). ADRBs are thought to play a central role in local tissue homeostasis, metabolism and function (Small et al. 2003); in the adipose tissue for example, ADRBs are involved in stimulating lipolysis and thermogenesis (Carpene et al. 1998),(Ravussin et al. 1999),(Bachman et al. 2002)) while in the heart the ADRB1 and ADRB2 receptors are primarily responsible for the manifestation of positive inotropic and chronotropic effects in response to catecholamines ((Bristow and Port 1998),(Brodde and Michel 1999). ADRBs mediate the activation by noradrenaline and adrenaline of adenylyl

cyclase, a key enzyme in intracellular signalling pathways; activation of adenylyl cyclase is achieved through the coupling of the receptor with the G_S (Collins et al. 2001) (figure 1.6). The signal transduction cascades of β-adrenergic receptors further involve the consequent activation activation of the cAMP and PKA pathways (Collins et al. 2001). Interestingly, the ADRB3 receptor is simultaneously coupled to both G_S and G_I (Soeder et al. 1999) resulting in the activation of both the PKA and MAP intracellular pathways allowing in this way a tighter regulation of certain transcriptional and metabolic responses. Although, in the adipose tissue at least, a degree of redundancy between the function of the receptors has been previously suggested (Rohlfs et al. 1995),(Susulic et al. 1995), the ADRBs maintain a level of non-overlapping function in tissues (Collins and Surwit 2001). Considering the adipose tissue in particular, previous investigations have indicated the presence of "cross-talk" between the ADRB1 and ADRB3 receptors (Susulic et al. 1995) (figure 1.6). SNPs have been described in all three ADRB genes that have been suggested to associate with pathophysiologies of the heart, lungs and obesity (for examples (D'amato et al. 1998),(Small et al. 2002),(Hao et al. 2004), albeit mainly in adult populations (for reviews (Rankinen et al. 2004),(Snyder et al. 2004)).

Two non-synonymous variants have been described in the ADRB1 gene resulting in two non-synonymous amino acid substitutions at positions 49 (Ser49Gly; rs1801252) and 389 (Arg389Gly; rs1801253). Codon 49 is located in the extracellular amino-terminus of the protein, an area known to affect receptor trafficking, while Codon 389 is located towards the C-terminus of the protein affecting the intracellular G-protein coupling (for a review (Small et al. 2003)). The Gly49 (rs1801252) variant, displaying higher agonist-promoted down-regulation (Small et al. 2003), has been associated with small but significant decreases of resting heart rate (Ranade et al. 2002), improved five-year survival risk

(Tesson et al. 1999) and positive influences on exercise capacity in patients with heart failure (Wagoner et al. 2002). The Arg389 (rs1801253) allele, conferring increased levels of adenylyl cyclase activity relative to the Gly389 (rs1801253) variant (Mason et al. 1999), has recently been associated with greater body weight and BMI as a result of increased fat mass in adult females (Dionne et al. 2002), an increased risk for heart failure (Small et al. 2002) and diastolic blood pressure (Bengtsson et al. 2001) and a positive influence on exercise capacity in patients with heart failure (Wagoner et al. 2002). In ADRB1, genotyping of the two commonest variants, Ser49Gly (rs1801252) and Arg389Gly (rs1801253), allows the inference of three haplotypes, namely Ser49Arg389, Ser49Gly (rs1801252)389 and Gly49Arg389, which represent the majority of variation along the complete length of the gene.

In ADRB2, thirteen SNPs have been identified in the 5'-UTR and coding regions, three of which, are non-synonymous (Drysdale et al. 2000). Historically, the two most commonly investigated SNPs are the non-synonymous changes at codons 16 and 27 located in the extracellular amino domain of the receptor. These changes result in a G to A transition at amino acid position 16 (Gly16Arg; (rs1042713)) and a G to C transversion at amino acid position 27 (Gln27Glu; (rs1042714)). The Gly16Arg (rs1042713) polymorphism is believed to regulate receptor function, with the Gly16 (rs1042713) variant being associated with lower receptor density, hence reduced receptor efficiency (McGraw et al. 1998). Interestingly, physical activity has been shown to interact with allelic variants of the Gly16Arg (rs1042713) and Gln27Glu (rs1042714) polymorphisms and modify their physiological effects; for example Gln27 homozygous males that partake physical activity show no difference in mean BMI relative to the Glu27 homozygotes and heterozygotes in contrast to Gln27 homozygotes that were hysically inactive (Meirhaege et al. 1999). In

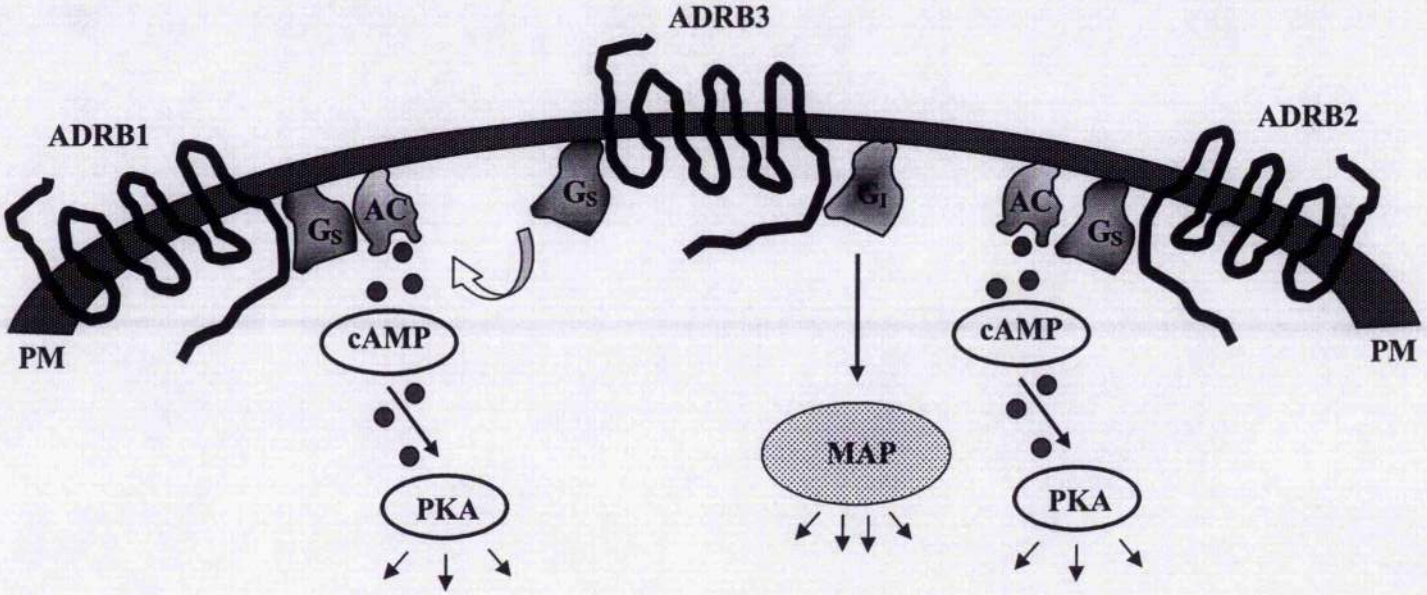


Figure 1.6: Intracellular signal transduction cascades of the ADRB receptors. (G_S) adenylyl cylcase stimulatory G protein, (G_I) adenylyl cylcase inhibitory G protein, (AC) adenylyl cyclase, (PM) plasma membrane. ADRB1 and ADRB2 receptors exert their effects via G_S while ADRB3 receptors interact with both G_S (including "cross-talk" with ADRB1 receptor) and G_I-linked signal transduction pathways. **(adapted from (Collins et al. 2001))**

contrast, (Corbalan 2002) have indicated that obese women that are carriers of the Glu27 allele may be more resistant to losing weight relative to their homozygote Gln27 counterparts when they participate in higher physical activity levels. All four alleles of the Gly16Arg (rs1042713) and Gln27Glu (rs1042714) polymorphisms have been previously associated with an increased risk for obesity. For example, (Large et al. 1997) indicated that Glu27 homozygous females were associated with increased body fat and that the Gly16Arg variant (rs1042713) did not influence the obese phenotype. In contrast, (Meirhaege et al. 2000) has suggested that the Gln27Gln genotype is associated with higher scores for obesity-related phenotypes in men but not in women and that if Gln27 homozygotes carried the Arg16 allele the aforementioned associations were stronger. Furthermore, (Hellstrom et al. 1999) indicated that the Glu27 variant positively associates with obesity in adult females but it is negatively associated with obesity in adult males. (Ellsworth et al. 2002) indicated that the Gly16 allele is associated with increased adiposity and obesity in males but not in females. (Moore et al. 2001) indicated that Gln27 carriers are associated with higher $\dot{V}o_{2\,max}$ values in postmenopausal women while (Wagoner et al. 2000) suggested that allelic SNP combinations in the ADRB2 gene determine $\dot{V}o_{2\,max}$ in patients with heart failure. The Gly16 allele and the Gly16Gln haplotype have been previously associated with nocturnal asthma and bronchial hyperresponsiveness (Turki et al. 1995),(D'amato et al. 1998). (Drysdale et al. 2000) has established twelve haplotypes in the ADRB2 gene that vary markedly in frequency between ethnic groups; typing of codons 16 and 27 allows the inference of three of these haplotypes (Gly16Glu27, Arg16Gln27 and Gly16Gln27 respectively) that account for 95% of the observed ADRB2 genetic variation in Caucasians. The Gly16Glu27 and Gly16Gln27 receptors exhibit similar levels of down-regulation between them and both higher when compared to the Arg16Gln27 receptors (Green et al. 1994).

A T to C transversion resulting in a non-synonymous mutation at codon 64 (Trp64Arg; (rs4994)) of the ADRB3 gene has been reported but with, as yet, unclear functional significance (for review (Small et al. 2003)). Amino acid 64 is positioned either to the most distal residue within the first transmembrane spanning domain or the most proximal residue of the first intra-cellular receptor loop. Several studies have reported positive associations and gender-specific effects of the Trp64Arg (rs4994) polymorphism on indices of obesity and type II diabetes (Ukkola et al. 2000),(Strazzulo et al. 2001),(Hao et al. 2004). The Trp64Arg (rs4994) polymorphism has also been suggested to interact synergistically with other obesity-related genes (Benecke et al. 2000),(Evans et al. 2000),(Corella et al. 2001) and a sedentary lifestyle (Marti et al. 2002) to influence obesity-related phenotypes. It is noteworthy that the very low allele frequency of the Arg64 (rs4994) allele may have significantly influenced the findings of previous investigations (for a review (Small et al. 2003)).

1.6 Aims and objectives

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

a. To assess the health and performance-related physical fitness of a large, representative cohort of adolescent Greek children. This was achieved by:

   i. Analysing the prevalence of overweight and obesity and compare the values obtained with the results of previous investigations in similar populations from Greece,

   ii. Assessing differences by gender and age in physical, physiological and skill-related phenotypes between the large, representative cohort of adolescent Greek children and normative data from Australian children of similar ages.

The Australian cohort was selected on the basis that it was the only large cohort readily available with data similar to those collected in the Greek adolescents,

iii. Discussing the aforementioned results relative to the findings of previous large and similarly aged Greek and international populations with emphasis on the overweight and obesity rates and physical fitness as indicated by $\dot{V}o_{2\,max}$ scores.

b. The ACE, ADRB1, ADRB2 and ADRB3 genes were selected as candidate genes to perform a series of cohort-based association studies. The selection of the aforementioned candidate genes was based on evidence from the published literature about:

- Their potential involvement in influencing human health and physical fitness,
- Knowledge of the LD levels present along their entire length in populations of Caucasian descent,

The aim of this series of cohort-based association studies was to assess whether variation within the selected candidate genes had an effect on a number of obesity and performance-related physical fitness phenotypes. This was achieved by:

i. Assessing genetic influences of the aforementioned candidate genes on obesity and performance-related physical fitness phenotypes in a large, representative and homogeneous population of Greek adolescent children. Younger individuals were preferable for this type of investigation; Greek adolescents were selected on the basis

that they were part of a large, representative and homogeneous cohort that was readily available and included data for a large number of physical, physiological and skill-related phenotypes,

ii. In addition to genotype-based approaches employ haplotype based approaches to maximise the genetic variation tested for association with the investigated traits,

iii. Considering gene-environment interactions with gender and participation in organised physical activity,

iv. Attempt to account for impact of multiple-testing effects on significant findings. Where such an approach is not realistically plausible other practical alternatives will be considered.

CHAPTER 2

GENERAL METHODS

2.1 Subject Population

A total of 1198 subjects provided written informed consent and parental approval prior to their participation in the study, which was approved by the University Ethics Committee and local Greek authorities (Appendix 1A). Subjects were comprised of 627 boys and 571 girls aged 11-18 years, all studying at schools in Greece. Schools were selected to include both urban and rural areas. Informed written consent was obtained from the parents or guardians of the child (Appendix 1B). The subjects were recruited voluntarily by letter through local schools (Appendix 1C). All subjects were in good health at the time of testing as assessed by completing a medical questionnaire (Appendix 1D).

2.2 Measurements

The amount of organised physical activity participation at and outside school was assessed by questionnaire (Appendix 1D). Habitual physical activity levels in children are inherently difficult to monitor and precisely quantify (Washburn and Montoye 1986) in such a large scale; hence, only organised physical activity was recorded as means of differentiating between levels of physical activity in this study cohort. All assessments were carried out by trained individuals after a period of protocol standardisation.

Height was measured to the nearest 0.1cm using a floor standing stadiometer (Leicester Height Measure, Child Growth Foundation, London). Body weight was measured to the nearest 0.1kg using electronic scales (SECA ALPHA model 770, Seca® Alpha, Model 770, Hamburg, Germany). Height and weight were converted to BMI using Quatelet's index [weight/height^2 (kg/m^2)]. Skinfold thickness was measured in triplicate using Harpenden callipers (British Indicators Ltd, John Bull, England) at two sites, triceps and subscapular, and the average of the three measures was used in the analysis. The validity of

Table 2.1: Sequences for the primers used for the genotyping of the ACE I/D, 7831 (rs4424958) and 8968 (rs4311) variants. The T_ms, probable secondary structure and GC content are as determined by the Sigma-Genosys primer calculator. None of the primers were predicted to form primer dimmers. refSNP identity codes of the polymorphisms are given in parenthesis.

ACE Polymorphism	Name	Sequence 5' to 3'	Length	Tm	Secondary Structure	% GC
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
G7831A (rs4424958)	**DCP1CM7517F**	CTTGGAATGGAGGAAGAAGGC	21	65.87	Very Weak	52.38
	DCP1CM7914R	ATGAAGCCGCAGTAGAAACAAG	22	64.25	None	45.45
C8968T (rs4311)	**DCP1CM8495F**	TCCCTGGCCTCACTTTCTG	19	65.39	None	57.89
	DCP1CM9086R	TCCAGCATCAAAGTGGGTTTC	21	66.05	Weak	47.62

the skinfolds thickness at two sites (triceps and subscapular) in predicting adiposity in children has been discussed elsewhere (Reilly et al. 1995),(Wells et al. 1999) while total skinfolds was estimated as the sum of skinfolds at the two sites. Skinfolds estimated % body fat was calculated using the age and gender-specific equations of (Slaughter et al. 1988) as described by (Reilly et al. 1995). For the purposes of selecting equations to estimate percentage body fat, males aged 11-13 were considered to be pre-pubescent, those aged 14 and 15 pubescent, and those 16 and older to be post-pubescent; these groupings were, on average, in agreement with findings for the timing of sexual maturity in other populations (Sun et al. 2002).

Subjects were also asked to complete a series of tasks designed to measure physical, physiological and skill-related attributes (Australian Sports Commission 1993). Detailed information with regards to the procedures for the measurement of physical, physiological and skill-related attributes are given in Appendix 2. $\dot{V}o_{2\,max}$ was estimated from the scores of 20m multistage shuttle run test as previously described (Leger et al. 1988). Sitting height was measured to the nearest 0.1cm using a floor standing stadiometer (Leicester Height Measure, Child Growth Foundation, London). Arm span was recorded with the arms outstretched at shoulder level to the nearest 0.5cm while sit and reach was measured to the nearest 0.5cm using a standard sit and reach box (Bodycare Products, Southam, Warwickshire, UK) as described in (ACSM 2000).Throw and catch was measured as the number of times that the subjects could throw under-arm and retrieve a tennis ball with their hands after successfully hitting a 30cm square target at shoulder level from 2.5m away. Each subject was allowed 10 tries with each hand and the sum of the two was recorded as the total throw and catch score after a short period of familiarisation with the test. Basketball throw was measured as the longest distance thrown (to the nearest 5 cm)

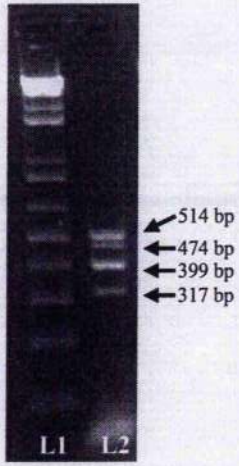
(a)

L1: 1 kb+ DNA ladder.

L2: II homozygote, identified by presence of 252 bp band and absence of 197 bp band. The 485 bp band can also be seen.

L3: ID heterozygote, showing amplification of all 3 bands.

L4: DD homozygote showing amplification of only the 197 bp band.



(b)

L1: 1 kb+ DNA ladder.

L2: Bands produced by a *Pst I* and *Mse I* double-digest of an individual heterozygous for both polymorphisms. The singly cut 8968C-allele is identified by the presence of the 514 bp band, and the 8968T-allele by the presence of the 474 bp band. The uncut 7831A-allele is identified by the uncut 399 bp band and the singly cut 7831G-allele is identified by the presence of the 317 bp band.

Figure 2.1: (a) 2% agarose gel, stained with ethidium bromide, of PCR I/D genotyping reactions; (b) 2% agarose gel, stained with ethidium bromide, of *Pst I* and *Mse I* double digested fragments of G7831A (rs4424958) and C8968T (rs4311) multiplex-PCR amplified products of the ACE gene.

using a standard size and weight senior basketball. Hand-grip strength was measured using a hand grip dynamometer (GRIP-D TKK 5101, Takei, Tokyo, Japan) that was individually adjusted to hand size; subjects performed two trials for each hand. The highest score for each arm was recorded in kg and the sum of the best tests for each arm was recorded as the total hand-grip strength. Vertical jump was measured in cm using a jump meter (Jump MD, TKK 5106; Takei); the subjects were standing in the centre of the test mat with the display unit attached around the subject's waist and the rope wound tight in the pulley in a vertical position. The subjects performed a counter movement jump, as high as possible, with free arm movement and landed with two feet on the mat. 40m sprinting times were measured to the nearest 0.1s; subjects were allowed two trials and the fastest time was recorded. Agility run times were recorded to the nearest 0.1s upon the successful completion of five cycles of a run-pivot-run trial across a distance of 5m; subjects were allowed two trials and the fastest time was recorded.

2.3 Genotype determination and haplotype inference

2.3.1 DNA extraction Buccal cell samples were collected by cytology brushes (Medical Packaging Corporation, Camarillo, CA, U.S.A.), were appropriately numbered, placed in 350μl of cell lysis solution (Gentra Systems, Minneapolis, U.S.A) and stored at -70 °C. Genomic DNA was subsequently extracted using PUREGENE® DNA purification kit standard buccal cell protocol (Gentra Systems, Minneapolis, U.S.A.) and stored at –20 °C. Prior to mass extraction of genomic DNA from the samples the DNA purification kit standard buccal cell protocol (Gentra Systems, Minneapolis, U.S.A.) was tested in 5 control samples. Assessment of the quality and the quantity of the extracted DNA was performed visually after separation of the genomic DNA on 1% agarose gels.

2.3.2 Genotyping reactions For genotyping all PCR reactions were carried out in 96-well Thermofast rigid semi-skirted plates (ABgene, Epsom, Surrey, UK) in 25 µl volumes with 2X ReddyMix™ mastermix containing 1.5mM $MgCl_2$ (AB Gene, ABgene® House, Surrey, UK), using as template genomic DNA at a concentration $1/100^{th}$ of the original stock (20-100ng of genomic DNA) and 10pmol of each primer. All digests were performed in 96-well Thermofast rigid semi-skirted plates (ABgene, Epsom, Surrey, UK). To address the possibility of mistyping during protocol standardisation, genotyping for each locus was initially optimised on control samples for which the allelic variants at each locus were confirmed by direct sequencing; 10 control samples were selected that represented all possible allelic combinations for the polymorphic loci considered. The validity of genotyping for each variant was assessed by repeating the genotyping reactions in a sample of thirty randomly selected individuals. Allele frequencies were calculated for the total, male and female participants. The accuracy of typing for all loci was further assessed by testing whether the obtained genotypes and inferred diplotypes were in HWE (Russel PJ 1994) within the total, male and female population groups; the agreement of the expected against the observed frequencies of genotypes and inferred diplotypes with HWE was assessed using appropriate X^2-tests.

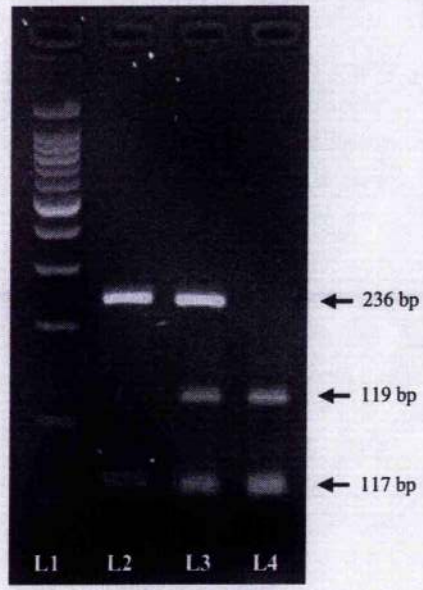
2.3.3 ACE gene New primers were designed for all genotyping reactions (table 2.1); the NCBI reference sequence used for primer design was AF118569. I/D genotypes were determined by PCR, using a three-primer system involving a forward primer recognising the deletion (D) sequence, a forward primer recognising the insertion (I) sequence and a common reverse primer. PCR was performed using 10pmol of each primer (Sigma-Genosys, Haverhill, Cambridgeshire, UK). Thermocycling consisted of 2.5min at 94°C followed by 35 cycles of 94°C, 56.5°C and 72°C each for 45s and was completed with

Table 2.2: Sequences of the primers used for the genotyping of the polymorphic variants of the ADRB1, ADRB2 and ADRB3 genes. The T_ms, probable secondary structure and GC content are as determined by the Sigma-Genosys primer calculator. None of the primers were predicted to form primer dimmers. The T_ms of Bar1CM33F and Bar1CM250R did not include the non-specific T-tail when calculated. The base marked in bold in Bar2CM26NcoF2 was used to introduce an alteration to the amplified fragment that created an *Nco I* site in conjunction with the codon 16 G allele of ADRB2 (discussed in text). rfSNP are given in parenthesis; Gly16Arg and Gln27Glu polymorphisms are abbreviated as (C16) and (C27), respectively.

SNP	Name	Sequence 5' to 3'	Length	Tm	Secondary Structure	% GC
ADRB1 Ser49Gly	**Bar1CM33F**	TTTTCGAGCCCGGTAACCTGTCG	23	69.99	Weak	56.52
(rs18011252)	**Bar1CM250R**	TTTTTTTTTTTTTTCGATGGCCACGATCACCAG	33	70.20	Weak	36.36
ADRB1 Arg389Gly	**Bar1CM1030F**	AACGTGGTGAAGGCCTTCC	19	66.27	Very weak	57.89
(rs1801253)	**Bar1CM1302R**	ATCGTCGTCGTCGTCGTCC	19	68.81	None	63.16
ADRB2 C16 & C27	**Bar2CM26NcoF2**	CCTTCTTGCTGGCACCC**C**AT	20	70.47	Weak	60.00
(rs1042713) & (rs1042713)	**Bar2CM196R**	TCTGCAGACGCTCGAACTTG	20	66.82	None	55.00
ADRB3 Trp64Arg	**Bar3CM62F**	CGCCCAATACCGCCAACAC	19	70.55	None	63.16
(rs4994)	**Bar3CM271R**	CCACCAGGAGTCCCATCACC	20	69.06	None	65.00

10min at 72°C. PCR products were separated on a 2% agarose gel. If present in the template, the D-allele yielded a product of 197 bp and the I-allele a product of 252 bp (figure 2.1a). 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.

To complete the haplotyping, restriction enzyme digest assays were set up for a G to A polymorphism at position 7831 (rs4424958) and a C to T polymorphism at position 8968 (rs4311); primer sequences are given in table 2.1. Nucleotide positions are as described by Rieder (Rieder et al. 1999). Neither variant was thought to be functional however in combination with the I/D polymorphism they allowed differentiation between the common European haplotypes (Rieder et al. 1999). Both loci were amplified by PCR, with all 4 primers present in a single reaction tube (10pmol of each primer). It should be noted, however, that the efficiencies of the 2 reactions were not dependent on each other, thus the intensity of the C8968T bands was independent of the intensity of the G7831A bands (figure 2.1b). Thermocycling consisted of 2.5 min at 94°C followed by 35 cycles of 94°C and 60°C each for 45s and 72°C for 1min; the reaction was completed with 10min at 72°C. The 7831G-allele PCR product contained a unique site for the restriction enzyme Pst I (New England Biolabs; NEB (UK) Ltd., Herts, UK), that was absent from the 7831A-allele, thus cleavage of the 399 bp product into fragments of 317 and 82 bp confirmed the presence of the 7831G allele. The 8968 PCR product contained a site for the restriction enzyme Mse I (New England Biolabs; NEB (UK) Ltd., Herts, UK) that is common to both the 8968T and 8968C alleles, however, the 8968T allele PCR product also contained a unique site for Mse I (New England Biolabs; NEB (UK) Ltd., Herts, UK), that was absent



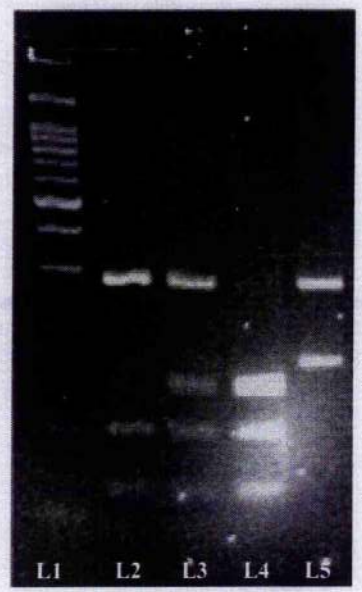
(a)

L1: 1 kb+ DNA ladder.

L2: Ser49 homozygote, identified by the 236 bp band.

L3: Ser49Gly heterozygote, identified by the 236 bp, 119 bp and 117 bp bands.

L4: Ser49 homozygote identified by the 119 bp and 117 bp bands.



(b)

L1: 1 kb+ DNA ladder.

L2: Arg389 homozygote, identified by the 273 bp band, and Trp64Trp homozygote identified by the the 97 bp and 61 bp bands.

L3: Arg389Gly heterozygote, identified by the 273 bp and 140 bp bands, and Trp64Trp homozygote identified by the 97 bp and 61 bp bands.

L4: Gly389 homozygote, identified by the 140 bp band, and Trp64Trp homozygote identified by the 97 bp and 61 bp bands.

L5: Arg389 and Arg64 double homozygote, identified by the 273 bp and the 158 bp bands, respectively.

Figure 2.2: (a) 2.5% agarose gel, stained with ethidium bromide, of the *Eco0109I* digested fragments for the ADRB1 Ser49Arg (rs1801252) variant; (b) 3.5% agarose gel, stained with ethidium bromide, of the *Bst NI* double digested fragments for the ADRB1 Arg389Gly (rs1801253) and the ADRB3 Trp64Arg (rs4994) variants.

from the 8968C-allele. Thus cleavage of the 592 bp product into fragments of 514 and 78 bp confirmed the presence of the 8968C allele, whilst cleavage into fragments of 474, 78 and 40 bp confirmed the presence of the 8968T allele (figure 2.1b). Neither product was cut at any other site by either of the enzymes, thus the reactions could be multiplexed. The double digests were performed using 7.5µl of the PCR reaction and 0.5U of Mse I and 1U of Pst I in a final volume of 10µl followed by incubation overnight at 37°C and were resolved on a 2% agarose gel. Haplotypes were inferred from the above results based on published literature (Rieder et al. 1999) with H1 being defined as 7831A, 8968T and D, H6 as 7831G, 8968C and I, H7 as 7831G, 8968T and D and H9 as 7831G, 8968C and D.

2.3.4 ADRB genes New primers were designed for all genotyping reactions (table 2.2); primer design was based on NCBI reference sequences NM_000684 (ADRB1), AC011354 (ADRB2) and X72861.1 (ADRB3).

For the ADRB1 gene, Bar1CM33F and Bar1CM250R amplified a 236 base-pair (bp) region including the Ser49Gly polymorphic site (rs1801252). The primers contained non-specific T-tails to facilitate their use in multiplexed reactions with the ADRB1 Arg389Gly primers, although this was not done here. Thermocycling for Ser49Arg consisted of 5min at 94°C followed by 35 cycles of 94°C and 59°C each for 45s and 72°C for 30s; the reaction was completed with 10min at 72°C. Amplification products were then digested with Eco0109I (New England Biolabs; NEB (UK) Ltd., Herts, UK) and separated on a 2.5% agarose gel. Digestions for Ser49Gly were performed using 5µl of the PCR reaction and 5u of Eco0109I in a final volume of 10µl followed by incubation overnight at 37°C. The Gly49 allele PCR product contained a unique site for Eco0109I yielding bands of 119 bp and 117 bp, whilst the Ser49 allele PCR product remained uncut (figure 2.2a).

Bar1CM1030F and Bar1CM1302R amplified a 273 bp fragment containing the Arg389Gly polymorphic variant (rs1801253) of ADRB1. The Gly389 allele PCR product contained a unique site for Bst NI (New England Biolabs; NEB (UK) Ltd., Herts, UK) yielding products of 140 bp and 133 bp, whilst the codon Arg389 allele PCR product remained uncut (figure 2.2b). ADRB1 Arg389Gly genotyping was multiplexed with the amplification and digestion of ADRB3 Trp64Arg variant (rs4994; see below) with all products being separated on a single 3.5% agarose gel (figure 2.2b). Thermocycling consisted of 5min at 94°C followed by 35 cycles of 94°C, 60°C and 72°C each for 30s; the reaction was completed with 10min at 72°C. The double digests for Arg389Gly and Trp64Arg were performed using 10μl of the PCR reaction (5μl from each PCR) and 5.1U of Bst NI in a final volume of 20μl followed by incubation overnight at 37°C. For ADRB1, individuals homozygous at both codon 49 and codon 389 were used to determine the common haplotypes which were Ser49Arg389, Ser49Gly389 and Gly49Arg389; this knowledge was then applied to heterozygous individuals.

For the ADRB2 gene, Bar2CM26NcoF2 and Bar2CM196R amplified a 171 bp region containing both the Gly16Arg (rs1042713) and the Gln27Glu (rs1042714) polymorphic sites. Thermocycling conditions consisted of 2.5 minutes at 94°C followed by 35 cycles of 94°C, 56.5°C and 72°C each for 30s; the reaction was completed with 10 minutes at 72°C. As shown in table 2.2, Bar2CM26NcoF2 contained a mismatched base that created an Nco I site in conjunction with the Gly16 allele only. This product was then used for two separate digests; one with Nco I (New England Biolabs; NEB (UK) Ltd., Herts, UK) to genotype the Gly16Arg polymorphism and one with Bbv I (New England Biolabs; NEB (UK) Ltd., Herts, UK) to genotype the Gln27Glu polymorphism (figure 2.3a and 2.3b).Digestions were performed using 5μl of the PCR reactions and 2U Nco I and 0.5U

Table 2.3: R-values for the Ryan-Joiner test assessing the normality of different transforms for the physiological, physical and skill-related phenotypes. The best transform for each measurement is in bold; note that higher R-values indicate higher levels of normality and better transform. Based on the R-values the $\log_{10}$ transformation was selected as the one giving, on average, the best transform for all measurements with the exception of throw and catch were the arcsine transformation was used.

	Transform Tested				
	No transform	**1/x**	**Log_{10} (x)**	**Squared (x^2)**	**Arcsine (x)**
BMI	0.9774	**0.9993**	0.9957	0.9467	0.9340
Triceps Skinfolds	0.9697	0.9469	**0.9972**	0.8787	0.9777
Subscapular Skinfolds	0.8978	0.9882	**0.9889**	0.7549	0.9169
Total Skinfolds	0.9432	0.9770	**0.9959**	0.8434	0.9526
Skinfolds % BF	0.9672	0.9528	**0.9984**	0.8600	0.9702
$\dot{V}O_{2\ max}$	0.9961	0.9857	**0.9969**	0.9851	0.9900
Sitting Height	0.9626	0.9729	0.9844	**0.9972**	0.9936
Arm Span	0.9612	0.9989	**0.9991**	0.9931	0.9849
Sit & Reach	0.9983	0.5937	**0.9989**	0.9736	0.9385
Basket Throw	0.9170	**0.9943**	0.9938	0.9055	0.9554
Throw & Catch	0.9920	0.5840	0.8818	0.9559	**0.9978**
Hand-grip	0.9719	0.9804	**0.9957**	0.9232	0.9685
Vertical Jump	0.9877	0.9751	**0.9970**	0.9590	0.9787
40m Sprint	0.8446	0.9911	**0.9962**	0.9912	0.9884
Agility Run	0.9919	0.9991	**0.9992**	0.9873	0.9808

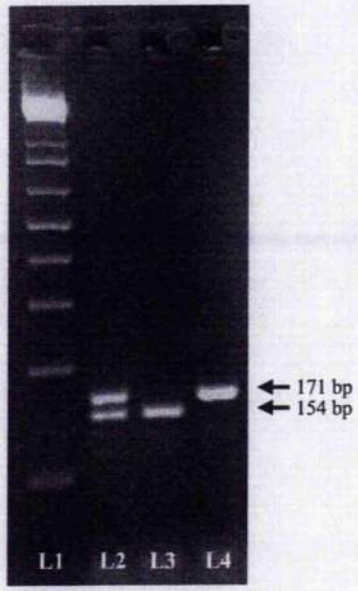
Bbv I to a final volume of 10μl followed by overnight incubation at 37°C; the digests and gels were carried out separately for reasons of clarity. The Gly16 allele yielded products of 154 bp and 17 bp, when digested with Nco I, which were separated on a 3.5% agarose gel, whilst the Arg16 allele remained uncut (figure 2.3a). The Gln27 allele yielded products of 109 bp and 62 bp, when digested with Bbv I, which were separated on a 2% agarose gel, whilst the codon Glu27 allele remained uncut (figure 2.3b). The genotype data was subsequently used to infer haplotypes based on the published literature (Drysdale et al. 2000).

For the ADRB3 gene, Bar3CM62F and Bar3CM271R amplified a 210 bp region which contained the Trp64Arg variant (rs4994); the PCR products were digested with Bst NI (New England Biolabs; NEB (UK) Ltd., Herts, UK). The codon Trp64 allele contained four Bst NI sites producing bands of 97, 61, 31, 15 and 6 bp, whilst the codon Arg64 allele contained three Bst NI sites producing bands of 158, 31, 15 and 6 bp. As described above these were separated on a 3.5% agarose gel together with the ADRB1 Arg389Gly variant (figure 2.2b).

2.4 Data analysis

Prior to any analysis, the electronic data entries of the results collected from the series of physical, physiological and skill-related tests were independently and comparatively assessed. Descriptive statistics and normal distribution plots were used to identify and concomitantly remove outliers from the dataset.

To accommodate for differences in growth and development the present series of experiments considered data stratified by age and by height for each gender separately. The



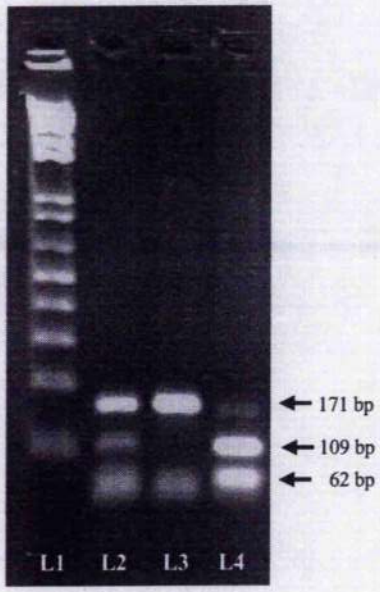
(a)

L1: 1 kb+ DNA ladder.

L2: Gly16Arg heterozygote, identified by presence of the 171 bp and the 154 bp band.

L3: Gly16 homozygote, identified by the presence of the 154 bp band.

L4: Arg16 homozygote showing amplification of only the 171 bp band.



(b)

L1: 1 kb+ DNA ladder.

L2: Gln27Glu heterozygote, identified by presence of the 171 bp, 109 bp and 62 bp bands.

L3: Glu27 homozygote, identified by the presence of the 171 bp band.

L4: Gln27 homozygote identified by the presence of the 109 bp and the 62 bp bands.

Figure 2.3: (a) 3.5% agarose gel, stained with ethidium bromide, of the *Nco I* digested fragments for the ADRB2 Gly16Arg (rs1042713) variant; (b) 2% agarose gel, stained with ethidium bromide, of the *Bbv I* digested fragments for the ADRB2 Gln27Glu (rs1042719) variant.

data were initially tested for normality using the Ryan-Joiner test (Minitab 13.32, Minitab Ltd., Coventry, UK). Using the R-value of the Ryan-Joiner test as reference, the $\log_{10}$ transformation was selected as the one providing, on average, the best transform for all measurements; exception was throw and catch for which the arcsine transformation was used (table 2.3). The transformed data was then standardised for age and for height by conversion to Z-scores that were used for all further parametric analysis; the data were expressed as SD from the phenotypic mean in age and height-specific groups. The use of Z-scores prior to parametric analysis when investigating physiological and physical fitness-related phenotypes, in children and adolescents, has been previously described (for example (Ferrari et al. 1998)). Conversion to Z-scores for age was performed within age groups; as the number of 11 and 18 year olds was small relative to the number of subjects in age groups 12-17, to maintain equally large numbers between age groups for Z-score correction, 11 to 12 year olds and 17 to 18 year olds were grouped together prior to $\log_{10}$ transformation and age standardisation (table 2.4). Conversion to Z-scores for height was performed within height groups. Height groups were obtained by dividing the height distribution in six equal groups for each gender separately (table 2.5); as the number of participants in the 1st and the 6th height groups was small relative to the number of subjects in the remaining groups, to maintain equally large numbers between height groups for Z-score correction, the 1st and 2nd height groups and the 5th and 6th height groups were grouped together prior to $\log_{10}$ transformation and age standardisation (table 2.5). No significant differences were observed between the results of the analysis stratified by age and the results of the analysis stratified by height (Appendix 3). As such, the results presented in Chapters 4-7 are for the age-stratified analysis. For ease of interpretation and figure presentation, reported Z-scores were back-converted to untransformed measurements as if derived from a single age group, namely 17 year olds.

For both genders, individual genotypes/diplotypes differences in physiological, physical and skill-related parameters were assessed in total, inactive and active groups using one-way ANOVA (Minitab 13.32, Minitab Ltd., Coventry, UK). Significant findings were further assessed by correlation analyses (Minitab 13.32, Minitab Ltd., Coventry, UK) of genotype values against phenotype values to determine the proportion of the phenotypic variance explained by various genetic models. The genetic models considered were additive and dominant models; other models were considered biologically less plausible so were not tested (Griffiths et al. 1996). Genotype values for the single locus additive model were 0, 0.5 and 1, where these represent homozygotes for one allele, heterozygotes and homozygotes for the other allele, respectively (table 2.6). An additive model explains the data best where the mean of the heterozygous group is exactly intermediate to the two homozygous allele groups. For the completely dominant genetic models, the corresponding values were 0, 0, 1 or 0, 1, 1, respectively depending on which allele was being tested for dominance (table 2.6). A dominance model explains the data best where the mean of the heterozygous group, is sufficiently close to the mean of one of the two homozygous allele groups to imply dominance of that allele. The proportion of the genetic variance explained by each of the genetic models was estimated by dividing the variance explained by correlation analyses (r_C^2) for each model by the variance explained by the appropriate ANOVA (r_A^2) that imposes no model on the data; the genetic model with the higher ratio (r_C^2/r_A^2) explained the higher proportion of phenotypic variance.

The influence of genotype on the distribution of phenotypes in the study population was assessed by calculating ORs. For significant gene-phenotype associations (as determined by

Table 2.4: Number of female and male subjects in each respective age group. To maintain equally large between age groups for z-score correction 11 to 12 year olds and 17 to 18 year olds were grouped together.

	Prior to Grouping		After Grouping	
	Females	**Males**	**Females**	**Males**
11	14	15	113	131
12	99	116		
13	78	123	78	123
14	74	140	74	140
15	117	109	117	109
16	106	99	106	99
17	81	87	83	88
18	2	1		

ANOVA), male and female populations (*i.e.* total, active and inactive) were divided into top (75^{th}-100^{th} centiles), pooled-middle (25^{th}-75^{th} centiles) and bottom (0-25^{th} centiles) quartiles of the phenotype distribution. ORs were calculated (Armitage P and Berry G 1994) as the proportional over or under-representation of a given genotype in a selected quartile. Significance of the association was calculated using 2x2 X^2-tests and 95% CIs.

2.4.1 Multiple testing Association studies involve multiple comparisons during testing and as such are subjected to type I (false positive) errors. In order to lower the probability of type I errors to a level where it will not exceed the selected probability of statistical significance α (usually $P<0.05$) several techniques have been developed; the majority of these methods have focused in establishing a global significance threshold for controlling the probability of making at least one false rejection, known as the FWER (Bender and Lange 2001),(Sabatti et al. 2003). Common examples of these methods include the Bonferroni, the Šidak and the Holm corrections (Holm 1979),(Sokal and Rohlf 2001). There is an ongoing discussion about the validity of correcting for multiple testing. A number of studies have suggested that such approaches are unnecessary (Rothman 1990),(Perneger TV 1998a),(Savitz and Olshan 1998) while others consider them as mandatory to differentiate between valid and biased associations (Sankoh et al. 1997),(Goodman 1998),(Bender and Lange 2001). Although appropriate to evaluate the locus with the strongest evidence, the aforementioned methods have a dependency for the interpretation of a finding on the number of loci and phenotypes considered (Perneger TV 1998),(Sabatti et al. 2003). The higher the number of the required comparisons, the more conservative these methods become and, while the probability for type I errors decreases, the likelihood of type II errors increases so that real associations are being missed (Sabatti et al. 2003). As such, many gene association studies neglect multiplicity control altogether

Table 2.5: Range and number of subjects for each respective height group. To maintain equally large between age groups for z-score correction the participants of the 1st and the 2nd height groups and the 5th and 6th height groups were grouped together. (x) is height value within group, (N) number of subjects in each group.

	Height Group Range				Height Group (N)			
	Prior to Grouping		After Grouping		Prior to Grouping		After Grouping	
	Females	Males	Females	Males	Females (N)	Males (N)	Females (N)	Males (N)
1st group	≤145,7	≤149,5	≤152,4	≤159	12	45	75	159
2nd group	145,7<(x)≤152,4	149,5<(x)≤159			63	114		
3rd group	152,4<(x)≤159,1	159<(x)≤168,5	152,4<(x)≤159,1	159<(x)≤168,5	172	157	172	157
4th group	159,1<(x)≤165,8	168,5<(x)≤178	159,1<(x)≤165,8	168,5<(x)≤178	198	220	198	220
5th group	165,8<(x)≤172,5	178<(x)≤187,5	165,8<(x)≤179	178<(x)≤197	115	81	124	90
6th group	172,5<(x)≤179	187,5<(x)≤197			9	9		

despite the potential risks (for examples (Alevizaki et al. 2000),(Ellsworth et al. 2002),(Strazzullo et al. 2003)). Several of the measurements considered in the present series of experiments are descriptive of the same phenotype; for example, BMI and skinfolds measurements are all indices of adiposity. This would suggest that the tests performed in the present population are not independent and corrections of the global significance threshold for controlling the probability of making at least one false rejection would be subject to partial correlations between measurements. Nevertheless, due to the multi-dimensionality of the dataset it would be difficult to realistically account for all the correlations present and concomitantly adjusting the level of α for such a large number of tests would still generate low values. As such, accounting for type I errors in datasets such as the present may require treating obesity and performance-related physical fitness phenotypes separately; such approaches have been suggested elsewhere (Bender and Lange 2001). For the experiments described in Chapters 4 and 6 that considered genetic influences on obesity-related phenotypes, a Šidak correction (Sokal and Rohlf 2001) in the presence of partial correlations was applied (Appendix 4). The mean correlation (r_{Obs}=0.776) was calculated for BMI and the triceps and subscapular skinfolds measurements (Minitab 13.32, Minitab Ltd., Coventry, UK). Total skinfolds and skinfolds estimated % body fat are directly estimated from the sum of skinfolds at the triceps and subscapular loci therefore they were considered as not adding to the data. The Šidak correction was performed using the SISA website (Uitenbroek 2005) and produced α values that were equal to 0.019 for the loci tested in Chapter 4, 0.017 for the loci tested in Chapter 6 and 0.016 when all the tested loci were considered simultaneously (Appendix 4). Irrespective of whether the Šidak correction was applied for the genetic loci tested for differences with BMI and skinfolds measurements in each study separately or for all genetic loci simultaneously the majority of the significant findings described in Chapters 4

Table 2.6: Genetic models tested to determine the type of genetic effect that best described the phenotypic variance for significant associations. The possible genotype values used for testing for each single locus genetic model are provided. (A) and (B) represent hypothetical additive allelic morphs. (C) and (D) represent hypothetical dominant and recessive allelic morphs; in Model I, (C) is considered dominant over (D) while in model II the (C) allele is considered recessive relative to (D).

	Additive Genetic Model			**Dominant Genetic Model**		
	AA	**AB**	**BB**	**CC**	**CD**	**DD**
Model I	0	0.5	1	1	1	0
Model II	1	0.5	0	0	1	1

and 6 (tables 4.1, 4.2, 6.1 and 6.2) remained significant. A similar correction, was performed for the genetic studies described in Chapters 5 and 7 (r_{Perf}=0.045), produced α values that were very low (Appendix 4). Considering the tests performed in Chapter 5 the α value calculated was 0.0002, for the tests performed in Chapter 7 the α obtained was 0.0001 while accounting for all genetic loci tested simultaneously produced an α equal to 0.0001. Such α values may be unrealistically low and may result in an inflation of type II errors hence an increase of the probability that real associations were being missed. Nevertheless, it is clear that caution must be exercised when interpreting results to avoid reporting biased associations. To counter these problems a practical alternative was considered irrespective of the α value produced by the Šidak correction (Sokal and Rohlf 2001). As suggested elsewhere (Perneger TV 1998),(Bender and Lange 2001),(Bacchetti 2002), the type and the reasoning behind the use of tests for significance were clearly reported. Furthermore, as data analysis and interpretation depends on factors such as the functional relevance and biological plausibility of the results (Royall 1997), the validity of significant findings was assessed relative to the number of potentially biased associations; this analysis was based on the number, the consistency across phenotypic groups and the relative strength of significant associations as well as on considerations with regards to the influence of the genetic effect across phenotypic distributions and its biological plausibility. In such situations, as indicated elsewhere (Bacchetti 2002), the results reinforce each other rather than detracting from each other as would be required by other methods.

CHAPTER 3

THE HEALTH AND PERFORMANCE-RELATED FITNESS OF THE GREEK ADOLESCENT COHORT: A COMPARISON WITH AUSTRALIAN NORMS.

3.1 Introduction

Health and fitness are positively influenced by factors such as diet (Heck et al. 2004) and physical activity participation (Philippaerts et al. 1999). In children and young adolescents of developed countries, the increased prevalence of inactivity and the endorsement of diets rich in saturated fat and starch have been well documented (Fox and Riddoch 2000),(Gibson 2000). In the USA for example, approximately 62% of children aged 9-13 years do not partake in organised physical activity outside school (CDC 2003) while white potatoes account for 50% of the vegetable intake in children and adolescents (Cavadini et al. 2000). Evidence of unbalanced diets and increasingly sedentary behaviours early in life have also been reported in developing countries like Greece (Mamalakis and Kafatos 1996),(Hassapidou and Fotiadou 2001),(Krassas et al. 2001). In Greece, the adoption of modern lifestyles has been previously linked with significant increases in the overweight and obesity rates over the last decades (Mamalakis et al. 2000),(Krassas et al. 2001b); Greek adolescents are considered to have one of the highest prevalences of overweight amongst western countries (Lissau et al. 2004). Despite this line of evidence, in the published literature, there is a lack of reports with regards to health and physical fitness-related phenotypes of Greek children outside studies assessing trends and prevalence in obesity (for examples (Mamalakis et al. 2000),(Krassas et al. 2001b)).

The aim of this study was to assess the general physical fitness of a large, representative adolescent population from Greece. This was achieved by comparing the rates of overweight and obesity in the present cohort of Greek adolescents with the findings of other published studies. The physical, physiological and skill-related fitness data of this Greek cohort were also compared with normative data from Australian children of similar age (Australian Sports Commission 1993).

Table 3.1: Overweight and obesity prevalence (%) in adolescents from Greece using the newly established IOTF cut-off points for BMI. (N) is number of subjects.

	N	Overweight (%)	Obese (%)
Females	571	22.8	4.6
Males	627	24.4	8.9
Total	1198	23.6	6.9

3.2 Methods

3.2.1 Subjects and Measurements For the present analysis, all information regarding subjects and measurements of physical, physiological and skill-related parameters in the Greek cohort were as described in General Methods (p. 43). Australian normative data were obtained from the *Talent Search* sports counselling program (1993) that is available from the Australian Sports Commission (Leverrier Cres Bruce ACT 2617, PO Box 176 Belconnen ACT 2616). The *Talent Search* program was part of the National Talent Identification and Development program and it included 4458 children, aged 12 to 17 years, all at urban, country, private and government schools across the Australian continent. The Australian normative data included measurements for height, % sitting height, arm span, body mass, sit and reach, hand-grip strength, vertical jump, basketball throw, throw and catch, agility run, 40 m sprint, and the multistage shuttle run (Australian Sports Commission 1993).

3.2.2 Data Analysis The cut-offs for BMI (Cole et al. 2000), recently adopted by IOTF, were used to analyse the prevalence of overweight and obesity in the Greek subject population; they were preferred over centile-based BMI cut-offs as they are considered less arbitrary and facilitate the comparison of findings between investigations (Cole et al. 2000). For the adolescents from Greece, mean and SD for the measured parameters were estimated within each age group and gender separately. Where appropriate, independent two-tailed *t*-tests were used to assess the significance of differences between the mean values of phenotypic traits; statistical significance was accepted at $P<0.05$. Comparisons between mean values for $\dot{V}o_{2\,max}$ were performed on level and shuttle number. However, for reasons of clarity of interpretation and presentation of the results, in the text, tables and figures the results for the comparison between shuttle-run level and shuttle-run number

Table 3.2: Mean values and SD for the measured parameters at different ages of females from Greece and Australia. Statistically significant differences (P<0.05) are indicated in bold; P<0.01 is indicated by (**) and P<0.001 by (***). (% Sit Height) stands for % sitting height and (Basket Throw) stands for basketball throw. Units are as follows: height in (m), arm span in (cm), % sitting height in (%), weight in (kg), $\dot{V}o_{2\ max}$ in ($ml kg^{-1} min^{-1}$), basketball throw in (m), throw and catch in (number of times), 40m sprint in (s), agility run in (s) and vertical jump (cm); note that for 40m sprint and agility run lower times are better.

Females		Mean ± SD					
		12	13	14	15	16	17
GREEK FEMALES	**Height**	1.55±0.06	1.60±0.06	1.60±0.06	**1.63±0.06**	**1.62±0.05****	**1.63±0.05****
	Arm Span	154.7±6.3	159.8±7.5	160.3±7.8	**162.2±7.4**	**162.0±7.1***	**162.0±6.6**
	% Sit Height	51.6±2.7	52.6±1.6	52.3±1.8	**52.5±1.4****	**52.9±1.6****	**52.6±1.7***
	Weight	**50.5±10.2****	**55.5±9.9****	**56.5±9.0***	**59.5±10.3***	59.1±9.2	58.8±7.3
	$\dot{V}o_{2\ max}$	**40.7±4.0****	**38.4±3.5****	**36.2±3.9****	**33.8±3.5****	**32.8±4.1****	**30.3±4.3****
	Basket Throw	**4.1±0.6****	**4.2±0.7****	**4.1±0.5****	**4.6±0.7****	**4.8±0.8****	**4.6±0.7****
	Throw&Catch	**5.4±4.5****	**6.8±4.6****	**5.8±3.9****	**6.3±4.7****	**6.7±4.7****	**6.8±4.8****
	40m Sprint	7.6±0.7	7.5±0.7	**7.6±0.5****	**7.7±0.6****	7.3±0.7	**7.5±0.6***
	Agility Run	21.9±1.5	**21.8±1.5**	**21.5±1.4**	**21.3±1.3***	21.0±7.3	**21.2±1.7***
	Vertical Jump	**29.1±1.5**	29.6±5.7	30.2±4.5	29.8±4.8	**30.6±4.2***	**31.2±4.7***
AUSTRALIAN FEMALES	**Height**	1.55±0.8	1.59±7.3	161.0±6.4	**164.2±6.7**	**165.1±6.6****	**165.1±6.6****
	Arm Span	155.6±9.4	159.4±9.0	161.8±8.9	**164.0±8.4**	**164.5±7.8***	**163.9±7.9**
	% Sit Height	51.8±2.0	52.3±1.9	52.4±1.9	**51.4±2.0****	**51.6±1.9****	**51.9±2.1***
	Weight	**46.3±9.4****	**50.5±9.1****	**53.0±8.6***	**56.4±8.9***	57.3±8.4	57.9±7.3
	$\dot{V}o_{2\ max}$	**44.7±4.6****	**43.8±4.5****	**42.1±4.5****	**40.5±4.9****	**38.9±4.7****	**37.2±5.2****
	Basket Throw	**5.0±0.7****	**5.24±0.8****	**5.6±0.9****	**5.8±0.8****	**6.0±0.9****	**6.2±1.0****
	Throw&Catch	**8.9±5.5****	**9.7±5.2****	**10.0±4.8****	**12.7±4.3****	**12.1±4.5****	**11.8±4.3****
	40m Sprint	7.6±0.8	7.4±0.7	**7.3±0.8****	**7.15±0.8****	7.2±0.9	**7.2±1.1***
	Agility Run	22.2±2.1	**21.31±2.1**	**21.0±2.4**	**20.8±2.6***	20.6±2.1	**20.6±2.0***
	Vertical Jump	**27.7±5.5**	29.2±5.9	29.3±6.0	30.7±6.6	**32.1±5.9***	**33.0±6.1***

mean scores and SD were converted to $\dot{V}o_{2\,max}$ values as described by Leger & Lambert (Leger and Lambert 1982),(Leger et al. 1988b). For the comparison of phenotypic traits between the adolescents from Greece and Australia, mean age group values were plotted for each gender separately. All statistical analyses were performed using Minitab statistical software (Minitab version 13.32, Minitab Ltd., Coventry, UK).

3.3 Results

3.3.1 Overweight and obesity prevalence A summary of the results for the prevalence of overweight and obesity in the Greek cohort is given in table 3.1. For the total population group, 23.6% of adolescents were overweight and 6.9% were obese (table 3.1). Overweight and obesity were more prevalent in males than in females; 24.4% of males and 22.8% of females were overweight while 8.9% of males and 4.6% of females were obese (table 3.1).

3.3.2 Mean values for Greek and Australian females A summary of the mean values and SD for the measured parameters in Greek and Australian females is given in table 3.2. Australians were significantly taller and had significantly longer arm span compared to their Greek counterparts at ages 15-17 (figure 3.1). In contrast, Greek females had significantly higher % sitting height values compared to Australians at ages 15 (52.5±1.4% vs. 51.4±2.0%, P<0.001), 16 (52.9±1.6% vs. 51.6±1.9%, P<0.001) and 17 (52.6±1.7% vs. 51.9±2.1%, P=0.003) (table 3.2; figure 3.1).

Although heavier between ages 12 and 15, Greek females did not differ significantly in mean body mass at ages 16 (59.1±9.2kg vs. 57.3±12.3kg, P=0.071) and 17 (58.8±7.3kg vs. 57.9±7.3kg, P=0.321) from Australians but had significantly lower mean $\dot{V}o_{2\,max}$ at all ages (table 3.2; figure 3.1). The highest mean $\dot{V}o_{2\,max}$ for both Greek and Australian females was observed at age 12 (40.7±4.0mlkg^{-1}min^{-1} and 45.4±4.4mlkg^{-1}min^{-1} respectively) and the lowest at age 17 (30.3±4.3mlkg^{-1}min^{-1} and 37.2±5.2mlkg^{-1}min^{-1} respectively) (table 3.2, figure 3.1).

Table 3.3: Mean values and SD for the measured parameters at different ages of males from Greece and Australia. Statistically significant differences ($P<0.05$) are indicated in bold; $P<0.01$ is indicated by (**) and $P<0.001$ by (***). (% Sit Height) stands for % sitting height and (Basket Throw) stands for basketball throw. Units are as follows: height in (m), arm span in (cm), % sitting height in (%), weight in (kg), $\dot{V}o_{2\ max}$ in ($ml kg^{-1} min^{-1}$), basketball throw in (m), throw and catch in (number of times), 40m sprint in (s), agility run in (s) and vertical jump (cm); note that for 40m sprint and agility run lower times are better.

Males		Mean ± SD					
		12	13	14	15	16	17
GREEK MALES	**Height**	**1.55±0.07**	**1.62±0.09**	1.68±0.08	1.73±0.07	1.76±0.07	**1.75±0.06***
	Arm Span	155.1±8.1	164.0±9.7	170.0±9.6	175.0±7.8	178.8±8.9	**177.2±7.3***
	% Sit Height	51.0±2.8	**51.7±1.9***	**51.8±0.7****	**51.5±1.7****	**51.9±1.5****	**52.2±1.6**
	Weight	**50.1±12.6****	**57.3±13.6****	**61.1±12.6***	**66.9±12.8**	**72.0±13.6****	**72.4±12.6***
	$\dot{V}o_{2\ max}$	**44.3±5.1****	**44.2±5.9****	**45.1±5.7****	**42.4±6.5****	**41.4±6.5****	**38.3±7.5****
	Basket Throw	**4.7±0.8****	**5.2±0.9****	**5.3±1.1****	**6.1±1.2****	**7.0±1.5****	**6.9±1.2****
	Throw&Catch	**9.1±4.6****	**11.3±5.2****	**11.0±4.7****	**12.2±4.4****	**12.2±4.2****	**11.9±4.7****
	40m Sprint	7.1±0.7	6.8±0.8	6.5±0.7	6.2±0.6	**6.0±0.5****	6.0±0.5
	Agility Run	21.0±1.6	**20.4±1.9***	**19.8±1.3**	19.2±1.2	18.8±1.4	**19.0±1.4***
	Vertical Jump	**34.0±6.0****	**35.6±7.3****	**39.4±5.9****	41.9±5.8	42.3±6.2	45.0±7.3
AUSTRALIAN MALES	**Height**	**153.1±7.6**	**160.5±8.9**	166.2±9.8	174.0±2.8	176.3±7.4	**177.5±7.6***
	Arm Span	155.1±9.9	162.8±10.4	167.7±10.8	176.2±9.1	178.3±8.5	**179.6±8.4***
	% Sit Height	51.2±2.4	**51.0±2.1***	**50.9±2.2****	**49.6±2.8****	**50.5±2.0****	**51.6±2.8**
	Weight	**43.7±7.8****	**50.9±10.8****	**56.0±12.5***	**63.4±11.2**	**66.6±10.9****	**68.5±9.7***
	$\dot{V}o_{2\ max}$	**49.0±5.6****	**49.0±5.8****	**50.2±5.6****	**48.8±6.0****	**47.4±5.7****	**46.0±5.4****
	Basket Throw	**5.6±0.8****	**6.3±1.2****	**6.9±1.2****	**8.0±1.2****	**8.5±1.3****	**9.0±1.3****
	Throw&Catch	**12.9±4.7****	**14.4±4.5****	**14.7±4.1****	**16.3±3.2****	**16.5±3.6****	**16.5±3.5****
	40m Sprint	7.1±0.7	6.9±0.7	6.58±0.7	6.4±0.9	**6.3±0.8****	6.1±0.8
	Agility Run	21.0±1.9	**19.8±2.1***	**19.3±1.9**	19.4±3.0	18.6±1.8	**18.5±1.6***
	Vertical Jump	**31.1±7.0****	**32.3±7.8****	**35.4±7.5****	42.3±9.6	41.5±8.8	45.1±9.7

Australian females performed better for basketball throw and throw and catch at all ages, were faster at ages 14 (7.3±0.8s vs. 7.6±0.5s, P<0.001), 15 (7.2±0.8s vs. 7.7±0.6s, P<0.001) and 17 (7.2±1.1s vs. 7.5±0.6s, P=0.003) and significantly more agile between ages 13-15 and at age 17 (table 3.2; figure 3.1). In contrast, Greek females jumped higher at age 12 (29.1±1.5cm vs. 27.7±5.5cm, P=0.015) but had significantly lower vertical jump scores at ages 16 (30.6±4.2cm vs. 32.1±5.9cm, P=0.002) and 17 (31.2±4.7cm vs. 33.0±6.1cm, P=0.006) (table 3.2; figure 3.1).

3.3.3 Mean values for Greek and Australian males Mean values and SD for the measured parameters in Greek and Australian males at different ages are summarised in table 3.3. Although taller at ages 12 (1.55±0.07m vs. 1.53±0.08m, P=0.037) and 13 (1.62±0.09m vs. 1.61±0.09m, P=0.028), Greek males were significantly shorter at age 17 (1.75±0.06m vs. 1.78±0.08m, P=0.004) and had significantly smaller arm span (177.2±7.3m vs. 179.6±8.4m, P=0.009) compared to their Australian counterparts. On the other hand, Greek boys had significantly higher % sitting height values at ages 13-17 compared to Australian boys of similar age (table 3.3; figure 3.2).

Mean body mass of Greek males was significantly higher at all ages; in contrast, mean $\dot{V}o_{2\,max}$ values of male Greek schoolchildren were significantly lower at all ages (table 3.3; figure 3.2). Mean $\dot{V}o_{2\,max}$ values for both Greek and Australians increased with age until 14 years (45.1±5.7ml$kg^{-1}min^{-1}$ and 50.2±5.6ml$kg^{-1}min^{-1}$ respectively) and declined thereafter reaching the lowest value at age 17 (table 3.3; figure 3.2).

Australians performed significantly better for basketball throw and throw and catch tests at all ages (table 3.3; figure 3.2). Australian males, although sprinting significantly slower at

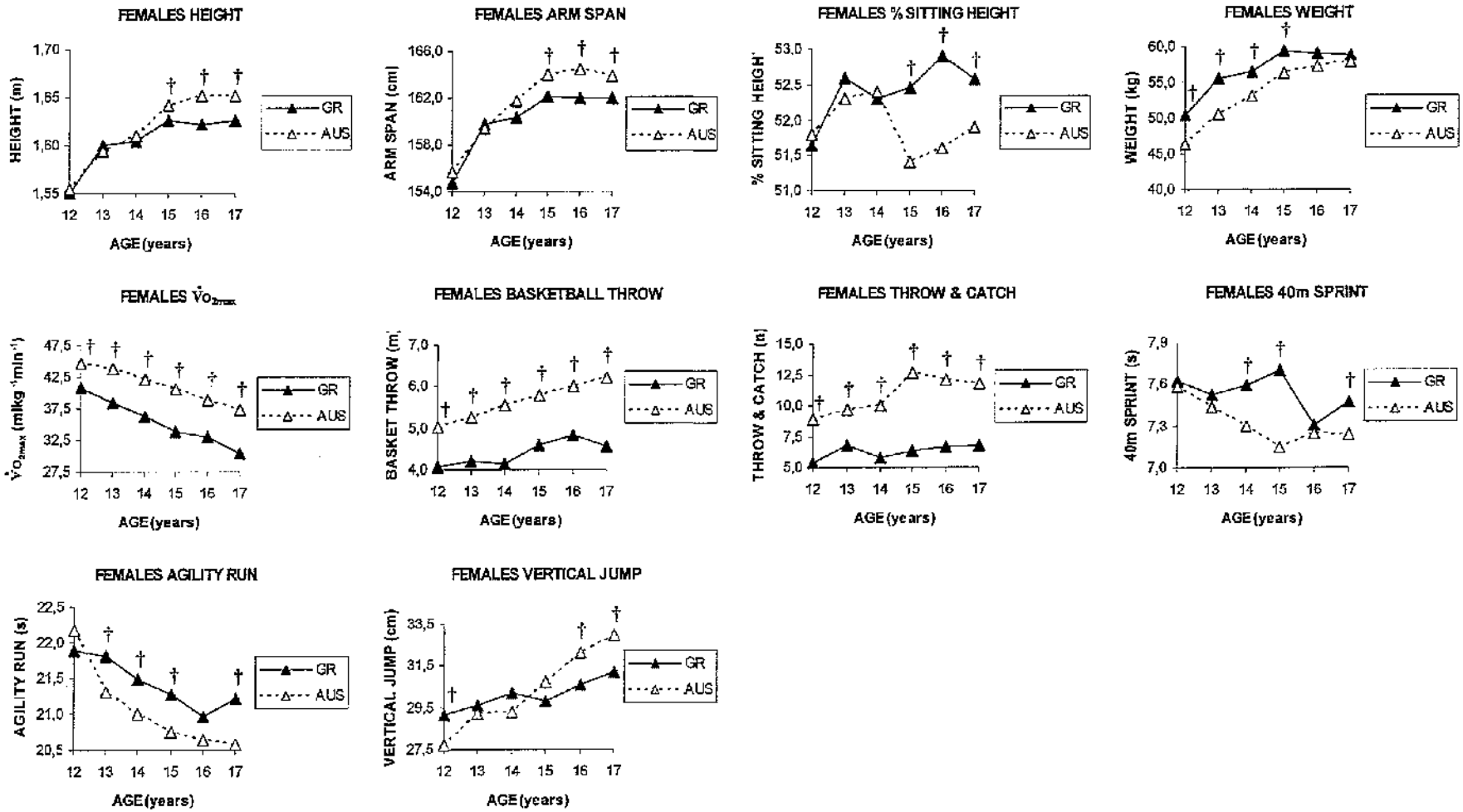


Figure 3.1: Comparison of mean values for the measured parameters between Greek and Australian females at different ages. (†) indicates statistically significant differences ($P<0.05$). Note that for 40m sprint and agility run lower times are better.

age 16 (6.3±0.8s vs. 6.0±0.5s, P<0.001), were significantly more agile at ages 13 (19.8±2.1s vs. 20.4±1.9s, P=0.004), 14 (19.8±1.3s vs. 19.3±1.9s, P=0.022) and 17 (18.5±1.6s vs. 19.0±1.4s, P=0.003) (table 3.3; figure 3.2). In contrast, Greek males jumped significantly higher at ages 12 (34.0±6.0cm vs. 31.1±7.5cm, P<0.001), 13 (35.6±1.5cm vs. 32.3±7.8cm, P<0.001) and 14 (39.4±5.9cm vs. 35.4±7.5cm, P<0.001) (table 3.3; figure 3.2).

Table 3.4: Differences in the reported prevalence of overweight and obese Greek children between the present and previous large and similarly aged Greek populations that have used the IOTF BMI cut-offs to establish overweight and obesity rates.

Location of Study	Age (years)	Females			Males		
		Number (*N*)	Overweight (%)	Obese (%)	Number (*N*)	Overweight (%)	Obese (%)
Northern Greece[1]	**11-17**	**608**	13.0	1.5	**624**	25.3	3.7
Greece (nationwide)[2]	**11-16**	**2205**	9.1	1.5	**2094**	21.7	2.5
Greece (present study)	**11-18**	**571**	22.8	4.6	**627**	24.4	8.9

[1]Data from Krassas et al. 2001, [2] Data from Karayiannis et al. 2003.

3.4 Discussion

In the present population, although the rates of overweight were similar between genders, the prevalence of obesity in males was nearly double that of females (4.6% and 8.9%, respectively), a finding that has been previously described in adolescents from Northern Greece (Krassas et al. 2001b). It is likely that females in Greece become conscious about their image earlier in life and make greater efforts to regulate their body size and shape relative to males; such behaviours could explain the aforementioned gender-specific differences in obesity rates. When compared to the findings from other large and similarly aged Greek populations that have used the IOTF BMI cut-offs (Cole et al. 2000) and for which data was available in the published literature (Krassas et al. 2001b),(Karayiannis et al. 2003) the participants of the present cohort had, on average and irrespective of gender, a higher prevalence of overweight and obesity (table 3.4). For females, the percentage of overweight and obese individuals was higher than previously reported (Krassas et al. 2001b),(Karayiannis et al. 2003); for males, although the prevalence of obesity was 5.2% to 6.4% higher, overweight rates were, on average, comparable to those from previous investigations (Krassas et al. 2001b),(Karayiannis et al. 2003). The largest differences between the overweight and obesity rates reported here and in previous investigations were observed with the nationwide study by (Karayiannis et al. 2003). (Karayiannis et al. 2003) reported overweight and obesity rates (table 3.4) that were based on self-reported data for height and body mass and as such, their results could be criticised as underestimates of the true overweight and obesity prevalence in Greece.

International surveys in developed (Magarey et al. 2001),(Hedley et al. 2004),(Jebb et al. 2004) and developing nations (Neutzling et al. 2000),(Chu 2001) indicate that overweight and obesity rates in children and adolescents have increased alarmingly. Considering the

participants of the *Talent Search* program (1993) (Australian Sports Commission 1993), the lack of available data for their BMI did not allow direct assessments of their overweight and obesity rates. However, relative to other reports in Australian populations (Magarey et al. 2001) (table 3.5) the prevalence of overweight and obesity was, at large, higher in the present cohort of Greek adolescents. A recent investigation by (Lobstein and Frelut 2003) has reported on the overweight rates in a number of European countries; the above study used the BMI cut-offs established by IOTF (Cole et al. 2000) to re-assess the results of previous studies that have employed different criteria to establish the prevalence of overweight in their subject populations. It must be noted that in their analysis (Lobstein and Frelut 2003) have only considered reports published in the international literature from surveys performed after 1990 in representative samples of children that had obtained their data from direct anthropometric measurements. In table 3.6, the overweight rates in the present population of Greek adolescents are presented relative to the prevalence of overweight reported in a number of European countries and Australia (Magarey et al. 2001),(Lobstein and Frelut 2003). The overall prevalence of overweight in the present cohort of adolescent children was 23.6%, a score that ranked second highest after Spain (*i.e.* 27.5%) and higher than the UK (*i.e.* 20.5%) between twelve countries. These results for Greek adolescents were in agreement with previous reports (Krassas et al. 2001b),(Lissau et al. 2004) suggesting the presence of a high prevalence of overweight in Greek children.

Irrespective of gender, Greek adolescents, had a higher mean body mass for most age groups when compared to Australian children from the *Talent Search* program (1993) (Australian Sports Commission 1993). It is well established that children normally increase their body mass and body fat content through puberty (Hergenroeder and Klish 1990). For

Table 3.5: Prevalence of overweight and obesity in the present population of adolescent Greeks relative to the overweight and obesity rates reported in Australians by (Magarey et al. 2001).

Location of Study	Age (years)	Females			Males			Total		
		Number (*N*)	Overweight (%)	Obese (%)	Number (*N*)	Overweight (%)	Obese (%)	Number (*N*)	Overweight (%)	Obese (%)
Australia	**2-18**	**1447**	15.8	5.3	**1515**	15.0	4.5	**2962**	15.4	4.9
Greece (present study)	**11-18**	**571**	22.8	4.6	**627**	24.4	8.9	**1198**	23.6	6.9

all participants, mean body mass, on average, increased with age; in females however, mean body mass remained relatively unchanged between 15 and 17 years of age, an observation that may be representative of gender-specific differences in image awareness, as already discussed. When compared to the Australian normative data, Greek adolescents, irrespective of gender and age, had a significantly lower mean $\dot{V}o_{2\,max}$ (tables 3.2 and 3.3); in females, $\dot{V}o_{2\,max}$ decreased linearly with age while, in males, $\dot{V}o_{2\,max}$ increased until 14 years of age and linearly decreased thereafter. $\dot{V}o_{2\,max}$ is inversely associated with sexual maturation (Meredith and Dwyer 1991) hence the gender-specific differences in the distribution of $\dot{V}o_{2\,max}$ may be consistent with an earlier entrance of females in puberty. Previous investigations have suggested that, relative to other published reports, the levels of the aerobic fitness of Australian adolescents range from poor to average (Tomkinson et al. 2003b). As such, the present findings would suggest that the aerobic fitness of Greek adolescents, as indicated by their $\dot{V}o_{2\,max}$, was poor. The data for the $\dot{V}o_{2\,max}$ of the Australian and Greek adolescents were compared to the EUROFIT $\dot{V}o_{2\,max}$ norms produced by the Council of Europe as part of an initiative to promote sport participation and physical fitness (Council of Europe 1988) (table 3.7). This comparison indicated that the age-specific mean $\dot{V}o_{2\,max}$ values of Australian adolescents were, at large, identical to the reciprocal values of the EUROFIT data while the $\dot{V}o_{2\,max}$ values of Greek adolescents were the lowest among the three. In light of the aforementioned findings, it is clear that the Australian normative data for $\dot{V}o_{2\,max}$ were consistent with average levels of aerobic fitness relative to other European populations while the aerobic fitness of Greek adolescents was lower than the Australian and European standards.

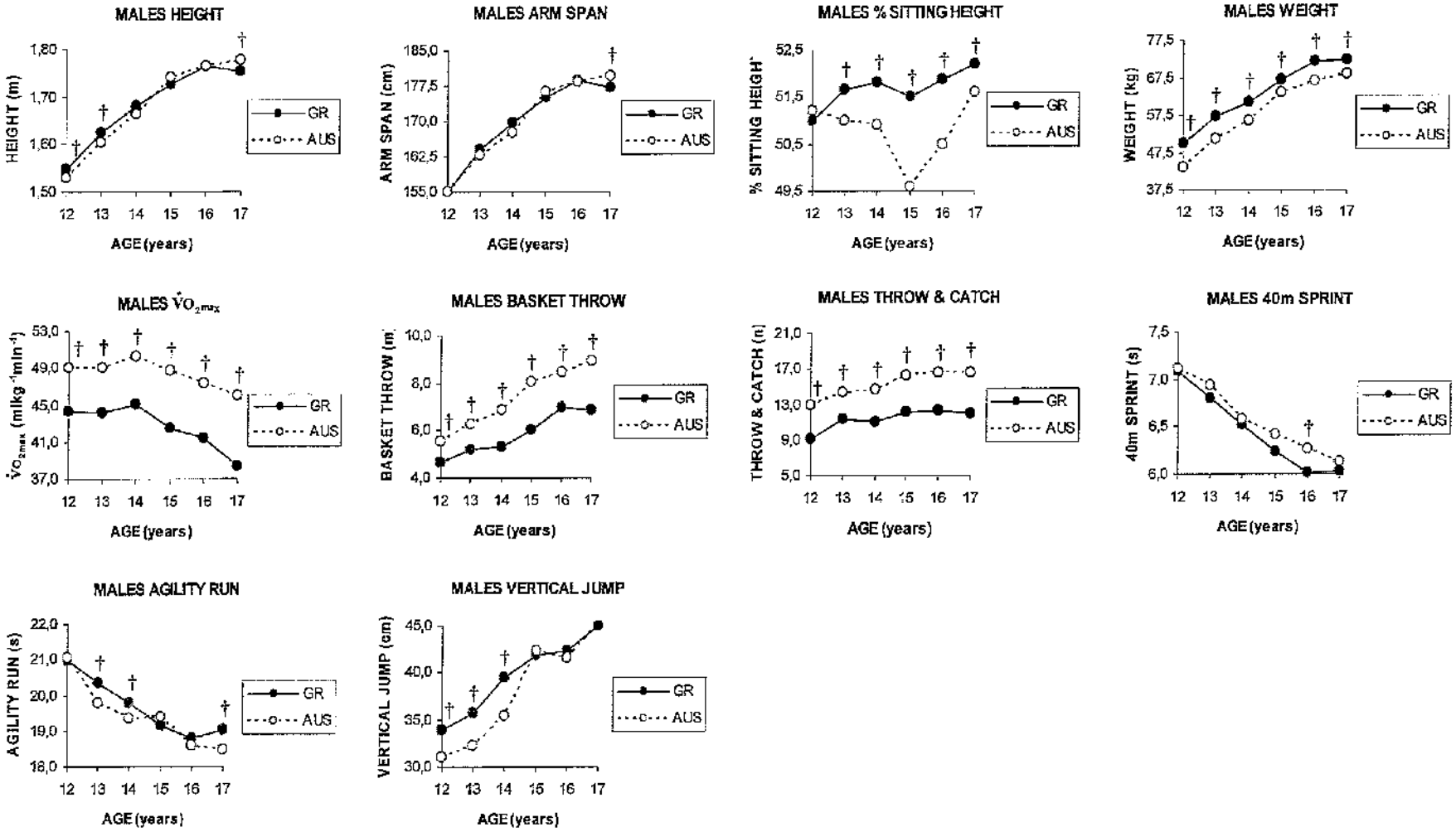


Figure 3.2: Comparison of mean values for the measured parameters between Greek and Australian males at different ages. (†) indicates statistically significant differences ($P \leq 0.05$). Note that for 40m sprint and agility run lower times are better.

As indicated by the age-specific distribution variations in mean arm span of Greek and Australian children (figures 3.1 and 3.2), arm span correlated strongly with variations in height irrespective of gender; these findings are in the same direction with the results of previous investigations (Cheng et al. 1998). In addition, Australian females grew taller and had longer arm span during the later stages of adolescence; for males, height and arm span differences were less pronounced. Nevertheless, Greek adolescents had, on average, higher mean % sitting height. The observed variations in % sitting height were not matched to reciprocal variations in stature hence the higher mean % sitting height of Greek children would be most likely attributed to a longer trunk length relative to their stature. As previous studies have indicated (Gasser et al. 2000),(Gasser et al. 2001), skeletal growth patterns are influenced by gender, age and environment; such effects, if present, could partly explain the differences in the distribution of mean height, arm span and % sitting height between Australian and Greek schoolchildren.

Australian adolescents, on average, performed better than their Greek counterparts in agility and upper-body strength and skill-related tests. Body composition, is inversely related to physical fitness phenotypes (Minck et al. 2000),(Deforche et al. 2003) hence, the higher mean body mass of Greek adolescents could explain, at least in part, their lower agility and upper-body strength-related performance. Motor fitness, among others, involves physical skill that is, in turn, influenced by factors such as development and physical activity (Volver et al. 2000); developmental effects and the lower physical activity of Greek adolescents, as suggested by their lower $\dot{V}o_{2\,max}$, may also serve to explain their lower agility and upper-body strength-related performance. Considering the inverse relationship between body composition and most weight-bearing and physical fitness-related phenotypes such as vertical jump trials (Minck et al. 2000),(Deforche et al. 2003),

Table 3.6: Prevalence of overweight in the present population of adolescent Greeks relative to the overweight rates reported in the internationally published literature for a number of European countries and Australia. Data for overweight rates in European countries were adapted from a recent review by Lobstein and Frelut 2003. Data for Australian were obtained from Magarey et al. 2001.

Location of Study	Age (years)	Number (*N*)	Overweight (%)
Australia	**2-18**	**2962**	15.4
Belgium	**6-12**	**1026**	18.0
Cyprus	**6-17**	**2467**	17.9
Denmark	**5-17**	**11218**	16.0
Germany	**1-17**	**32429**	14.5
The Nederlands	**0-21**	**14377**	11.5
Poland	**0-17**	**10654**	15.0
Russia	**6-18**	**2688**	9.5
Spain	**5-17**	**1637**	27.5
Sweden	**9-11**	**6700**	18.0
UK	**5-17**	**2882**	20.5
Greece (present study)	**11-18**	**1198**	23.6

Australians would have been expected to outperform Greek adolescents in tests of lower-body strength-related performance. Nevertheless, Australians were not outright better performers in lower-body strength-related tests; Australian males in particular had a mean lower-body strength-related performance that was comparable, and on occasion lower, relative to their Greek counterparts. The most likely explanation for the observed variation in lower-body strength-related performance would be the presence of small numbers of phenotypically extreme Greek or Australian individuals exerting an influence on the mean phenotypic values of certain age groups. For example, the presence of a number of extremely high-scoring Greek or low-scoring Australian males could explain the significant differences in mean vertical jump for 12, 13 and 14 year-olds.

The number of the observed significant differences between the phenotypic means of Australian and Greek adolescents, stratified for age and gender, may have been subjected to type I (false positive) errors. Accounting for multiple testing by the Bonferroni or a similar correction would have allowed for such effects however, as indicated in General Methods (p. 50-54), due to the large number of tests involved such approaches may have been overly conservative (Perneger TV 1998),(Sokal and Rohlf 2001),(Sabatti et al. 2003). For each gender, 66 *t*-tests were performed (6 age groups x 11 phenotypes) and approximately 3 of these tests would be expected to be statistically significant by chance at a probability level of 0.05 (0.05x66=3.3). However, the number of significant differences found here (41 for females and 40 for males) was far greater than what would be expected by chance alone. It can be suggested therefore with some conviction that the significant differences found here represented more than an effect of multiple testing.

Table 3.7: Age-specific mean values for $\dot{V}o_{2\ max}$ of females and males from Greece and Australia relative to the EUROFIT norms (Council of Europe 1988). Units for $\dot{V}o_{2\ max}$ are ($ml kg^{-1} min^{-1}$). Irrespective of gender, the age-specific mean normative data for $\dot{V}o_{2\ max}$ from Australian children are, at large, identical to the EUROFIT data. In contrast, the age-specific mean $\dot{V}o_{2\ max}$ scores of Greek children are, irrespective of gender, the lowest recorded.

		Mean $\dot{V}o_{2\ max}$					
		12	**13**	**14**	**15**	**16**	**17**
FEMALES	**EUROFIT**	45.4	43.8	39.4	40.5	38.9	37.2
	AUSTRALIAN	44.7	43.8	42.1	40.5	38.9	37.2
	GREEK	40.7	38.4	36.2	33.8	32.8	30.3
MALES	**EUROFIT**	50.5	49.0	47.5	48.8	47.4	48.9
	AUSTRALIAN	49.0	49.0	50.2	48.8	47.4	46.0
	GREEK	44.3	44.2	45.1	42.4	41.4	38.3

3.5 Conclusion

The present population, thought to be representative of adolescent children from Greece, is the only cohort of Greek adolescents that includes such a wide range of physiological, physical and skill-related physical fitness data. The prevalence of overweight and obesity in the present cohort of Greek adolescents was higher relative to other Australian and Greek populations and, considering overweight rates alone, among the highest relative to other populations in Europe. Compared to normative data from Australian children of similar age, Greek adolescents had higher mean body mass and were, at large, outperformed in agility and upper-body strength and skill-related trials. In addition, the aerobic fitness of Greek adolescents was lower than the Australian and European norms. The findings for the physical, physiological and skill-related physical fitness of Greek relative to Australian children were most likely reflective of their higher adiposity and lower than average aerobic fitness.

CHAPTER 4

EFFECTS OF INTERACTION BETWEEN ACE POLYMORPHISMS AND LIFESTYLE ON OBESITY-RELATED PHENOTYPES IN ADOLESCENT GREEKS

4.1 Introduction

ACE is a key component of the endocrine RAS; in humans, RAS systems have been described in several tissues, significantly including the adipose tissue (Engeli et al. 1999) where ACE has been suggested to influence growth, differentiation (Negrel et al. 1989) and metabolism (Hennes et al. 1996). Although the ACE gene is highly polymorphic, historically work has centred on an Insertion/Deletion polymorphism characterised by the presence or absence of a 287 base-pair (bp) *Alu* sequence in the 16th intron of the gene (Rigat et al. 1990). In Caucasians, the I-allele has been associated with low circulating ACE activity levels (Rigat et al. 1990), lower ACE gene expression (for example (Davis et al. 2000)) and increased endothelial cell survival (Hamdi and Castellon 2003). The genetic variation in the ACE gene defines 13 haplotypes; only four of these are found in Caucasians with the I-allele associating with haplotype H6 and the D-allele associating with the remaining three (Rieder et al. 1999). Previous studies have sought to associate the I/D polymorphism with disease phenotypes such as myocardial infarction (Schelleman et al. 2004) and elite athlete performance (for example (Scanavini et al. 2002)). A recent cross-sectional longitudinal study (Strazzullo et al. 2001) of Italian adult men reported a significant interaction of the ACE DD genotype with increased BMI, waist circumference and diastolic blood pressure in relation to age.

Given its involvement with adipose tissue, and association with obesity in middle-aged Italian men, the present study wished to elucidate the effects of individual polymorphisms and haplotypes of the ACE gene on obesity-related phenotypes in an adolescent population from Greece. Genotype-environment interactions with gender and participation in organised physical activity were also considered.

4.2 Methods

4.2.1 Subjects and measurements For the present analysis, all information regarding subjects and measurements of physical, physiological and skill-related parameters in the Greek cohort were as described in General Methods (p. 33-37). The parameters assessed here included: BMI, skinfolds at the triceps and subscapular sites, total skinfolds and skinfolds estimated % body fat.

4.2.2 DNA extraction, genotype determination and haplotype inference DNA extraction, quantification and storage as well as ACE I/D, G7831A (rs4424958) and C8968T (rs4311) genotype determination and ACE haplotype inference were performed as described in General Methods (p. 37-42).

4.2.3 Data Analysis All data and statistical analyses were carried out as described in General Methods (p. 45-54).

Table 4.1: Analysis of associations between genotypes/diplotypes and obesity-related phenotypes in total males and females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that subscapular skinfolds has been abbreviated to (Subscap Skinfolds).

		Total Females					Total Males				
		BMI	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**	**BMI**	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**
Polymorphism	**I/D**	P=0.169 V=0.7% N=480	**P=0.019** **V=1.7%** **N=481**	**P=0.028** **V=1.5%** **N=481**	**P=0.010** **V=1.9%** **N=481**	**P=0.012** **V=1.8%** **N=481**	P=0.461 V=0.3% N=537	P=0.164 V=0.7% N=536	P=0.494 V=0.3% N=535	P=0.307 V=0.4% N=535	P=0.303 V=0.4% N=535
	G7831A	P=0.309 V=0.5% N=461	**P=0.025** **V=1.6%** **N=462**	P=0.186 V=0.7% N=462	**P=0.036** **V=1.4%** **N=462**	**P=0.033** **V=1.5%** **N=462**	P=0.192 V=0.6% N=515	P=0.489 V=0.3% N=514	P=0.741 V=0.1% N=513	P=0.541 V=0.2% N=513	P=0.532 V=0.2% N=513
	C8968T	P=0.431 V=0.4% N=461	**P=0.004** **V=2.4%** **N=462**	*P=0.053* *V=1.3%* *N=462*	**P=0.009** **V=2.0%** **N=462**	**P=0.008** **V=2.1%** **N=462**	P=0.245 V=0.5% N=515	P=0.142 V=0.8% N=514	P=0.410 V=0.3% N=513	P=0.242 V=0.6% N=513	P=0.233 V=0.6% N=513
	Diplotypes	P=0.921 V=0.6% N=448	*P=0.083* *V=2.8%* *N=449*	P=0.392 V=1.6% N=449	P=0.187 V=2.2% N=449	P=0.179 V=2.3% N=449	P=0.496 V=1.3% N=502	P=0.761 V=0.8% N=501	P=0.790 V=0.8% N=501	P=0.720 V=0.9% N=501	P=0.728 V=0.9% N=501

4.3 Results

4.3.1 Genotyping Genotyping was successful in 1019 individuals (537 males and 482 females). All populations and subpopulations were in Hardy-Weinberg equilibrium (HWE) for all polymorphic loci tested; no significant differences were found in allele or haplotype frequencies. Overall allele frequencies were $f_{(D)}$=0.57 and $f_{(I)}$=0.43 for the I/D polymorphism, $f_{(G)}$=0.60 and $f_{(A)}$=0.40 for the G7831A (rs4424958) polymorphism and $f_{(C)}$=0.48 and $f_{(T)}$=0.52 for the C8968T (rs4311) polymorphism. Diplotypes (haplotype allelic combinations) were inferred for 952 individuals; haplotype frequencies were $f_{(H1)}$=0.40, $f_{(H6)}$=0.42, $f_{(H7)}$=0.13 and $f_{(H9)}$=0.06. Only 25 out of 952 individuals showed evidence of rare recombinant haplotypes even after re-genotyping. Re-genotyping of thirty individuals gave identical results on each occasion. Together with the HWE of the population these provided strong validation of the genotyping methods used.

4.3.2 Genotype associations with phenotypes. For the group of total females, statistically significant differences were observed between the means of the I/D genotypes and the triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat (table 4.1). II individuals had the lowest mean values for all phenotypes in contrast to DD individuals that had the highest (data not shown). Statistically significant differences in total females were also observed between the means of the G7831A (rs4424958) genotypes for the triceps skinfolds, the total skinfolds and skinfolds estimated % body fat and for the means of the C8968T (rs4311) genotypes and the triceps skinfolds, the total skinfolds and skinfolds estimated % body fat (table 4.1). For the G7831A (rs4424958) genotype, G homozygotes had the lowest mean skinfolds values relative to A homozygotes and heterozygotes while for the C8968T (rs4311) genotype, C homozygotes had the lowest

mean skinfolds values relative to T homozygotes and heterozygotes (data not shown). No statistically significant differences were detected in the groups of total males (table 4.1).

4.3.3 Analysis by level of regular activity A summary of the female ANOVA results is shown in table 4.2. No associations were observed for active females; however, several associations were detected between obesity-related phenotypes and each of the ACE polymorphisms in the inactive female population (table 4.2). The I/D polymorphism showed the strongest associations with the smallest *P*-values and largest % of the variance explained and was significantly associated with each of the phenotypes; BMI, triceps skinfolds, subscapular skinfolds, total skinfolds and percentage body fat (table 2). II homozygotes had the lowest mean BMI, skinfolds and skinfolds estimated % body fat values in contrast to DD individuals that had the highest (for example figure 4.1).The G7831A (rs4424958) polymorphism showed associations with total skinfolds and skinfolds estimated % body fat (table 4.2) with G homozygotes having the lowest mean values relative to A homozygotes and heterozygotes (data not shown). The C8968T (rs4311) polymorphism showed associations with triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat (table 4.2) with the C homozygotes having the lowest mean values relative to T homozygotes and heterozygotes for all phenotypes (data not shown). No statistically significant differences were detected in the groups of active and inactive males (table 4.3).

4.3.4 Determination of the type of genetic effect observed for associations with the I/D polymorphism In all cases, where significant associations had been identified by the ANOVA tests, an additive genetic model, where the heterozygote was exactly halfway between the two homozygotes, could explain almost all the variance. It accounted for

Table 4.2: Analysis of associations between genotypes/diplotypes and obesity-related phenotypes in inactive and active females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that subscapular skinfolds has been abbreviated to (Subscap Skinfolds).

		Inactive Females					Active Females				
		BMI	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**	**BMI**	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**
Polymorphism	**I/D**	**P=0.041** **V=3.1%** **N=206**	**P=0.012** **V=4.3%** **N=206**	**P=0.001** **V=6.6%** **N=206**	**P=0.001** **V=6.6%** **N=206**	**P=0.001** **V=6.5%** **N=206**	P=0.741 V=0.2% N=274	P=0.511 V=0.5% N=275	P=0.950 V<0.1% N=275	P=0.749 V=0.2% N=275	P=0.787 V=0.2% N=275
	G7831A	P=0.197 V=1.7% N=196	P=0.110 V=2.3% N=196	*P=0.071* *V=2.7%* *N=196*	**P=0.042** **V=3.2%** **N=196**	**P=0.039** **V=3.3%** **N=196**	P=0.556 V=0.4% N=265	P=0.189 V=1.3% N=266	P=0.911 V<0.1% N=266	P=0.479 V=0.6% N=266	P=0.468 V=0.6% N=266
	C8968T	P=0.321 V=1.2% N=196	**P=0.015** **V=4.3%** **N=196**	**P=0.013** **V=4.4%** **N=196**	**P=0.006** **V=5.2%** **N=196**	**P=0.005** **V=5.4%** **N=196**	P=0.841 V=0.1% N=265	P=0.160 V=1.4% N=266	P=0.805 V=0.2% N=266	**P=0.419** V=0.7% N=266	P=0.427 V=0.6% N=266
	Diplotypes	P=0.624 V=2.8% N=190	P=0.325 V=4.3% N=190	*P=0.067* *V=6.9%* *N=190*	P=0.111 V=6.1% N=190	P=0.104 V=6.3% N=190	P=0.943 V=0.9% N=258	P=0.323 V=3.1% N=259	P=0.977 V=0.6% N=259	P=0.780 V=1.6% N=259	P=0.733 V=1.7% N=259

95%, 99%, 99% and 99% respectively, of the variance, when considering the I/D polymorphism versus triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat, for all females. When considering the I/D polymorphism versus BMI, triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat, for inactive females, an additive model would explain 100%, 97%, 99%, 98% and 99% of the variance respectively. A small dominance effect and other environmental effects would account for the remaining variance.

4.3.5 Associations of ACE genetic variants with extreme phenotypes The odds ratios (table 4.4) were broadly consistent with the phenotypic means reported in the ANOVAs and the genetic models identified by the correlation analyses. The significant ANOVA results above could either be due to all II individuals getting thinner (and DD individuals getting fatter), or due to a small number of very thin II (and very fat DD) individuals. For most of the phenotypes below, the II individuals were over-represented in the low quartile and under-represented in the high quartile (table 4.4). To a lesser extent, in several cases the DD individuals were also over-represented in the high quartile. This suggests that all II individuals were a little thinner (and DD individuals a little fatter), rather than a small number of extreme individuals influencing the ANOVAs. Figure 4.2 shows a comparison of inactive and active females for one of the phenotypes shown to be associated with the ACE polymorphisms above.

When considered as total females, II homozygotes were significantly over-represented in the low group for triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat (table 4.4). II homozygotes were, concomitantly, significantly under-

represented in the high group for subscapular skinfolds, total skinfolds and skinfolds estimated % body fat (table 4.4). DD homozygotes were over-represented in the high group for total skinfolds and skinfolds estimated % body fat (table 4.4).

For inactive females, II homozygotes were significantly over-represented in the low group for BMI, triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat (table 4.4). They were, concomitantly, significantly under-represented in the high group for BMI, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat (table 4.4) and the middle group for triceps skinfolds (P=0.007, OR=0.37; 95% CI, 0.17 to 0.78). DD homozygotes were significantly over-represented in the high group for BMI, total skinfolds and skinfolds estimated % body fat and under-represented in the low group for triceps skinfolds (table 4.4).

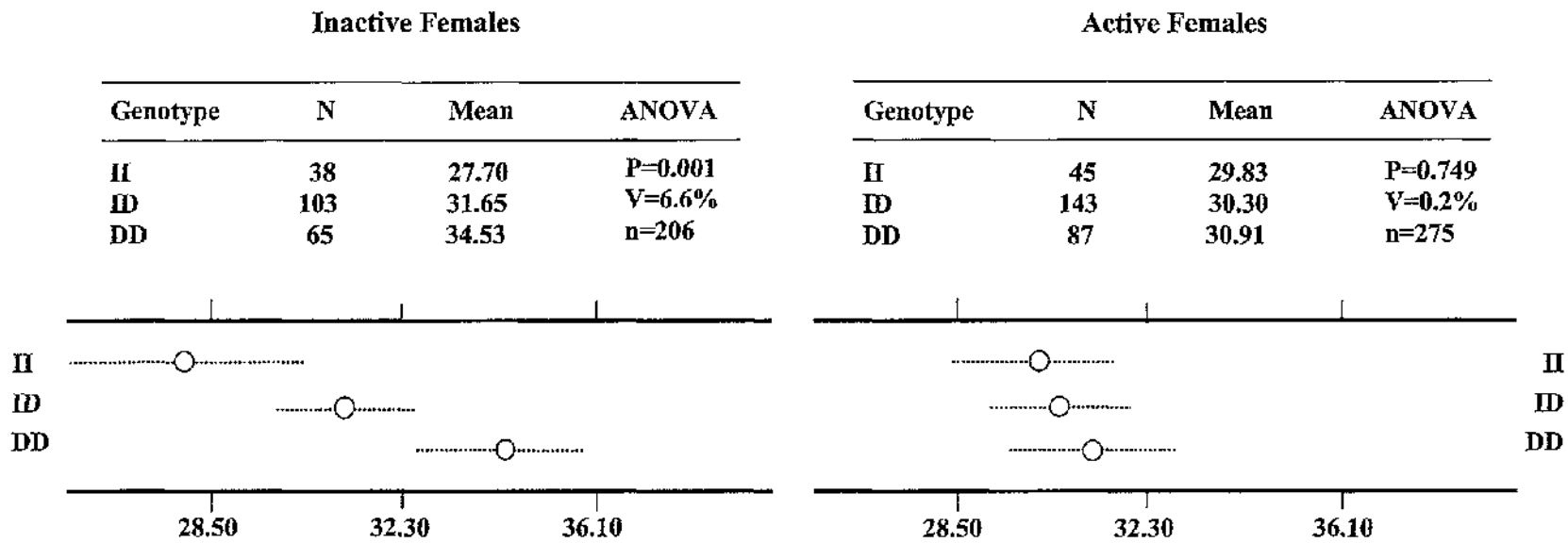


Figure 4.1: ID genotype differences in total skinfolds of inactive and active females. Inactive II homozygotes have significantly lower total skinfolds (mm) when compared to the ID and DD individuals. (P)=probability, (V)=observed variance explained, (N)=number within group.

4.4 Discussion

This study has been among the first few to investigate the effects of ACE genetic variants in combination with lifestyle choices in adolescent children. Associations were found only in female subjects, suggesting that ACE has a gender-specific effect when influencing obesity-related phenotypes in adolescents. In particular, the I-allele associated with lower obesity-related scores in contrast to the D-allele that, in the homozygote state, associated with increased BMI and adiposity. Considering participation in organised physical activity, these associations were only found in inactive females suggesting that lifestyle can modify the effects of ACE.

No previous studies have attempted to associate the G7831A (rs4424958) or C8968T (rs4311) variants with adiposity. As suggested in General Methods (p. 40), these polymorphisms are thought not to be functional and, as such, they are not obvious candidate loci; they were simply selected because they allowed differentiation between the four Caucasian haplotypes (Rieder et al. 1999) in the adolescent cohort. Haplotyping allows the investigation of how a number of SNPs work in concert, and also infers information about untested SNPs that associate with the given haplotypes. As such, several previous studies have advocated the use of haplotype-based methods to better capture gene effects that may mediate susceptibility to disease or complex traits (for examples (Akey et al. 2001),(Pritchard and Cox 2002),(Weiss and Clark 2002)). Although an association was observed between the G7831A (rs4424958) and C8968T (rs4311) markers and several of the obesity-related phenotypes investigated here, no functional argument can be made for their involvement in mediating the functional effects of the ACE gene. The significant associations observed in total females indicated that the C8968T (rs4311) variant explained a higher % of variance than the I/D polymorphism however when participation in

organiscd physical activity was taken into account it was the I/D polymorphism that, on average, explained the higher % of variance. These findings would indicate a stronger genetic effect on phenotype for the I/D, relative to the C8968T (rs4311), variant that in the group of total females was masked by influences from organised physical activity participation. For significant associations, the G7831A (rs4424958) polymorphism explained a lower % of variance when compared to both the I/D and the C8968T (rs4311) polymorphisms. It is more likely therefore, that the G7831A (rs4424958) and C8968T (rs4311) were simply in LD with a functional polymorphism(s).

The use of ACE haplotypes to analyse a possible ACE gene-obesity-environment interaction did not reveal any significant differences between the means of the adiposity indices for individual haplotypes. Such a finding was disappointing, although if the putative functional polymorphism does not associate with a single haplotype in Greeks, haplotype analysis may have been subdividing the crucial groups. This highlights the importance of identifying candidate SNPs and their haplotypes on a functional basis. Another possible explanation for the lack of association with haplotypes may be the increased number of degrees of freedom (relative to the genotype association tests). This allows an increase in the variance explained, but it may also have sufficiently reduced the power of the statistical analysis to identify the significant but rather small influence of the ACE gene on adiposity, as this is indicated by the % of the total variance explained for each phenotype. The findings with regards to haplotype use are not evidence against the importance of haplotype-based approaches when analysing complex traits. The use of haplotypes does offer more detailed analytical ability than individual SNP approaches but is directly linked to the statistical detecting power of the cohort under investigation and the strength of the effect. Thus it is possible that the present study population was not large

Table 4.3: Analysis of associations between genotypes/diplotypes and obesity-related phenotypes in inactive and active males. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that subscapular skinfolds has been abbreviated to (Subscap Skinfolds).

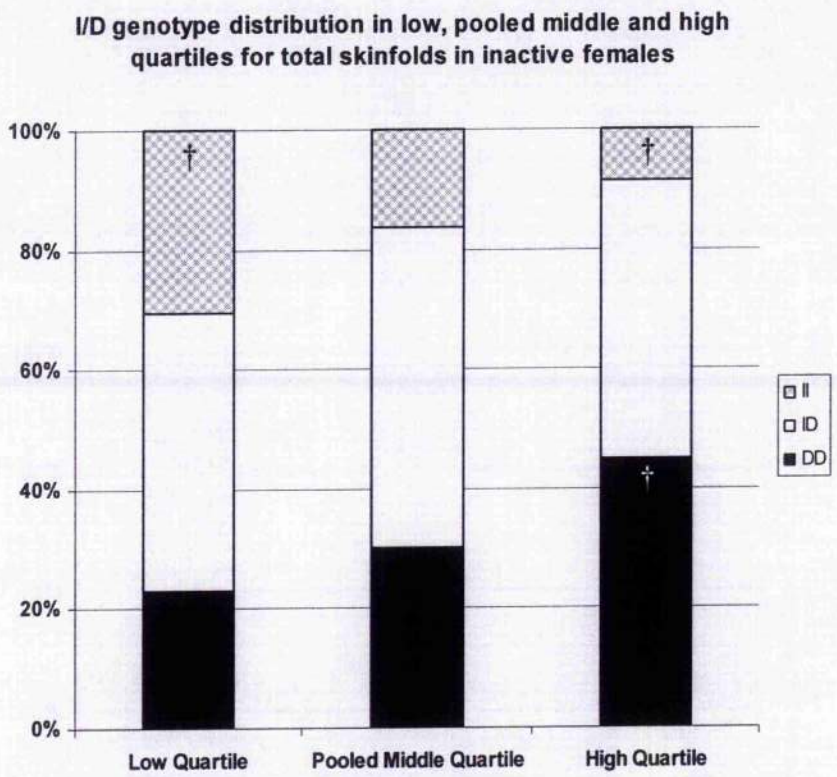
		Inactive Males					**Active Males**				
		BMI	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**	**BMI**	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**
Polymorphism	**I/D**	P=0.976 V<0.1% N=113	P=0.796 V=0.4% N=113	P=0.687 V=0.7% N=113	P=0.707 V=0.6% N=113	P=0.715 V=0.6% N=113	P=0.237 V<0.1% N=424	P=0.150 V=0.9% N=423	P=0.436 V=0.4% N=422	P=0.246 V=0.7% N=422	P=0.243 V=0.7% N=422
	G7831A	P=0.944 V=0.1% N=108	P=0.295 V=2.3% N=108	P=0.757 V=0.5% N=108	P=0.467 V=1.4% N=108	P=0.470 V=1.4% N=108	P=0.275 V<0.1% N=413	P=0.853 V<0.1% N=412	P=0.960 V<0.1% N=411	P=0.844 V<0.1% N=411	P=0.852 V<0.1% N=411
	C8968T	P=0.749 V=0.6% N=108	P=0.624 V=0.9% N=108	P=0.858 V=0.3% N=108	P=0.839 V=0.3% N=107	P=0.857 V=0.3% N=108	P=0.302 V<0.1% N=413	P=0.316 V=0.6% N=412	P=0.551 V=0.3% N=411	P=0.412 V=0.4% N=411	P=0.421 V=0.4% N=411
	Diplotypes	P=0.125 V=10.6% N=107	P=0.536 V=5.8% N=107	P=0.353 V=7.4% N=107	P=0.385 V=7.1% N=107	P=0.407 V=6.9% N=107	P=0.728 V=1.1% N=408	P=0.901 V=0.7% N=407	P=0.877 V=0.8% N=406	P=0.868 V=0.8% N=406	P=0.884 V=0.7% N=406

enough to support the investigation of a gene like ACE with such modest effects on phenotype when there were so many different genetic groupings. Such problems have been discussed in the literature (for a review (Pritchard and Cox 2002)) and may also be one of the major reasons for the shortcomings of other investigations. At the same time, the inability of the present study to show an effect of the ACE gene using a haplotype based approach does not mean that ACE has no role in determining adiposity levels in young females and does not contradict the findings from multiple individual SNPs.

The significant phenotypic differences between genotypes were best explained by additive genetic models. An additive genetic model is, biologically, the most parsimonious model where increasing the dosage of a particular allele directly increases the size of its effect. A good example of this is shown in figure 4.1, where, for total skinfolds, the mean of the ID group is approximately half way between the means of the two homozygote groups. Where significant, the risk of being in the low (thin) quartile for the phenotypes tended to be raised for IIs and reduced for DDs, and for being in the middle or bottom (fat) quartile the risk tended to be reduced for IIs and raised for DDs. These risks were consistent with ACE having a modest effect on the whole inactive female population, rather than a large effect on a few extreme individuals. Once split into appropriate groups the associations between the obesity-related phenotypes and the I/D polymorphism were much stronger than the others. Previous studies have also shown that the I/D polymorphism associates with circulating ACE activity (Rigat et al. 1990),(Tiret et al. 1992). Furthermore, the I/D polymorphism has been suggested to associate with the levels of ACE expression (Davis et al. 2000),(Mizuiri et al. 2001) and with endothelial cell survival (Hamdi and Castellon 2003). These findings would suggest that the I/D polymorphism may be the causal variant of the observed effects on adiposity. Although possible that the insertion or deletion of an

Alu sequence in an intron (*i.e.* the I/D polymorphism) could influence the function of the ACE gene, it is unlikely that such a polymorphism may be reflected in post-transcriptional regulation; the I/D polymorphism is not known to be closely associated with amino acid changes hence alterations of protein function. Nevertheless, the association of the I/D polymorphism with the levels of ACE gene expression (Davis et al. 2000),(Mizuiri et al. 2001) may suggest an influence of the *Alu* insert on transcriptional regulation. However, it may be more probable that another polymorphism(s) in strong LD with the *Alu* insert is responsible for the observed effects (Tiret et al. 1992). Two previous studies (Zhu et al. 2000),(Cox et al. 2002) have suggested that such a functional polymorphism is A22982G (rs4363) given that this polymorphism is in much stronger association with circulating ACE levels in Africans where it is not in complete LD with the I/D polymorphism. This would also help explain why no association was observed between the obesity-related phenotypes and the diplotypes since, as suggested above, one of the functionally different groups (D) would have been split into three parts (H1, H7 and H9) in that analysis. However, in spite of its probable lack of functionality, the I/D polymorphism, in Caucasians at least, is a sufficiently good marker of circulating ACE activity to use in prospective association studies.

Association studies may be subjected to type I errors caused by multiple testing. For example, in the group of total females a sum of 20 tests were conducted (5 phenotypes x 4 genotypes/diplotypes); one of these tests would be expected to be statistically significant ($P<0.05$) by chance, given the number of tests performed for each gender (0.05 x 20 tests=1). Of these tests, 10, uncorrected for multiple testing effects, were found to be statistically significant ($P<0.05$). The number of tests actually found to be statistically significant was greatly in excess of what would be expected from chance alone hence, the



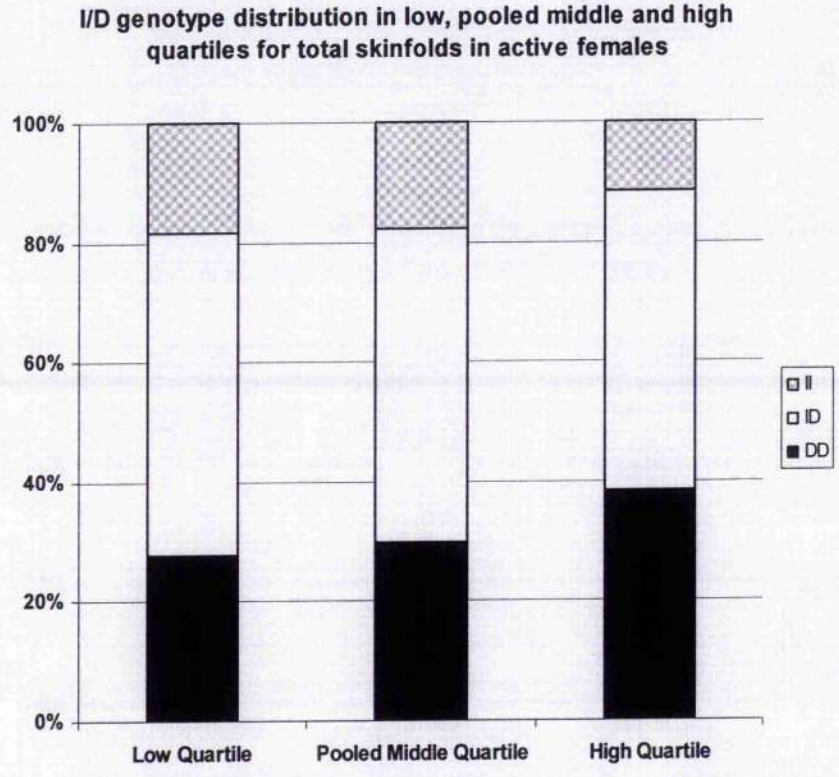


Figure 4.2: ID genotype distributions in the low, pooled middle and high quartiles for total skinfolds in inactive and active males. Within quartiles, significantly over or under-represented genotypes (table 4.4) are indicated by (†). II homozygotes are significantly over-represented in the low quartile group and under-represented in the high quartile group, while DD homozygotes are over-represented in the high quartile group.

influence of ACE genetic variants on obesity-related phenotypes in total females must have represented more than an effect of multiple testing. Considering the relative strength and consistency of the effects over a number of phenotypes and across activity-divided population groups in addition to the significant genetic effects, as indicated by the ORs, across the phenotypic distributions of the measured parameters served to increase confidence in the validity of significant findings for the ACE genotype in adolescent females. Irrespective of these considerations, as indicated in General Methods (p. 52-54), a Šidak correction for multiple testing in the presence of partially correlated phenotypes was performed on the data for BMI, triceps and subscapular skinfolds; total skinfolds and skinfolds estimated % body fat measurements have been excluded from the correction process as they were both derived from the individual skinfolds measurements and were not adding to the data (Appendix 4). This approach produced an α equal to 0.019 (α=0.019); considering the findings for the I/D polymorphism in females (tables 4.1 and 4.2) would indicate that the significant associations for triceps skinfolds in total females and for triceps and subscapular skinfolds in inactive females, remained significant even after the correction. These findings further increased confidence in the aforementioned arguments about an influence of ACE genetic variants on obesity-related phenotypes in young females.

The Olivetti Heart Study (Strazzullo et al. 2001), which tested only males, reported a significant association between the ACE DD genotype and increased BMI, waist circumference and diastolic blood pressure in relation to age. Nevertheless, several other studies have failed to consistently associate the ACE I/D polymorphism with BMI (for example (Cooper et al. 1997)). In addition, as (Strazzullo et al. 2001) indicated, the prevalence of overweight or abdominal adiposity was significantly greater in their DD

Table 4.4: ID genotype odds ratios for phenotypes significant by ANOVA (tables 4.1 and 4.2). 95% confidence intervals are given in parentheses. Probability (P) <0.05 for all tests shown, * indicates P≤0.01 and ** indicates P≤0.001. Low means lower BMI, lower skinfold thickness and lower skinfolds estimated % body fat. High indicates the converse. (n.s.) indicates X^2 tests that were not statistically significant.

		Total Females		Inactive Females	
		Low	High	Low	High
BMI	**II**	ANOVA not significant		2.40 (1.12-5.15)	0.36 (0.13-0.98)
	ID			n.s.	n.s.
	DD			n.s.	2.10 (1.10-3.99)
Triceps Skinfolds	**II**	2.32** (1.41-3.80)	n.s.	4.95** (2.31-10.62)	n.s.
	ID	n.s.	n.s.	n.s.	n.s.
	DD	n.s.	n.s.	0.41 (0.18-0.95)	n.s.
Subscap Skinfolds	**II**	1.99* (1.2-3.29)	0.44 (0.23-0.85)	3.45** (1.65-7.21)	0.28* (0.10-0.76)
	ID	n.s.	n.s.	n.s.	n.s.
	DD	n.s.	n.s.	n.s.	n.s.
Total Skinfolds	**II**	1.79 (1.08-2.98)	0.49 (0.26-0.92)	2.89* (1.37-6.09)	0.39 (0.15-0.98)
	ID	n.s.	n.s.	n.s.	n.s.
	DD	n.s.	1.73 (1.13-2.65)	n.s.	2.26 (1.21-4.22)
Skinfolds % BF	**II**	1.89 (1.07-3.32)	0.40 (0.19-0.84)	2.89* (1.37-6.09)	0.39 (0.15-0.98)
	ID	n.s.	n.s.	n.s.	n.s.
	DD	n.s.	1.71 (1.07-2.73)	n.s.	2.26* (1.21-4.22)

group than in their ID plus II group; this finding, however, applied only to participants 54 years of age and older. Although the present data are in agreement with the direction of this effect, a significant effect in young males was not detected. It is clear from the data in the present and Olivetti studies (Strazzullo et al. 2001) that the effects of the ACE gene on adiposity are modest, as would be expected for such a multi-factorial condition. It is also clear that selecting a cohort of the "wrong" gender, age or activity level could easily mask these modest effects of the ACE gene on adiposity.

4.5 Conclusion

In young females, polymorphic variants within the ACE gene had a significant, albeit modest, influence on the adiposity levels of the present population of adolescent children. In particular, the ACE I-allele, linked with lower ACE expression and activity, was associated with low and moderate BMI and adiposity while the D-allele was associated with increased obesity-related scores. The observed ACE genotype-specific associations were influenced by participation in organised physical activity and were more apparent in inactive females. These findings highlighted the impact of non-genetic sources of variation on multi-factorial phenotypes and indicated that selecting a cohort of the "wrong" gender, age or activity level could easily mask the modest effects of the ACE gene on obesity-related phenotypes.

CHAPTER 5

EFFECTS OF INTERACTION BETWEEN ACE POLYMORPHISMS AND LIFESTYLE ON PHYSIOLOGICAL, PHYSICAL AND SKILL-RELATED PHENOTYPES IN ADOLESCENT GREEKS

5.1 Introduction

The ACE gene, coding for the ACE peptidase, is one of the most studied putative performance genes. ACE is a key component of the circulating and tissue RAS; it is involved in vascular homeostasis (for review (Crisan and Carr 2000)) and the metabolism of tissues such as skeletal muscle (for review (Jones and Woods 2003)). Historically, research has centred on an I/D polymorphism characterised by the presence or absence of a 287 bp *Alu* repeat in intron 16 (Rigat et al. 1990). In Caucasians, the I-allele associates with low ACE expression and activity (Davis et al. 2000),(Rigat et al. 1990) and increased endothelial cell survival (Hamdi and Castellon 2003). The polymorphic variation in the ACE gene defines 13 haplotypes; only four of these haplotypes are found in Caucasians with the I-allele associating exclusively with haplotype H6 and the D-allele associating with the remaining three (Rieder et al. 1999). The I–allele has been linked with elite endurance performance (Montgomery et al. 1998), greater $\dot{V}o_{2\,max}$ in postmenopausal females (Hagberg et al. 1998) and improved responses to physical training (Woods et al. 2001). Correspondingly, the D-allele has been linked with elite power-oriented performance (Nazarov et al. 2001), quadriceps strength in patients with CLD (Hopkinson et al. 2004) and responses to physical training in male subjects (Folland et al. 2000).

Given the associations with physical performance in elite athletes and selected adult populations, the present study aimed to investigate whether variation in the ACE genotype had an effect on physical, physiological and skill phenotype parameters in a large, representative sample of young individuals using both genotype and haplotype-based approaches. Furthermore, genotype-environment interactions with gender and participation in organised physical activity were also considered.

5.2 Methods

5.2.1 Subjects and measurements For the present analysis, all information regarding subjects and measurements of physical, physiological and skill-related parameters in the Greek cohort were as described in General Methods (p. 33-37). The parameters assessed here included: $\dot{V}o_{2\,max}$, sitting height, arm span, sit and reach, basketball throw, throw and catch, hand-grip strength, vertical jump, agility run and the 40 m sprint.

5.2.2 DNA extraction, genotype determination and haplotype inference DNA extraction, quantification and storage as well as ACE I/D, G7831A (rs4424958) and C8968T (rs4311) genotype determination and ACE haplotype inference were performed as described in General Methods (p. 37-42).

5.2.3 Data Analysis All data and statistical analyses were carried out as described in the General Methods (p. 45-54).

Table 5.1: Analysis of associations between genotypes/diplotypes and physical, physiological and skill-related phenotypes in total females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that basketball throw has been abbreviated to (Basket Throw).

		Total Females									
		$\dot{V}o_{2\ max}$	**Sitting Height**	**Arm Span**	**Sit & Reach**	**Basket Throw**	**Throw & Catch**	**Hand-Grip**	**Vertical Jump**	**40m Sprint**	**Agility Run**
Polymorphism	**I/D**	P=0.176 V=0.8% n=454	**P=0.001** **V=2.7%** **n=480**	P=0.111 V=0.9% n=482	**P=0.112** V=0.9% n=481	**P=0.011** **V=1.9%** **n=481**	P=0.183 V=0.7% n=480	**P<0.001** **V=4.0%** **n= 480**	**P<0.001** **V=4.5%** **n= 482**	**P=0.004** **V=2.4%** **n= 464**	P=0.389 V=0.4% n=476
	G7831A	P=0.290 V=0.6% n=439	*P=0.080* *V=1.1%* *n=463*	P=0.409 V=0.4% n=465	P=0.748 V=0.1% n=465	P=0.328 V=0.5% n=465	P=0.382 V=0.4% n=464	P=0.216 V=0.7% n=465	**P=0.013** **V=1.9%** **n= 466**	**P=0.034** **V=1.5%** **n= 450**	P=0.610 V=0.2% n=460
	C8968T	P=0.187 V=0.8% n=439	**P=0.009** **V=2.0%** **n=463**	P=0.233 V=0.6% n=465	P=0.442 V=0.4% n=464	P=0.241 V=0.6% n=464	P=0.555 V=0.3% n=464	**P=0.003** **V=2.5%** **n=465**	**P=0.001** **V=2.9%** **n=465**	*P=0.092* *V=1.1%* *n=450*	P=0.730 V=0.1% n=460
	Diplotypes	P=0.490 V=1.5% n=424	**P=0.034** **V=3.4%** **n=448**	P=0.201 V=2.2% n=450	P=0.448 V=1.5% n=449	P=0.189 V=2.2% n=449	P=0.133 V=2.5% n=449	**P=0.002** **V=5.1%** **n=449**	**P<0.001** **V=6.4%** **n=450**	**P=0.008** **V=4.4%** **n=435**	P=0.369 V=1.7% n=445

5.3 Results

5.3.1 Genotyping Genotyping was successful in 1019 individuals (537 males and 482 females). All populations and subpopulations were in Hardy-Weinberg equilibrium (HWE) for all polymorphic loci tested; no significant differences were found in allele or haplotype frequencies. Overall allele frequencies were $f_{(D)}$=0.57 and $f_{(I)}$=0.43 for the I/D polymorphism, $f_{(G)}$=0.60 and $f_{(A)}$=0.40 for the G7831A (rs4424958) polymorphism and $f_{(C)}$=0.48 and $f_{(T)}$=0.52 for the C8968T (rs4311) polymorphism. Diplotypes (haplotype allelic combinations) were inferred for 952 individuals; haplotype frequencies were $f_{(H1)}$=0.40, $f_{(H6)}$=0.42, $f_{(H7)}$=0.13 and $f_{(H9)}$=0.06. Only 25 out of 952 individuals showed evidence of rare recombinant haplotypes even after re-genotyping. Re-genotyping of thirty individuals gave identical results on each occasion. Together with the HWE of the population these provided strong validation of the genotyping methods used.

5.3.2 Genotype associations with phenotypes In females, several performance measures were associated with the ACE genotype (table 5.1). Relative to ID and DD individuals, II homozygotes had the longest trunk, were stronger (further basketball throw and greater hand-grip scores), jumped higher and were faster in the 40m sprint compared to the other genotypes (data not shown). Significant differences were also observed for the G7831A (rs4424958) variant with vertical jump and 40m sprint scores and for the C8968T (rs4311) variant with sitting height, hand-grip and vertical jump trials (table 5.1). 7831GG homozygous individuals jumped higher (vertical jump) and were faster over 40m than their 7831GA and 7831AA counterparts, whilst for the C8968T (rs4311) polymorphism, 8968CC homozygotes had the longest trunk and were stronger and jumped higher than those of 8968CT or 8968TT genotype (data not shown). For the inferred diplotypes, significant differences in females were observed for sitting height, hand-grip, vertical jump

Table 5.2: Analysis of associations between genotypes/diplotypes and physical, physiological and skill-related phenotypes in total males. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that basketball throw has been abbreviated to (Basket Throw).

		Total Males									
		$\dot{V}_{O_2\,max}$	Sitting Height	Arm Span	Sit & Reach	Basket Throw	Throw & Catch	Hand-Grip	Vertical Jump	40m Sprint	Agility Run
Polymorphism	I/D	P=0.386 V=0.4% n=519	P=0.981 V<0.1% n=535	P=0.822 V<0.1% n=533	P=0.287 V=0.5% n=531	P=0.153 V=0.7% n=536	P=0.163 V=0.7% n=536	P=0.285 V=0.5% n=536	P=0.282 V=0.5% n=535	P=0.701 V=0.1% n=522	P=0.717 V=0.1% n=528
	G7831A	P=0.348 V=0.4% n=503	P=0.918 V<0.1% n=519	P=0.894 V=0.4% n=519	P=0.174 V=0.7% n=514	P=0.733 V=0.1% n=520	P=0.221 V=0.6% n=520	P=0.929 V<0.1% n=520	P=0.211 V=0.6% n=519	P=0.166 V=0.7% n=506	P=0.154 V=0.7% n=512
	C8968T	P=0.697 V=0.1% n=503	P=0.826 V=0.1% n=519	P=0.987 V<0.1% n=519	P=0.611 V=0.2% n=514	P=0.609 V=0.2% n=520	**P=0.009** **V=1.8%** **n=520**	P=0.291 V=0.5% n=520	P=0.417 V=0.3% n=519	P=0.548 V=0.2% n=506	P=0.773 V=0.1 % n=512
	Diplotypes	P=0.906 V=0.5% n=484	P=0.803 V=0.8% n=500	P=0.873 V=0.6% n=500	P=0.398 V=1.5% n=496	P=0.225 V=1.9% n=501	*P=0.067* *V=2.6%* *n=501*	P=0.518 V=1.2% n=501	P=0.794 V=0.8% n=500	P=0.669 V=1.0% n=487	P=0.584 V=1.1% n=493

and 40m sprint (table 5.1). H6H6 (associated with the II, 7831GG, 8968CC genotypes) homozygous females had the longest trunk, were stronger and jumped higher compared to other diplotypes; they also sprinted faster than all the other diplotypes combined although individually H1H9 individuals were as quick (data not shown). Performance characteristics of males were largely independent of genotype (table 5.2). The sole exception was for throw and catch (table 5.2), in which those individuals homozygous for the T-allele at position 8968 performed better than 8968CT or 8968CC individuals (data not shown).

5.3.3 Analysis by level of regular activity Analysis was performed for the active and inactive groups separately as previous studies have shown effects of training on the association between the I/D polymorphism and cardiovascular phenotypes (Montgomery et al. 1998; Woods et al. 2001). Significant associations were found for the I/D polymorphism, C8968T (rs4311) polymorphism and diplotypes in both active and inactive females (table 5.3, for example figure 5.1).

For inactive females, I/D genotype was significantly associated with predicted $\dot{V}o_{2\,max}$, vertical jump and 40m sprint (table 3). Amongst inactive females, II homozygotes had the highest mean $\dot{V}o_{2\,max}$ (figure 5.1), vertical jump scores, and also sprinted faster than their ID and DD counterparts (data not shown). Considering diplotypes, significant differences in inactive females were also detected for vertical jump and 40m sprint (table 5.3); H6H6 homozygotes jumped higher and ran faster than the other diplotypes combined although, individually, H1H9 individuals were as quick in the 40m sprint (data not shown). Among active females, the I/D polymorphism showed associations with sitting height, sit and reach, hand-grip strength and vertical jump (table 5.3). Active II homozygotes had the longest trunk, had a higher mean sit and reach score, were stronger and jumped higher than

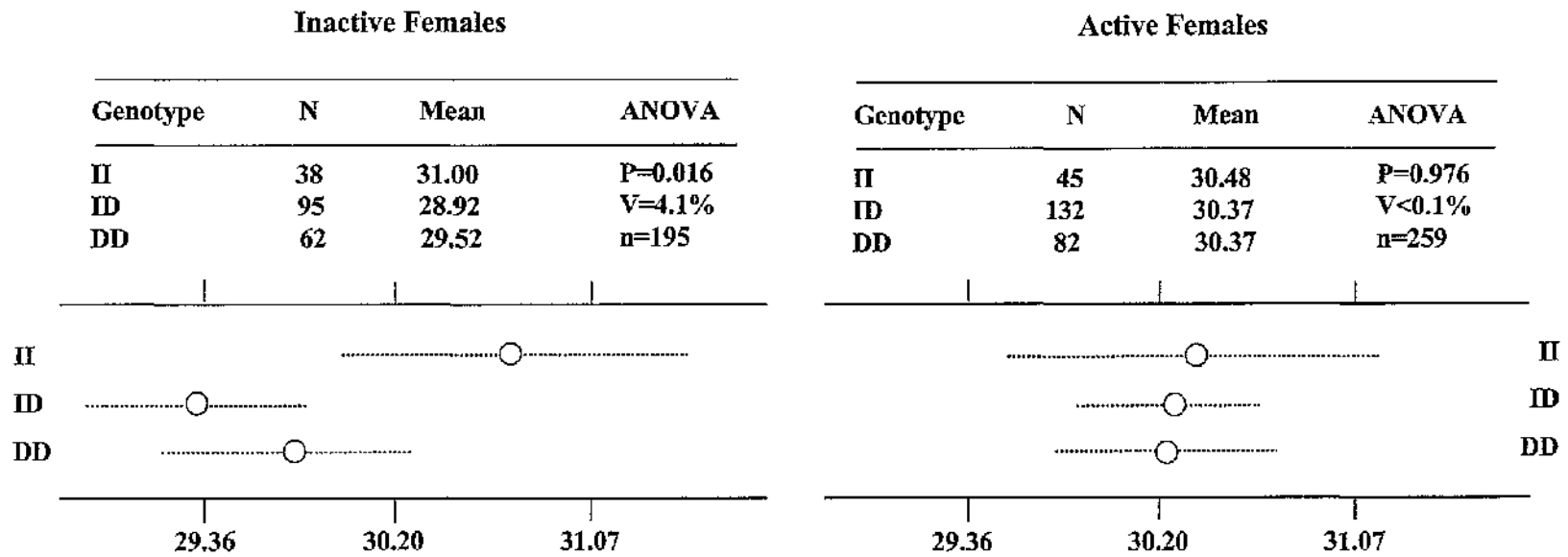


Figure 5.1: I/D genotype differences in $\dot{V}o_{2\ max}$ of active and inactive females; inactive II homozygotes have significantly higher mean $\dot{V}o_{2\ max}$ while no significant differences by genotype were observed for active females. $\dot{V}o_{2\ max}$ is measured in ($ml kg^{-1} min^{-1}$). (P)=probability, (V)=observed variance explained, (N)=number within group.

ID and DD individuals (data not shown). For C8968T (rs4311), significant genotype differences among active females were found for sitting height, hand-grip strength and vertical jump (table 5.3); active 8968CC individuals had the longest trunk, were stronger and jumped higher compared to 8968CT and 8968TT individuals (data not shown). Diplotypes also showed associations with sitting height, sit and reach, hand-grip strength and vertical jump (table 5.3). Active H6H6 individuals had the longest trunk, were stronger and jumped higher than the other diplotypes combined although, individually, H1H7 individuals had equally high sit and reach scores (data not shown).

Among inactive males, the I/D genotype was associated with basketball throw while, among active males, the C8968T (rs4311) polymorphism was associated with throw and catch (table 5.4). Inactive DD homozygotes had the highest mean scores for basketball throw; while active 8968TT males had the highest mean score for throw and catch (data not shown). No significant associations were detected in the G7831A (rs4424958) variant for any of the physical, physiological and skill parameters among active or inactive males.

5.3.4 Determination of the type of genetic effect observed for associations with the I/D polymorphism A model in which the phenotypic effects of the D-allele are completely dominant over those of the I-allele explained 96%, 75%, 96%, 81% and 64% respectively, of the genetic variance for sitting height, basketball throw, hand grip strength, vertical jump and 40m sprint of total females, 87%, 97% and 80% respectively, of the variance for $\dot{V}o_{2\,max}$, vertical jump and 40m sprint times of inactive females and 81%, 95% and 68% respectively, of the variance for hand grip strength, sit and reach and vertical jump of active females. 97% of the observed variance for sitting height of active females could be explained by an additive model, and 81% of the variance for basketball throw in inactive

Table 5.3: Analysis of associations between genotypes/diplotypes and physical, physiological and skill-related phenotypes in inactive and active females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance ($0.05<P<0.1$) and where they are given in bold the tests were significant ($P<0.05$). Note that basketball throw has been abbreviated to (Basket Throw).

		$\dot{V}O_{2\ max}$	Sitting Height	Arm Span	Sit & Reach	Basket Throw	Throw & Catch	Hand-Grip	Vertical Jump	40m Sprint	Agility Run
		Inactive Females									
Polymorphism	**I/D**	**P=0.016** **V=4.1%** **n=195**	P=0.143 V=1.9% n=206	P=0.653 V=0.4% n=207	P=0.993 V<0.1% n=206	P=0.170 V=1.7% n=207	P=0.548 V=0.6% n=206	*P=0.076* *V=2.5%* *n=206*	**P=0.015** **V=4.0%** **n=207**	**P=0.041** **V=3.2%** **n=197**	P=0.546 V=0.6% n=203
	G7831A	P=0.210 V=1.7% n=187	P=.461 V=0.8% n=197	P=0.939 V=0.1% n=200	P=0.754 V=0.3% n=197	P=0.497 V=0.7% n=198	P=0.481 V=0.7% n=198	P=0.899 V=0.1% n=198	P=0.152 V=1.9% n=198	P=0.178 V=1.8% n=190	P=0.326 V=1.2% n=194
	C8968T	P=0.290 V=1.3% n=187	P=0.357 V=1.1% n=197	P=0.949 V<0.1% n=198	P=0.911 V=0.1% n=199	P=0.302 V=1.2% n=198	P=0.658 V=0.4% n=198	P=0.334 V=1.1% n=198	*P=0.064* *V=2.8%* *n=198*	P=0.594 V=0.6% n=190	P=0.598 V=0.5% n=194
	Diplotypes	P=0.115 V=6.4% n=180	P=0.585 V=3.0% n=190	P=0.346 V=4.1% n=191	P=0.944 V=1.2% n=190	P=0.617 V=2.8% n=191	P=0.685 V=2.6% n=191	P=0.103 V=6.2% n=191	**P=0.005** **V=10.3%** **n=191**	**P=0.011** **V=9.8%** **n=183**	P=0.497 V=3.5% n=187
		Active Females									
Polymorphism	**I/D**	P=0.976 V<0.1% n=259	**P=0.001** **V=5.1%** **n=274**	P=0.118 V=1.6% n=275	**P=0.024** **V=2.7%** **n=275**	*P=0.063* *V=2.0%* *n=274*	P=0.317 V=0.8% n=274	**P<0.001** **V=6.1%** **n=274**	**P<0.001** **V=5.8%** **n=275**	*P=0.064* *V=2.1%* *n=267*	P=0.507 V=0.5% n=273
	G7831A	P=0.768 V=0.2% n=252	P=0.165 V=1.4% n=266	P=0.139 V=1.5% n=267	P=0.835 V=0.1% n=267	P=0.323 V=0.9% n=266	P=0.556 V=0.4% n=266	*P=0.087* *V=1.8%* *n=266*	*P=0.063* *V=2.1%* *n=267*	P=0.166 V=1.4% n=260	P=0.941 V<0.1% n=266
	C8968T	P=0.482 V=0.6% n=252	**P=0.004** **V=4.1%** **n=266**	P=0.120 V=1.6% n=267	P=0.208 V=1.2% n=2667	P=0.325 V=0.8% n=266	P=0.654 V=0.3% n=266	**P=0.003** **V=4.3%** **n=266**	**P=0.013** **V=3.2%** **n=266**	*P=0.064* *V=2.1%* *n=260*	P=0.977 V<0.1% n=266
	Diplotypes	P=0.916 V=1.1% n=244	**P=0.015** **V=6.7%** **N=258**	P=0.364 V=3.0% n=259	**P=0.038** **V=5.7%** **n=259**	P=0.113 V=4.5% n=258	P=0.288 V=3.3% n=258	**P=0.027** **V=6.1%** **n=258**	**P=0.008** **V=7.2%** **n=259**	P=0.172 V=4.1% n=252	P=0.839 V=1.4% n=258

males could be explained by a D-recessive (I-dominant) model. For each parameter, the remaining variance would be accounted for by appropriately considered partial dominance models.

5.3.5 Associations of ACE genetic variants with extreme phenotypes The odds ratios (tables 5.5 and 5.6) were broadly consistent with the phenotypic means reported in the ANOVAs and the genetic models identified by the correlation analyses. II homozygotes, and their associated H6H6 diplotypes, tended to be over-represented in the high quartile and/or under-represented in the low quartile groups for the performance phenotypes; in contrast, carriers of the dominant D-allele (ID heterozygotes and DD homozygotes), and their associated diplotypes, were mostly over-represented in the low quartile and/or under-represented in the high quartile groups for the performance phenotypes (for example figure 5.2). Note that these results are reversed for the 40m sprint since the high quartile represents slow times. For males, significant odds ratios were observed only in the inactive group; DD homozygous males were significantly over-represented (P=0.011, OR: 3.28, 95%CI: 1.28-8.38) whereas the ID heterozygotes were significantly under-represented (P=0.007, OR: 0.27, 95%CI: 0.10-0.73) in the high quartile for basketball throw.

Table 5. 4: Analysis of associations between genotypes/diplotypes and physical, physiological and skill-related phenotypes in inactive and active males. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that basketball throw has been abbreviated to (Basket Throw).

		$\dot{V}O_{2\ max}$	Sitting Height	Arm Span	Sit & Reach	Basket Throw	Throw & Catch	Hand-Grip	Vertical Jump	40m Sprint	Agility Run
		Inactive Males									
Polymorphism	I/D	P=0.615 V=1.0% n=102	P=0.414 V=1.7% n=106	P=0.350 V=2.0% n=106	P=0.918 V=0.2% n=106	**P=0.022** **V=7.1%** **n=107**	P=0.595 V=0.4% n=107	P=0.341 V=2.1% n=107	P=0.362 V=2.0% n=105	P=0.284 V=2.5% n=102	P=0.903 V=0.2% n=106
	G7831A	P=0.156 V=3.9% n=97	P=0.655 V=0.9% n=101	P=0.799 V=0.5% n=101	P=0.887 V=0.2% n=101	P=0.294 V=2.4% n=102	P=0.897 V=0.2% n=102	P=0.327 V=2.2% n=102	P=0.820 V=0.4% n=100	P=0.618 V=1.0% n=97	P=0.607 V=1.0% n=101
	C8968T	P=0.591 V=1.1% n=97	P=0.813 V=0.4% n=101	P=0.961 V=0.1% n=101	P=0.827 V=0.4% n=101	P=0.127 V=4.1% n=102	P=0.401 V=1.8% n=102	P=0.607 V=1.0% n=102	P=0.504 V=1.4% n=100	P=0.647 V=0.1% n=97	P=0.751 V=0.6% n=101
	Diplotypes	P=0.337 V=7.5% n=93	P=0.562 V=5.1% n=97	P=0.667 V=4.3% n=97	P=0.979 V=1.3% n=97	P=0.333 V=7.1% n=98	P=0.465 V=6.9% n=98	P=0.527 V=5.4% n=98	P=0.328 V=7.3% n=96	P=0.441 V=6.4% n=93	P=0.318 V=7.4% n=97
		Active Males									
Polymorphism	I/D	P=0.588 V=0.3% n=417	P=0.893 V=0.1% n=429	P=0.469 V=0.4% n=427	P=0.128 V=1.0% n=425	P=0.598 V=0.2% n=429	P=0.112 V=1.0% n=429	P=0.506 V=0.3% n=429	P=0.114 V=1.0% n=430	P=0.469 V=0.4% n=420	P=0.655 V=0.2% n=422
	G7831A	P=0.457 V=0.4% n=406	P=0.989 V<0.1% n=418	P=0.876 V=0.1% n=418	P=0.195 V=0.8% n=413	P=0.980 V<0.1% n=418	P=0.178 V=0.8% n=418	P=0.962 V<0.1% n=418	P=0.248 V=0.7% n=419	P=0.219 V=0.7% n=409	P=0.394 V=0.5% n=411
	C8968T	P=0.536 V=0.3% n=406	P=0.822 V=0.1% n=418	P=0.998 V<0.1% n=418	P=0.403 V=0.4% n=413	P=0.906 V<0.1% n=418	**P=0.012** **V=2.1%** **n=418**	P=0.399 V=0.4% n=418	P=0.176 V=0.8% n=419	P=0.294 V=0.6% n=409	P=0.571 V=0.3% n=411
	Diplotypes	P=0.916 V=0.7% n=390	P=0.915 V=0.7% n=402	P=0.306 V=2.1% n=402	P=0.319 V=2.1% n=398	P=0.649 V=1.3% n=402	P=0.102 V=3.0% n=402	P=0.335 V=2.0% n=402	P=0.566 V=1.4% n=403	P=0.734 V=1.1% n=393	P=0.856 V=0.8% n=395

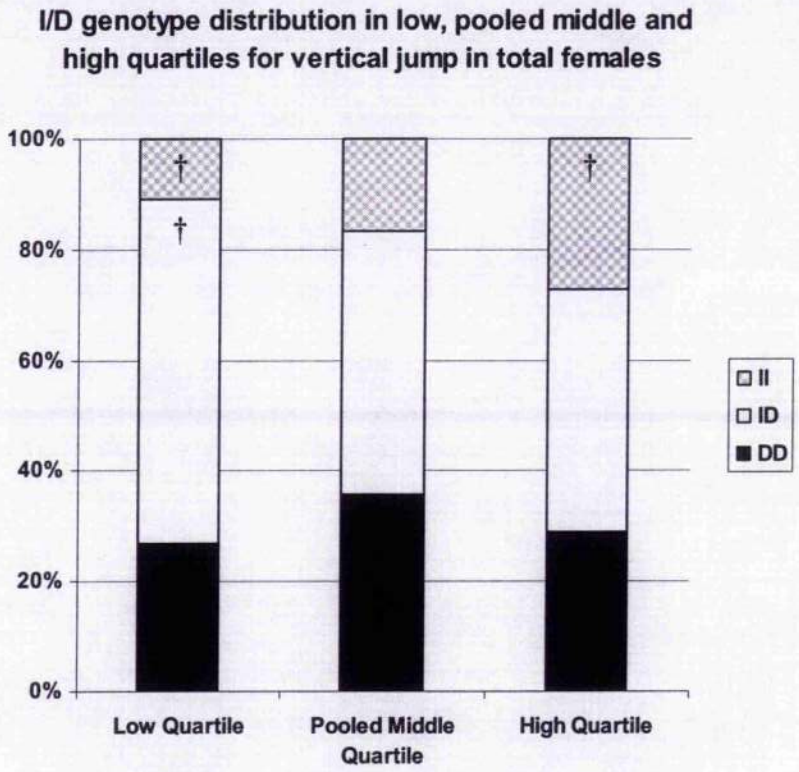
5.4 Discussion

Polymorphisms within the ACE gene were significantly associated with several of the physical, physiological and skill-related phenotypes measured in the present cohort of adolescent children. Although many studies have shown associations of the ACE genotype with performance in adults (for example (Montgomery et al. 1998)), this is the first study to investigate such associations in children. Significant associations were predominantly found in females; in particular, homozygotes for the I-allele or the corresponding H6 haplotype were associated with enhanced physical, physiological and skill parameter scores, mainly in a recessive manner. In contrast, the D-allele, in homozygous or heterozygous form, was associated with low or moderate physical, physiological and skill phenotype scores. The observed significant associations were influenced by participation in organised physical activity.

As indicated in General Methods (p. 40), G7831A (rs4424958) and C8968T (rs4311) polymorphisms are thought not to be functional and were merely selected since, when combined with the I/D polymorphism, they allow the inference of haplotypes in Caucasians (Rieder et al. 1999). Haplotyping allows the investigation of how a number of SNPs work in concert, and also infers information about untested SNPs that associate with the given haplotypes. As such, haplotype-based methods have been advocated to better assess genetic effects mediating susceptibility to disease or complex traits (Akey et al. 2001). Associations between the G7831A (rs4424958) and C8968T (rs4311) markers and performance-related physical fitness phenotypes were observed however, as indicated by the lower % of variance explained for these associations relative to the I/D polymorphism, these findings were most likely the result of LD between these markers and a functional variant(s). Furthermore, the results of haplotype analysis, as indicated by the *P*-values,

suggested that the genotyping of additional ACE polymorphisms did not produce any phenotypic associations markedly stronger than those associated with the I/D polymorphism although diplotypes explained a larger % of the observed variance. The increase in variance explained by the haplotype analysis was no larger than that expected by random sampling effects, suggesting that the majority of the genetic effects are accounted for by the I/D polymorphism or a variant in strong LD with it.

Previous findings (Davis et al. 2000),(Mizuiri et al. 2001) associating the ACE I/D polymorphism with ACE gene expression advocate a functional role of the *Alu* insert. Although possible that the insertion or deletion of an *Alu* sequence in an intron (*i.e.* the I/D polymorphism) could influence the function of the ACE gene, it is unlikely that such a polymorphism may be reflected in post-transcriptional regulation as the I/D polymorphism is not known to be closely associated with amino acid changes hence alterations of protein function. Nevertheless, the association of the I/D polymorphism with the levels of ACE gene expression (Davis et al. 2000),(Mizuiri et al. 2001) may suggest an influence of the *Alu* insert on transcriptional regulation. However, it is more likely that another polymorphism(s) in strong linkage LD with the *Alu* insert is responsible for the observed effects. Two previous studies (Zhu et al. 2000),(Cox et al. 2002) have suggested that such a functional polymorphism may be A22982G (rs4363), given that this polymorphism is in much stronger association with circulating ACE levels in Africans (Zhu et al. 2000),(Cox et al. 2002) where it is not in complete LD with the I/D polymorphism. Nevertheless, in Caucasian populations the I/D polymorphism is in almost complete linkage disequilibrium with A22892G (rs4363) (Rieder et al. 1999),(Soubrier et al. 2002), and, in spite of its probable lack of functionality, is therefore a sufficiently good marker of circulating ACE activity to use in prospective association studies.



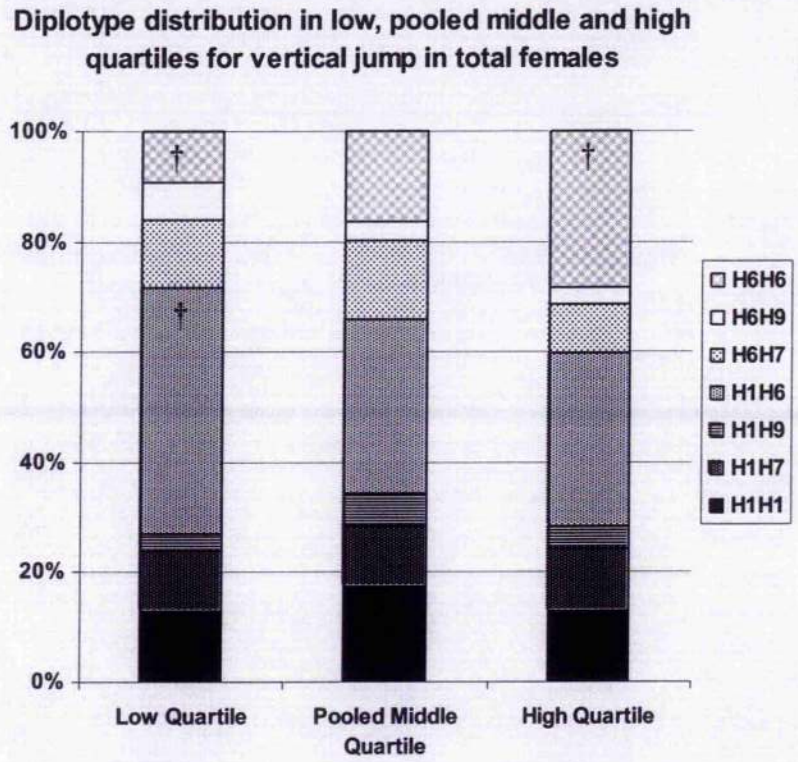


Figure 5.2: ACE I/D genotype and diplotype distributions in the low, pooled middle and high quartiles of vertical jump in the group of total females. Within quartiles, significantly over or under-represented genotypes/diplotypes (tables 5.5 and 5.6) are indicated by (†). II and H6H6 homozygotes are over-represented in the high and under-represented in the low quartile. In contrast, ID and H1H6 heterozygotes are over-represented in the low quartile for vertical jump.

Association studies are at risk of type I (false positive) errors caused by multiple testing. The Bonferroni and related corrections allows for this but, considering the large number of tests involved, may be very conservative and result in real associations being missed (Perneger TV 1998),(Sokal and Rohlf 2001),(Sabatti et al. 2003). The number of significant associations found here was compared to the number of significant associations expected by chance, given the number of statistical tests performed on each gender. Within each (sub)population (considered independently) 40 tests were conducted (10 phenotypes x 4 genotypes/diplotypes) and would expect two to show significant association ($P<0.05$) by chance (0.05 x 40=2). Considering the group of total females as an example, 16 tests were found to be significant, a number greatly in excess of what would be expected by chance alone. It can be suggested therefore that the significant results observed represented more than just an effect of multiple testing. Considering the relative strength and consistency of the effects over a number of phenotypes and across activity-divided population groups served to strengthen the confidence in the validity of significant findings for the ACE genotype in adolescent females. In contrast, in young males, significant results (uncorrected for multiple tests) were observed in only three of the analyses and these three associations were most likely the product of type I errors.

The significant phenotypic differences between genotypes were largely best explained by a dominant genetic model with the D-allele being dominant to the I-allele (or the I-allele being recessive to the D-allele), in which the heterozygote mean is nearly identical to one or other of the two homozygote means. This is shown for hand-grip strength in total females. Only one association (sitting height of active females) was best explained by an additive model, in which the heterozygote mean was exactly intermediate between the two homozygote means. However the D-allele dominant model could account for over 75%

Table 5.5: I/D genotype odds ratios for phenotypes significant by ANOVA (tables 5.1 and 5.2). 95% confidence intervals are given in parentheses. Probability (P) <0.05 for all tests shown, * indicates P≤0.01, ** indicates P≤0.001. Low means less tall (sitting height), less strong (hand-grip strength), smaller jump (vertical jump), longer sprint time (40m sprint), lower shuttle score ($\dot{V}o_{2\ max}$) and shorter distance (sit and reach). High indicates the converse. (n.s.) indicates X^2 tests that were not statistically significant.

I/D Genotype	Phenotype	Females					
		Total		Inactive		Active	
		Low Quartile	High Quartile	Low Quartile	High Quartile	Low Quartile	High Quartile
II	VO2max	ANOVA not Significant		0.39 (0.15-0.99)	2.44 (1.09-5.46)	ANOVA not Significant	
	Sitting Height	0.40* (0.20-0.72)	n.s			0.12** (0.03-0.51)	n.s
	Sit & Reach	ANOVA not Significant		ANOVA not Significant		n.s	2.40* (1.22-4.71)
	Hand-Grip	0.28 (0.14-0.59)	2.42** (1.47-4.00)			0.13** (0.03-0.53)	3.42** (1.76-6.65)
	Vertical Jump	0.50 (0.28-0.90)	2.21* (1.32-3.70)	0.41 (0.18-0.94)	n.s	n.s	2.38* (1.22-4.63)
	40m Sprint	2.03* (1.22-3.39)	n.s	2.72 (1.20-6.14)	n.s	ANOVA not Significant	
ID	Hand-Grip	1.69 (1.12-2.55)	0.62 (0.40-0.94)	ANOVA not Significant		1.89 (1.06-3.63)	0.56 (0.33-0.97)
	Vertical Jump	1.88* (1.26-2.79)	n.s	1.86 (1.04-3.30)	n.s	1.97 (1.12-3.44)	n.s
	40m Sprint	0.61 (0.40-0.93)	1.73* (1.40-2.64)	0.48 (0.22-1.00)	n.s	ANOVA not Significant	
DD	Sitting Height	1.53 (1.01-2.33)	n.s	ANOVA not Significant		2.62** (1.48-2.62)	n.s

(data not shown) of the genetic variance in this case. The additive genetic model can be reconciled with models of molecular mechanism acting via a linear dosage effect. One association (basketball throw for inactive males) was best explained by an I-allele dominant genetic model, however few effects were observed in males in this study suggesting that this may be a type I error. Two cases were not well (*i.e.* <75% of variance explained) explained by any of the models tested, suggesting that they were cases of under or over-dominance, in which the heterozygote mean is more extreme in phenotype than either of the homozygote means. Although genetic models for under or over-dominance can be formulated, they are difficult to reconcile with molecular mechanisms and the detected underdominance was most likely caused by a few outliers shifting the mean of ID heterozygotes lower than that of both homozygotes. This argument is further supported by the marginal difference in the percentage of variance explained by the underdominant and dominant genetic models (data not shown).

Most previous studies have concentrated on elite athletes, linking the ACE I-allele to endurance performance (Myerson et al. 1999) and the D-allele to muscle strength and power oriented performance (Nazarov et al. 2001). The I-allele has also been shown to associate with $\dot{V}o_{2\,max}$ (Hagberg et al. 1998) and with functional responses to physical training in postmenopausal women (Woods et al. 2001) and adult male subjects (Folland et al. 2000). The present findings advocate a broader, although modest, role for the ACE gene in human fitness and physical performance than previously described. In this investigation, the I-allele was associated with improved physical, physiological and skill parameter values in female subjects from a large representative sample of young individuals. This association was modified by participation in organised physical activity. Although this finding conflicts with several previous studies, the present study was not performed in a

Table 5.6: Diplotype odds ratios for phenotypes significant by ANOVA (tables 5.3 and 5.4). 95% confidence intervals are given in parentheses. P<0.05 for all tests shown, * indicates P≤0.01, ** indicates P≤0.001. Low means less tall (sitting height), shorter distance (sit and reach), less strong (hand-grip strength), smaller jump (vertical jump) and longer sprint time (40m sprint). (n.s.) indicates X^2 tests that were not statistically significant. Note that no H1H9 individuals were observed in the high quartile for sitting height of active females, thus no meaningful odds ratio or confidence interval could be calculated [indicated by (-)].

		Females					
Diplotype	**Phenotype**	**Total**		**Inactive**		**Active**	
		Low Quartile	**High Quartile**	**Low Quartile**	**High Quartile**	**Low Quartile**	**High Quartile**
H6H6	**Sitting Height**	0.38* (0.19-0.76)	n.s.			0.13* (0.03-0.56)	n.s
	Sit & Reach	ANOVA not Significant		ANOVA not Significant		n.s	2.10 (1.03-4.28)
	Hand Grip	0.24** (0.11-0.54)	2.66** (1.57-4.49)			0.14* (0.03-0.59)	3.44** (1.72-6.88)
	Vertical Jump	0.41* (0.21-0.79)	2.53** (1.48-4.31)	0.27* (0.10-0.73)	2.89 (1.13-6.41)	n.s	2.56* (1.28-5.12)
	40m Sprint	2.21* (1.30-3.78)	n.s	3.21* (1.38-7.43)	n.s	ANOVA not Significant	
H6H7	**Hand Grip**	n.s	0.41 (0.18-0.93)	ANOVA not Significant		n.s	n.s
H6H9	**Hand Grip**	2.59 (1.02-6.53)	n.s			n.s	n.s
H1H6	**Vertical Jump**	1.79* (1.18-2.71)	n.s	n.s	n.s	2.17* (1.22-3.86)	n.s
	40m Sprint	0.49* (0.30-0.81)	1.81* (1.17-2.82)	0.40 (0.16-0.97)	n.s	ANOVA not Significant	
H1H9	**Sitting Height**	n.s	2.68 (1.08-6.66)	ANOVA not Significant		n.s	-
	40m Sprint	n.s	n.s	n.s	n.s	ANOVA not Significant	
H1H7	**Sitting Height**	n.s	n.s	ANOVA not Significant		2.24 (1.07-4.71)	n.s
H1H1	**Sitting Height**	n.s	n.s			2.46 (1.10-5.49)	n.s

highly selected elite population, nor did it assess intra-individual responses to a highly structured training program in adults. In addition, sample sizes in both the previous types of study were limited by their very nature and may have had inherently low power to detect small effects such as the ones described here. Clearly, further investigations are required to offer greater insight into the molecular mechanisms of the involvement of the ACE gene in human performance.

5.5 Conclusion

In young females, polymorphic variants within the ACE gene were significantly associated with several of the measured phenotypes; this is the first study to investigate such associations in adolescent children. In particular, the ACE I-allele, and the corresponding H6 haplotype (linked with lower ACE expression and activity) were associated with improved physical, physiological and skill parameter phenotypes. The observed ACE genotype-specific associations were strongly dependant on gender and on participation in physical activity. These findings were consistent with a broader influence of the ACE gene on human physical performance than the previously described, limited associations with elite athletic status and responses to structured training regimes but indicated that this influence is modest.

CHAPTER 6

EFFECTS OF INTERACTION BETWEEN ADRB POLYMORPHISMS AND LIFESTYLE ON OBESITY-RELATED PHENOTYPES IN ADOLESCENT GREEKS

6.1 Introduction

Among the promising candidate genes for obesity are the ADRB1, ADRB2 and ADRB3 genes. All three code for 7-transmembrane GPCRs that are differentially expressed throughout the body (for reviews (Small et al. 2003),(Leineweber and Brodde 2004)). GPCRs mediate the activation by catecholamines of adenylyl cyclase, a key enzyme in intracellular signalling pathways and are thought to play a central role in local tissue metabolism and function (for a review (Small et al. 2003)) significantly including the adipose tissue where ADRBs are involved in stimulating lipolysis and thermogenesis (for examples (Carpene et al. 1998),(Ravussin and Gautier 1999),(Bachman et al. 2002)). SNPs have been described in all three ADRB genes that have been suggested to associate with pathophysiologies of the heart, lungs and obesity (Small et al. 2003),(Leineweber and Brodde 2004),(Snyder et al. 2004), albeit mainly in adult populations; for example, the ADRB3 Arg64 variant has been associated with higher BMI in adult males from Spain (Corella et al. 2001). Genotype determination of the ADRB1 Ser49Gly (rs1801252) and Arg389Gly (rs1801253) variants allows the inference of 3 haplotypes that represent the majority of variation along the complete length of the gene. For the ADRB2 gene, genotyping of the Gly16Arg (rs1042713) and Gln27Glu (rs1042714) variants allows the inference of three haplotypes that account for 95% of the observed ADRB2 genetic variation in Caucasians (Drysdale et al. 2000).

Given the previously described associations with obesity in adults the present study wished to investigate the effect of variation in the ADRB genes on obesity-related phenotypes in a large, representative sample of young individuals using both genotype and haplotype-based approaches. Furthermore, genotype-environment interactions with gender and participation in organised physical activity were also considered.

Table 6.1: Analysis of associations between genotypes/diplotypes and obesity-related phenotypes in total males and females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that subscapular skinfolds has been abbreviated to (Subscap Skinfolds).

		Total Males					Total Females				
		BMI	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**	**BMI**	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**
Polymorphism	**ADRB1 Ser49Gly**	**P=0.015** **V=1.7%** **N=504**	P=0.477 V=0.3% N=503	P=0.132 V=0.8% N=502	**P=0.256** V=0.5% N=502	P=0.279 V=0.5% N=502	P=0.699 V=0.2% N=448	P=0.175 V=0.8% N=449	P=0.686 V=0.2% N=449	P=0.339 V=0.5% N=449	P=0.318 V=0.5% N=449
	ADRB1 Arg389Gly	P=0.338 V=0.4% N=506	*P=0.062* *V=1.1%* *N=505*	P=0.150 V=0.8% N=504	*P=0.065* *V=1.1%* *N=504*	*P=0.068* *V=1.1%* *N=504*	P=0.614 V=0.2% N=453	P=0.580 V=0.2% N=454	P=0.805 V=0.1% N=454	P=0.928 V<0.1% N=454	P=0.950 V<0.1% N=454
	ADRB1 Diplotypes	P=0.154 V=1.8% N=499	P=0.190 V=1.5% N=498	P=0.139 V=1.7% N=497	P=0.132 V=1.7% N=497	P=0.148 V=1.6% N=497	P=0.678 V=0.7% N=440	P=0.476 V=1.0% N=441	P=0.842 V=0.5% N=441	P=0.770 V=0.6% N=441	P=0.760 V=0.6% N=441
	ADRB2 Gly16Arg	**P=0.002** **V=2.5%** **N=487**	**P=0.011** **V=1.9%** **N=486**	**P=0.013** **V=1.8%** **N=485**	**P=0.010** **V=1.9%** **N=485**	**P=0.010** **V=1.9%** **N=485**	P=0.456 V=0.4% N=439	P=0.357 V=0.5% N=440	P=0.735 V=0.1% N=440	P=0.442 V=0.4% N=440	P=0.417 V=0.4% N=440
	ADRB2 Gln27Glu	P=0.818 V=0.1% N=489	P=0.167 V=0.7% N=488	P=0.502 V=0.3% N=487	P=0.366 V=0.4% N=487	P=0.363 V=0.4% N=487	P=0.174 V=0.8% N=442	P=0.210 V=0.7% N=443	P=0.509 V=0.3% N=443	P=0.269 V=0.6% N=443	P=0.263 V=0.6% N=443
	ADRB2 Diplotypes	**P=0.009** **V=3.2%** **N=482**	**P=0.024** **V=2.7%** **N=481**	**P=0.036** **V=2.5%** **N=480**	**P=0.027** **V=2.6%** **N=480**	**P=0.026** **V=2.6%** **N=480**	P=0.675 V=0.7% N=436	P=0.626 V=0.8% N=437	P=0.826 V=0.5% N=437	P=0.676 V=0.7% N=437	P=0.658 V=0.8% N=437
	ADRB3 Trp64Arg	P=0.533 V=0.3% N=493	P=0.565 V=0.2% N=492	P=0.575 V=0.2% N=491	P=0.569 V=0.2% N=491	P=0.589 V=0.2% N=491	*P=0.062* *V=0.8%* *N=451*	P=0.490 V=0.1% N=452	P=0.617 V=0.1% N=452	P=0.490 V=0.1% N=452	P=0.431 V=0.1% N=452

6.2 Methods

6.2.1 Subjects and measurements For the present analysis, all information regarding subjects and measurements of physical, physiological and skill-related parameters in the Greek cohort were as described in General Methods (p. 33-37). The parameters assessed here included: BMI, skinfolds at the triceps and subscapular sites, total skinfolds and skinfolds estimated % body fat.

6.2.2 DNA extraction, genotype determination and haplotype inference DNA extraction, quantification and storage as well as ADRB genotype determination and haplotype inference were performed as described in General Methods (p. 37-38 and 42-45).

6.2.3 Data Analysis All data and statistical analyses were carried out as described in General Methods (p. 45-54).

6.3 Results

6.3.1 Genotyping All populations and subpopulations were in Hardy-Weinberg equilibrium (HWE) for all polymorphic loci tested. Genotypes for 953 individuals (504 males and 449 females) were successfully obtained for the ADRB1 Ser49Gly (rs1801252) polymorphism and for 960 individuals (506 males and 454 females) for the ADRB1 Arg398Gly polymorphism. Allele frequencies for the ADRB1 Ser49Gly (rs1801252) variant were, in both genders, $f_{(Ser49)}$=0.91 and $f_{(Gly49)}$=0.09 and for the ADRB1 Arg398Gly variant were $f_{(Arg389)}$=0.66 and $f_{(Gly389)}$=0.34 for males and $f_{(Arg389)}$=0.63 and $f_{(Gly389)}$=0.37 for females. Haplotypes were inferred for 940 individuals (499 males and 441 females); haplotype frequencies were $f_{(Ser49Arg389)}$=0.57, $f_{(Ser49Gly389)}$=0.34 and $f_{(Gly49Arg389)}$=0.09 for males and $f_{(Ser49Arg389)}$=0.54, $f_{(Ser49Gly389)}$=0.37 and $f_{(Gly49Arg389)}$= 0.09 for females. For the ADRB2 gene, 927 individuals (487 males and 440 females) were genotyped for the Gly16Arg (rs1042713) polymorphism and 932 individuals (489 males and 443 females) for the Gln27Glu (rs1042714) polymorphism. Allelic frequencies for Gly16Arg (rs1042713) were $f_{(Gly16)}$=0.59 and $f_{(Arg16)}$=0.41 for males and $f_{(Gly)}$=0.61 and $f_{(Arg16)}$=0.39 for females. For the Gln27Glu (rs1042714) variant, allelic frequencies were $f_{(Gln27)}$=0.66 and $f_{(Glu27)}$=0.34 for males and $f_{(Gln27)}$=0.65 and $f_{(Glu27)}$=0.35 for females. Based on published literature (Drysdale et al. 2000) diplotypes (haplotype allelic combinations) for 919 individuals (482 males and 437 females) were inferred; haplotype frequencies for males were $f_{(Arg16Gln27)}$=0.41, $f_{(Gly16Gln27)}$=0.25 and $f_{(Gly16Glu27)}$=0.34, and for females were $f_{(Arg16Gln27)}$=0.39, $f_{(Gly16Gln2)}$=0.27 and $f_{(Gly16Glu27)}$=0.34. For the ADRB3 Trp64Arg (rs4994) polymorphism, 945 genotypes were established (493 males and 452 females); allelic frequencies were $f_{(Trp64)}$=0.94 and $f_{(Arg64)}$=0.06 for males and $f_{(Trp64)}$=0.95 and $f_{(Arg64)}$=0.05 for females. Thirty individuals were re-sampled giving identical genotypes on each occasion. Together with the Hardy-

Table 6.2: Analysis of associations between genotypes/diplotypes and obesity-related phenotypes in inactive and active males. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that subscapular skinfolds has been abbreviated to (Subscap Skinfolds).

		Inactive Males					Active Males				
		BMI	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**	**BMI**	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**
Polymorphism	**ADRB1 Ser49Gly**	P=0.674 V=0.8% N=99	P=0.986 V<0.1% N=99	P=0.922 V=0.2% N=99	P=0.974 V<0.1% N=99	P=0.961 V=0.1% N=99	**P=0.013** **V=2.2%** **N=405**	P=0.320 V=0.6% N=404	*P=0.087* *V=1.2%* *N=403*	P=0.157 V=0.9% N=402	P=0.180 V=0.9% N=403
	ADRB1 Arg389Gly	P=0.649 V=0.9% N=98	P=0.753 V=0.6% N=98	P=0.330 V=2.3% N=98	P=0.449 V=1.7% N=98	P=0.494 V=1.5% N=98	P=0.488 V=0.4% N=408	*P=0.064* *V=1.4%* *N=407*	P=0.335 V=0.5% N=406	P=0.120 V=1.0% N=406	P=0.116 V=1.1% N=406
	ADRB1 Diplotypes	P=0.912 V<0.1% N=98	P=0.975 V=0.9% N=98	P=0.401 V=5.3% N=98	P=0.724 V=3.0% N=98	P=0.762 V=2.7% N=98	P=0.158 V=2.2% N=401	P=0.118 V=2.2% N=400	P=0.190 V=1.9% N=399	P=0.117 V=2.2% N=399	P=0.134 V=2.1% N=399
	ADRB2 Gly16Arg	*P=0.073* *V=5.6%* *N=94*	P=0.986 V<0.1% N=94	P=0.608 V=1.1% N=94	P=0.839 V=0.4% N=94	P=0.887 V=0.3% N=94	**P=0.022** **V=1.9%** **N=393**	**P=0.005** **V=2.7%** **N=392**	**P=0.022** **V=1.9%** **N=391**	**P=0.009** **V=2.4%** **N=391**	**P=0.007** **V=2.5%** **N=391**
	ADRB2 Gln27Glu	P=0.285 V=2.7% N=95	P=0.883 V=0.3% N=95	P=0.996 V<0.1% N=95	P=0.993 V<0.1% N=95	P=0.993 V<0.1% N=95	P=0.811 V=0.1% N=394	P=0.146 V=1.0% N=393	P=0.340 V=0.6% N=392	P=0.251 V=0.7% N=392	P=0.249 V=0.7% N=392
	ADRB2 Diplotypes	P=0.175 V=8.4% N=92	P=0.860 V=2.2% N=92	P=0.293 V=6.8% N=92	P=0.544 V=4.5% N=92	P=0.614 V=4.0% N=92	*P=0.067* *V=2.6%* *N=390*	**P=0.014** **V=3.7%** **N=389**	*P=0.064* *V=2.7%* *N=388*	**P=0.024** **V=3.3%** **N=388**	**P=0.020** **V=3.4%** **N=388**
	ADRB3 Trp64Arg	P=0.724 V=0.1% N=98	**P=0.046** **V=4.1%** **N=98**	P=0.130 V=2.4% N=98	*P=0.061* *V=3.6%* *N=98*	*P=0.055* *V=3.8%* *N=98*	P=0.420 V=0.4% N=395	P=0.469 V=0.4% N=394	P=0.263 V=0.7% N=393	P=0.302 V=0.6% N=393	P=0.336 V=0.6% N=393

Weinberg equilibrium of the population these provided strong validation of the genotyping methods used.

6.3.2 Genotype associations with phenotypes For the ADRB1 gene, in total males (table 6.1), significant differences were observed between the means of the individual Ser49Gly (rs1801252) genotypes for BMI with Ser49Gly heterozygotes having the lowest mean BMI values (data not shown). No significant differences were observed for the ADRB1 genotypes/diplotypes in the group of total females (table 6.1).

For the ADRB2 gene, in the group of total males, significant differences were observed between the means of Gly16Arg (rs1042713) genotypes for BMI, triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat (table 6.1). As indicated by their mean scores (data not shown), where significant, the Gly16Gly homozygotes were the fattest in contrast to Arg16 homozygotes that were the slimmest. In the group of total males, significant differences were also observed between the means of inferred diplotypes for BMI, triceps skinfolds, subscapular skinfolds, total skinfolds and for skinfolds estimated % body fat (table 6.1). Arg16Gln27/Arg16Gln27 homozygotes had the lowest mean BMI values relative to the remaining diplotypes (data not shown). In contrast, for all remaining obesity-related phenotypes Arg16Gln27/Gly16Glu27 individuals were the slimmest (data not shown). No other significant differences were observed for the ADRB2 genotypes/diplotypes in the group of total males or females (table 6.1).

No significant differences were observed between the means of the ADRB3 Trp64Arg (rs4994) genotypes for any of the obesity-related phenotypes in the group of total males and females (table 6.1).

6.3.3 Analysis by level of regular activity For the ADRB1 gene, irrespective of regular activity participation, significant differences were observed only between the Ser49Gly (rs1801252) genotypes for BMI in the group of active males (table 6.2); Ser49Gly heterozygotes had the lowest mean BMI values. No significant differences were observed for the ADRB1 genotypes/diplotypes in inactive or active females (table 6.3).

In the group of active males, significant differences were observed for the ADRB2 Gly16Arg (rs1042713) variant with BMI, triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat (table 6.2). Gly16 homozygotes had the highest mean BMI values (data not shown) and were the fattest relative to Arg16 homozygotes and heterozygotes (for example figure 6.1). In active males, significant differences were also detected for the inferred ADRB2 diplotypes with triceps skinfolds, total skinfolds and skinfolds estimated % body fat (table 6.2); Arg16Gln27/Gly16Glu27 individuals had the lowest mean skinfolds and skinfolds estimated % body fat (data not shown). No significant differences were observed for the ADRB2 genotypes/diplotypes in the groups of inactive or active females (table 6.3).

For the ADRB3 gene, a sole significant difference was detected for the Trp64Arg (rs4994) polymorphism with triceps skinfolds in the group of inactive males (table 6.2); Trp64Arg heterozygotes had the lowest mean triceps skinfolds values relative to Trp64Trp homozygotes (data not shown). No significant differences were observed for the ADRB3

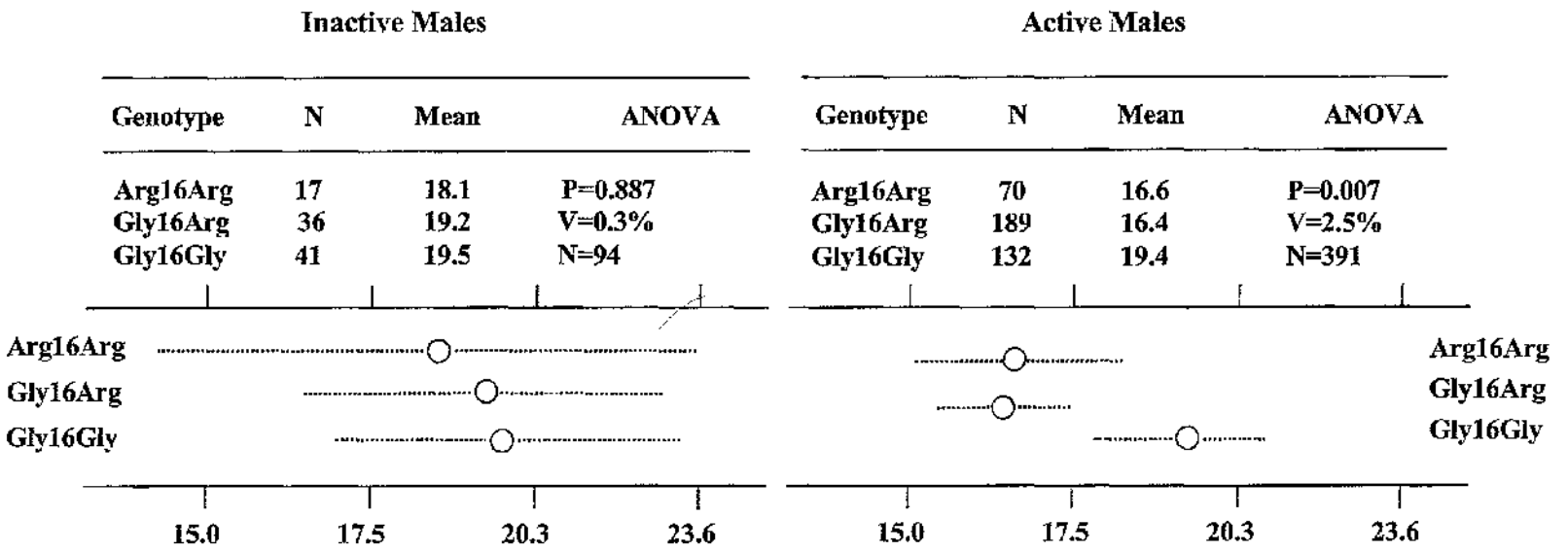


Figure 6.1: Gly16Arg genotype differences in skinfolds estimated % body fat of active and inactive males. Inactive Gly16Gly homozygotes have significantly higher skinfolds estimated % body fat when compared to the Arg16Arg and Gly16Arg individuals. (P)=probability, (V)=observed variance explained, (N)=number within group.

genotypes in active males (table 6.2) and in females irrespective of organised physical activity participation (table 6.3).

6.3.4 Determination of the type of genetic effect observed for associations with the ADRB polymorphisms Considering the phenotypic effects of the Ser49Gly (rs1801252) genotype a dominant effect of the Ser49 allele explained 67% and 80% of the data for BMI in total and active males, respectively. For the ADRB2 Gly16Arg (rs1042713) genotype, an additive genetic model accounted for 95% of the observed genetic variance for BMI in total and active males. In contrast, an Arg16 dominance model explained 95%, 97%, 99% and 100% of the genetic variance for triceps skinfolds, subscapular skinfolds, total skinfolds and skinfolds estimated % body fat, respectively. For the ADRB3 gene, the lack of Arg64Arg homozygotes in inactive males did not allow the determination of the type of the genetic effect of the Trp64Arg (rs4994) variant on triceps skinfolds.

6.3.5 Associations of ADRB genetic variants with extreme phenotypes The odds ratios were broadly consistent with the phenotypic means reported in the ANOVAs and the genetic models identified by the correlation analyses. ADRB1 Ser49Gly heterozygotes were significantly under-represented in the high quartile for BMI in both total (P=0.021, OR: 0.48, 95% CIs: 0.26 to 0.91) and active males (P=0.007, OR: 0.33, 95% CIs: 0.15 to 0.76). In active males, Ser49Ser homozygotes were significantly over-represented in the high quartile for BMI (P=0.019, OR: 2.37, 95% CIs: 1.13 to 4.99).

ADRB2 Arg16Arg homozygotes were significantly over-represented in the low quartile groups for BMI and subscapular skinfolds in active males (table 6.4). In total and active males, ADRB2 Gly16Arg heterozygotes were significantly over-represented in the low

quartile group for triceps skinfolds and significantly under-represented in the high quartile groups for BMI and total skinfolds (table 6.4). ADRB2 Gly16Arg heterozygotes were also significantly under-represented in the high quartile group of triceps skinfolds and skinfolds estimated % body fat in total males (table 6.4). In contrast, ADRB2 Gly16 homozygotes were significantly under-represented in the low quartile groups for triceps skinfolds, total skinfolds and skinfolds estimated % body fat in total and active males and significantly under-represented in the low quartile group for subscapular skinfolds in active males (table 6.4; for example figure 6.2). ADRB2 Gly16 homozygotes were concomitantly significantly over-represented in the high quartile for all measurements (table 6.4; for example figure 6.2).

Considering ADRB2 haplotypes for total males, Arg16Gln27/Arg16Gln27 individuals were significantly over-represented in the low quartile for BMI in contrast to Gly16Glu27/Gly16Glu27 individuals that were significantly under-represented in the low quartile and significantly over-represented in the high quartile for BMI (table 6.5). ADRB2 Arg16Gln27/Gly16Glu27 males were significantly under-represented in the high group for BMI (table 6.5). For all the remaining phenotypes in total males, ADRB2 Arg16Gln27/Gly16Glu27 individuals were significantly over-represented in the low quartile and significantly under-represented in the high quartile phenotypic groups while the opposite was true for Gly16Glu27/Gly16Glu27 individuals (table 6.5). For total skinfolds and skinfolds estimated % body fat in the active males, Arg16Gln27/Arg16Gln27 individuals were significantly over-represented in the low quartile groups while Gly16Gln27/Gly16Glu27 individuals were significantly over-represented in the high quartile groups (table 6.5).

Table 6.3: Analysis of associations between genotypes/diplotypes and obesity-related phenotypes in inactive and active females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that subscapular skinfolds has been abbreviated to (Subscap Skinfolds).

		Inactive Females					Active Females				
		BMI	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds %BF**	**BMI**	**Triceps Skinfolds**	**Subscap Skinfolds**	**Total Skinfolds**	**Skinfolds % BF**
Polymorphism	**ADRB1 Ser49Gly**	P=0.957 V<0.1% N=192	P=0.105 V=2.4% N=192	P=0.767 V=0.3% N=192	P=0.310 V=1.2% N=192	P=0.293 V=1.3% N=192	P=0.450 V=0.6% N=256	P=0.841 V=0.1% N=257	P=0.938 V<0.1% N=257	P=0.899 V=0.1% N=257	P=0.881 V=0.1% N=257
	ADRB1 Arg389Gly	P=0.799 V=0.2% N=196	P=0.806 V=0.2% N=196	P=0.973 V<0.1% N=196	P=0.976 V<0.1% N=196	P=0.985 V<0.1% N=196	P=0.585 V=0.4% N=257	P=0.639 V=0.4% N=258	P=0.723 V=0.3% N=258	P=0.904 V=0.1% N=258	P=0.919 V=0.1% N=258
	ADRB1 Diplotypes	P=0.926 V=0.7% N=189	P=0.324 V=3.1% N=189	P=0.980 V=0.4% N=189	P=0.731 V=1.5% N=189	P=0.721 V=1.5% N=189	P=0.255 V=2.6% N=251	P=0.898 V=0.7% N=252	P=0.874 V=0.7% N=252	P=0.964 V=0.4% N=252	P=0.968 V=0.4% N=252
	ADRB2 Gly16Arg	P=0.338 V=1.2% N=186	*P=0.090* *V=2.6%* *N=186*	P=0.435 V=0.9% N=186	P=0.143 V=2.1% N=186	P=0.138 V=2.1% N=186	P=0.879 V=0.1% N=253	P=0.773 V=0.2% N=254	P=0.931 V=0.1% N=254	P=0.871 V=0.1% N=254	P=0.854 V=0.1% N=254
	ADRB2 Gln27Glu	P=0.221 V=1.6% N=186	P=0.288 V=1.4% N=186	P=0.698 V=0.4% N=186	P=0.401 V=1% N=186	P=0.421 V=0.9% N=186	P=0.450 V=0.6% N=256	P=0.635 V=0.4% N=257	P=0.665 V=0.3% N=257	P=0.617 V=0.4% N=257	P=0.588 V=0.4% N=257
	ADRB2 Diplotypes	P=0.606 V=2.0% N=184	P=0.306 V=3.3% N=184	P=0.858 V=1.1% N=184	P=0.481 V=2.5% N=184	P=0.480 V=2.5% N=184	P=0.892 V=0.7% N=252	P=0.677 V=1.3% N=253	P=0.771 V=1% N=253	P=0.726 V=1.1% N=253	P=0.718 V=1.2% N=253
	ADRB3 Trp64Arg	P=0.100 V=1.4% N=196	P=0.749 V=0.1% N=196	P=0.272 V=0.6% N=196	P=0.409 V=0.4% N=196	P=0.394 V=0.4% N=196	P=0.221 V=0.6% N=255	P=0.510 V=0.2% N=256	P=0.711 V=0.1% N=256	P=0.847 V<0.1% N=256	P=0.763 V<0.1% N=256

For inactive males, ADRB3 Trp64 homozygotes were significantly under-represented (P=0.007, OR=0.24, 95% CI=0.08 to 0.72) in the low quartile for triceps skinfolds in contrast to Trp64Arg heterozygotes that were significantly over-represented (P=0.007, OR=4.25, 95% CI=1.40 to 12.95) in the same quartile group.

6.4 Discussion

Polymorphisms within the ADRB2 gene significantly associated with several of the obesity-related phenotypes measured in the present cohort of adolescent children. These associations were gender-specific and influenced by participation in organised physical activity. The present findings were consistent with a previously unreported, protective effect of the ADRB2 Arg16Gln27/Gly16Glu27 diplotype over the obese phenotype that was dominantly mediated by the Arg16 variant. In contrast, the ADRB2 Gly16 allele and the corresponding Gly16Glu27/Gly16Glu27 diplotype were associated with increased adiposity. In addition, the Arg16Gln27/Arg16Gln27 and the Gly16Gln27/Gly16Glu27 diplotypes associated with lower and higher, respectively, levels of adiposity however this association was primarily observed in active males.

Several studies have advocated the use of haplotype-based methods rather than individual SNPs to assess genetic effects mediating susceptibility to disease or complex traits (for examples (Akey et al. 2001),(Pritchard and Cox 2002),(Weiss and Clark 2002)). Considering the *P*-values, haplotype analysis did not produce markedly stronger associations compared to individual genotypes although diplotypes explained a larger % of the observed variance. Nevertheless, for significant associations, the increase in the % of variance explained by haplotype analysis was no larger than what would be expected by random sampling effects. The mechanics of haplotype analysis, requiring splitting of the data into smaller groups relative to genotypes, may have underpowered this study to detect certain effects (for example ADRB2 diplotypes with BMI in active males; table 6.2). These results however, are not evidence against the greater analytical power of haplotypes or their use in the investigation of complex traits. If, for example, SNPs at positions 16 and 27 had been independently assessed, the protective effect over the obese phenotype of a

Table 6.4: ADRB2 Gly16Arg genotype odds ratios for phenotypes significant by ANOVA (tables 6.1 and 6.2). 95% confidence intervals are given in parentheses. Probability (P) <0.05 for all tests shown, * indicates P≤0.01. Low means lower BMI, lower skinfold thickness and lower skinfolds estimated % BF. High indicates the converse. (n.s.) indicates X^2 tests that were not statistically significant. Note that subscapular skinfolds has been abbreviated to (Subscap Skinfolds).

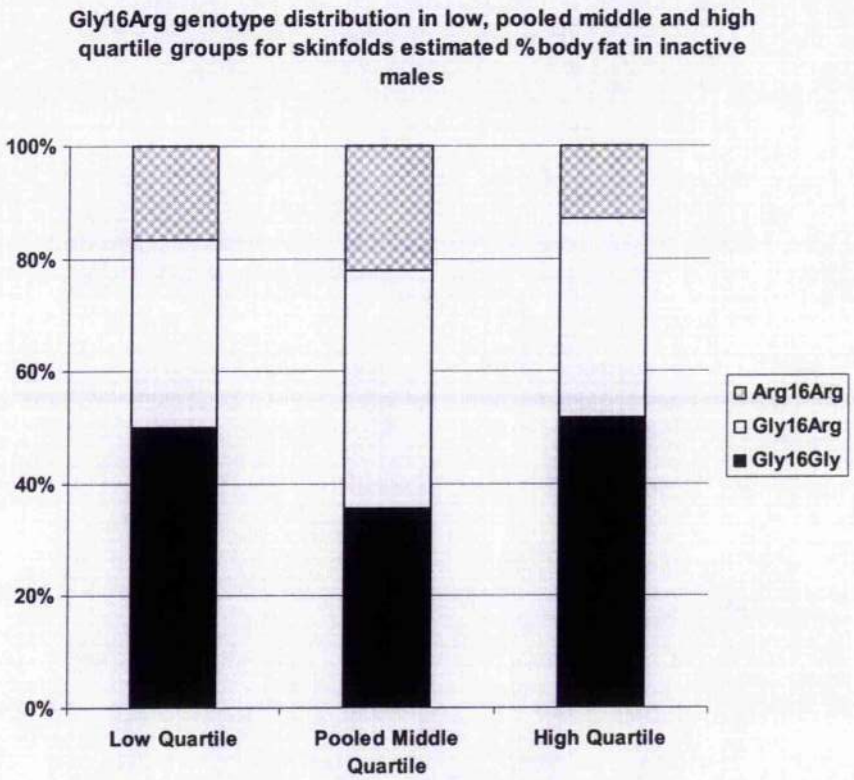
ADRB2 Gly16Arg		Total Males		Active Males	
		Low	High	Low	High
BMI	**Arg16Arg**	2.02* (1.23 to 3.33)	n.s.	1.97 (1.13 to 3.44)	n.s.
	Gly16Arg	n.s.	0.63 (0.42 to 0.95)	n.s.	0.55 (0.34 to 0.88)
	Gly16Gly	n.s.	1.75* (1.16 to 2.64)	n.s.	1.90* (1.19 to 3.04)
Triceps Skinfolds	**Arg16Arg**	n.s.	n.s.	n.s.	n.s.
	Gly16Arg	1.57 (1.03 to 2.38)	0.65 (0.43 to 0.99)	1.68 (1.06 to 2.68)	n.s.
	Gly16Gly	0.57 (0.36 to 0.90)	1.73* (1.14 to 2.62)	0.46* (0.27 to 0.80)	1.84 (1.15 to 2.94)
Subscap Skinfolds	**Arg16Arg**	n.s.	n.s.	1.82 (1.04 to 3.18)	n.s.
	Gly16Arg	n.s.	n.s.	n.s.	n.s.
	Gly16Gly	n.s.	1.63 (1.08 to 2.46)	0.53 (0.31 to 0.89)	1.67 (1.04 to 2.69)
Total Skinfolds	**Arg16Arg**	n.s.	n.s.	n.s.	n.s.
	Gly16Arg	n.s.	0.62 (0.41 to 0.94)	n.s.	0.59 (0.37 to 0.95)
	Gly16Gly	0.50 (0.34 to 0.88)	1.81* (1.20 to 2.74)	0.44* (0.25 to 0.76)	1.82 (1.13 to 2.92)
Skinfolds % BF	**Arg16Arg**	n.s.	n.s.	n.s.	n.s.
	Gly16Arg	n.s.	0.65 (0.43 to 0.99)	n.s.	n.s.
	Gly16Gly	0.55 (0.34 to 0.88)	0.73* (1.14 to 2.62)	0.44* (0.25 to 0.76)	1.71 (1.07 to 2.76)

genetic subdivision of the Gly16Arg (rs1042713) variant, the Arg16Gln27/Gly16Glu27 diplotype, would, most likely, not have been detected.

Association studies are at risk of type-I (false positive) errors caused by multiple testing. The number of significant associations found here was compared to the number of significant associations expected by chance, given the number of statistical tests performed on each gender. Within each (sub)population 35 tests were conducted (5 phenotypes x 7 genotypes/diplotypes) and would expect approximately two to show significance ($P<0.05$) by chance ($0.05x35=1.75$). Considering as an example the group of total males the number of tests actually found to be statistically significant (*i.e.* 11) was greatly in excess of what would be expected from chance alone and, as such, the significant effects observed must have represented more than an effect of multiple testing. The significant effects for the ADRB2 gene persisted across activity-divided phenotypic groups and, considering the results for the ORs, significant genotypic influences were detected across the phenotypic distributions of the obesity-related parameters. These findings increased confidence in the validity of the significant findings for the ADRB2 gene. In contrast, the low number of significant associations detected for the ADRB1 and ADRB3 genes (two and one, respectively) would suggest that these findings should be interpreted with caution. In addition, as described in General Methods (p. 52-54), a Šidak correction in the presence of partially correlated phenotypes was applied that produced an α value equal to 0.017; total skinfolds and skinfolds estimated % body fat were excluded from the correction as they were calculated from the skinfolds measurements at the triceps and subscapular sites hence they were not adding to the data (Appendix 4). Considering the significant findings for the ADRB2 Gly16Arg (rs1042713) variant in total and active males (tables 6.1 and 6.2) would suggest that the significant associations observed for BMI, triceps and subscapular skinfolds in total males and the significant associations observed for triceps and

subscapular skinfolds in active males remained significant even after the Šidak correction. In a similar fashion the significant findings for ADRB2 haplotypes with BMI in total males and with triceps skinfolds in active males remained significant even after the α has been lowered to 0.017. These findings serve to increase confidence in the aforementioned suggestions with regards to an influence of the ADRB2 gene on obesity-related phenotypes. Furthermore, adjusting for multiple testing indicated that the significant association of the Trp64Arg (rs4994) genotype with triceps skinfolds in inactive males (table 6.2) was most likely the result of a type I error, as previously suggested. However, the significant associations for the ADRB1 Ser49Gly (rs1801252) variant with BMI in total and active males (tables 6.1 and 6.2) remained significant even after the Šidak correction. Nevertheless, as previously indicated, an effect for the Ser49Gly variant was observed for only two tests therefore caution must be exercised when interpreting these results.

Previous investigations have associated polymorphisms in the ADRB1 gene with pathophysiologies of the heart (for review (Small et al. 2003)), greater body mass and BMI (Dionne et al. 2002). Irrespective of gender and participation in organised physical activity, the present investigation did not detect significant associations between variants of the ADRB1 gene and obesity-related phenotypes in Greek adolescents. In males, a significant effect was detected for the Ser49Gly (rs1801252) variant with BMI that was influenced by participation in physical activity but was not reflected on significant associations with other obesity-related phenotypes. This association was best explained by a Ser49-allele dominant genetic model that, for the group of total males, did not explain the observed phenotypic variance well (*i.e.* <75% of variance explained). This finding suggested that this was a case of under or over-dominance, in which the heterozygote mean was more extreme in phenotype than either of the homozygote means. Genetic models for



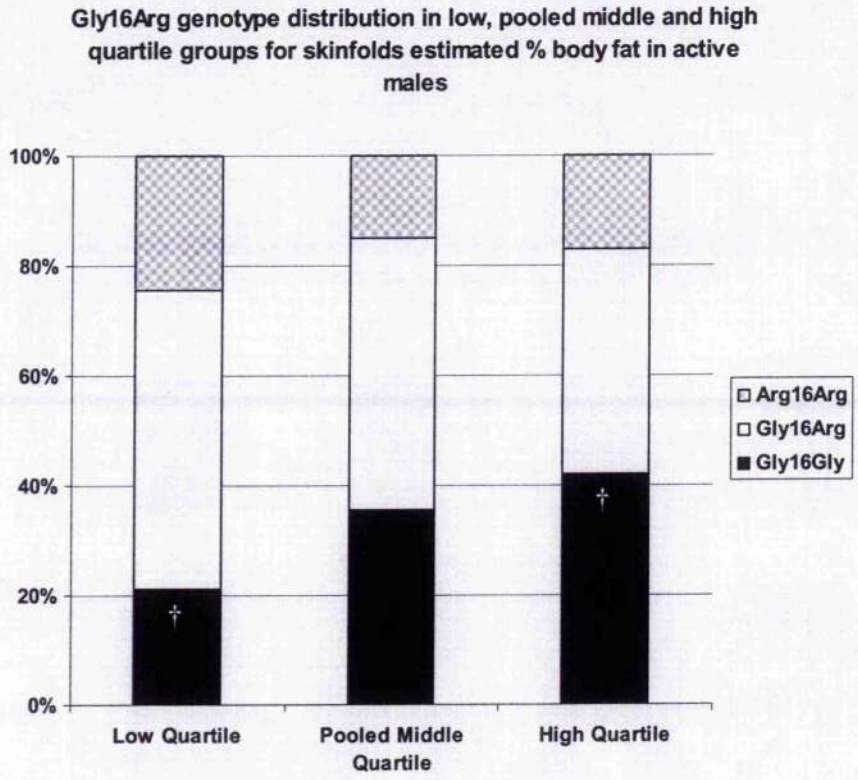


Figure 6.2: Gly16Arg genotype distributions in the low, pooled middle and high quartiles for skinfolds estimated % body fat in active and inactive males. Within quartiles, significantly over or under-represented genotypes (table 6.4) are indicated by (†). Active Gly16 homozygotes were significantly over-represented in the high quartile group for skinfolds estimated % body fat and concomitantly significantly under-represented in the low quartile group for skinfolds estimated % body fat.

under or over-dominance are difficult to reconcile with molecular mechanisms. The detected underdominance was most likely caused by outliers shifting the mean of Ser49Gly heterozygotes lower than that of both homozygotes. Irrespective of considerations for the functional plausibility of a Ser49Gly (rs1801252) effect on BMI and the persistency of this effect across activity-divided groups, the lack of a consistent association with other phenotypes would question these results. Population-based variations in allelic frequencies could have influenced, in part, these findings however considering all the available evidence indicates that the significant association of the Ser49Gly (rs1801252) genotype with BMI must be interpreted with caution.

A number of studies (Fujisawa et al. 1998),(Oizumi et al. 2001),(Strazzullo et al. 2001) have reported associations between the ADRB3 Trp64Arg (rs4994) polymorphism and indices of obesity; others have failed to reproduce similar findings (Moriarty et al. 1997). Here, an influence of the Trp64Arg (rs4994) genotype on obesity-related phenotypes was not observed, irrespective of gender and organised activity participation. A single significant association was detected between the Trp64Arg (rs4994) genotypes and triceps skinfolds in inactive males. As previously suggested (for a review (Snyder et al. 2004)), the low allelic frequency of the Arg64 variant coupled to the small number of subjects in the group of inactive males may have influenced the reported findings. Nevertheless, the presence of a single significant association and, as indicated by the results for the ORs, the lack of a consistent genetic effect across phenotypic distributions would suggest that the influence of the Trp64Arg (rs4994) genotype on triceps skinfolds was most likely the result of a type I error.

Most previous studies have concentrated on adult populations, linking ADRB2 genotypes with obesity-related phenotypes (for examples (Snyder et al. 2004)). All four alleles of the

Gly16Arg (rs1042713) and Gln27Glu (rs1042714) polymorphisms have been previously associated with an increased risk for obesity. For example, (Large et al. 1997) indicated that adult women homozygous for the Glu27 allele were associated with increased body fat and that the Gly16Arg variant (rs1042713) is associated with ADRB2 function but has no bearing on the obese phenotype. In contrast, (Meirhaeghe et al. 2000) has suggested that the Gln27Gln genotype is associated with higher scores for obesity-related phenotypes in men but not in women and that if Gln27 homozygotes carried the Arg16 allele the aforementioned associations were stronger. Furthermore, (Hellstrom et al. 1999) indicated that the Glu27 variant positively associates with obesity in adult females but it is negatively associated with obesity in adult males while (Ellsworth et al. 2002), in a 24-year longitudinal study, associated the Gly16 allele with age-dependent increases adiposity and obesity in males but not in females. Previous reports have also indicated that, in adult males, the genetic influences of ADRB2 variants on obesity are modified by participation physical activity (for example (Meirhaeghe et al. 1999). Here, the Gly16Arg polymorphism (rs1042713), but not the Gln27Glu (rs1042714) variant, was associated with obesity-related phenotypes in a gender-specific manner; Gly16 homozygous males had higher levels of adiposity in contrast to Arg16 carriers that generally associated with low and moderate adiposity. These associations were influenced by participation in organised physical activity; active Arg16 carriers had lower BMI and adiposity compared to Gly16 homozygotes while no significant differences were observed in inactive males. The significant phenotypic differences between genotypes were best explained by an Arg16 allele dominant genetic model in which the heterozygote mean was nearly identical to one of the two homozygote means. The results for individual SNPs and haplotypes were consistent with previous reports (McGraw et al. 1998) of a dominant influence of the Gly16Arg variant on receptor function and indicated the presence of a previously

Table 6.5: ADRB2 diplotype odds ratios for phenotypes significant by ANOVA (tables 6.1 and 6.2). 95% confidence intervals are given in parentheses. Probability (P) <0.05 for all tests shown, * indicates P≤0.01, ** indicates P≤0.001. Low means lower BMI, lower skinfold thickness and lower skinfolds estimated % body fat. High indicates the converse. (n.s.) indicates X^2 tests that were not statistically significant. Note that subscapular skinfolds has been abbreviated to (Subscap Skinfolds).

ADRB2 Diplotypes		Total Males		Active Males	
		Low	High	Low	High
BMI	Arg16Gln27/Arg16Gln27	2.10* (1.27 to 3.47)	n.s.	ANOVA not significant	
	Arg16Gln27/Gly16Glu27	n.s.	0.54* (0.34 to 0.87)		
	Gly16Glu27/Gly16Glu27	0.37* (0.17 to 0.79)	2.87** (1.74 to 4.76)		
	Gly16Gln27/Gly16Glu27	n.s.	n.s.		
Triceps Skinfolds	Arg16Gln27/Arg16Gln27	n.s.	n.s.	n.s.	n.s.
	Arg16Gln27/Gly16Glu27	2.11** (1.35 to 3.28)	0.58 (0.36 to 0.92)	n.s.	n.s.
	Gly16Glu27/Gly16Glu27	0.33* (0.15 to 0.75)	3.02** (1.80 to 5.06)	n.s.	n.s.
	Gly16Gln27/Gly16Glu27	n.s.	n.s.	n.s.	n.s.
Subscap Skinfolds	Arg16Gln27/Arg16Gln27	n.s.	n.s.	ANOVA not significant	
	Arg16Gln27/Gly16Glu27	1.62 (1.03 to 2.54)	0.58 (0.37 to 0.93)		
	Gly16Glu27/Gly16Glu27	0.43; (0.21 to 0.90)	2.91** (1.76 to 4.82)		
	Gly16Gln27/Gly16Glu27	n.s.	n.s.		
Total Skinfolds	Arg16Gln27/Arg16Gln27	n.s.	n.s.	1.93 (1.03 to 3.63)	n.s.
	Arg16Gln27/Gly16Glu27	1.79 (1.14 to 2.81)	0.53* (0.33 to 0.86)	n.s.	n.s.
	Gly16Glu27/Gly16Glu27	0.40 (0.19 to 0.87)	2.98** (1.79 to 4.97)	n.s.	n.s.
	Gly16Gln27/Gly16Glu27	n.s.	n.s.	n.s.	2.25 (1.15 to 4.41)
Skinfolds % BF	Arg16Gln27/Arg16Gln27	n.s.	n.s.	1.93 (1.03 to 3.63)	n.s.
	Arg16Gln27/Gly16Glu27	1.79 (1.14 to 2.81)	0.53* (0.33 to 0.86)	n.s.	n.s.
	Gly16Glu27/Gly16Glu27	0.40 (0.19 to 0.87)	2.98** (1.79 to 4.97)	n.s.	n.s.
	Gly16Gln27/Gly16Glu27	n.s.	n.s.	n.s.	2.25 (1.15 to 4.41)

unreported, protective effect of the ADRB2 Arg16Gln27/Gly16Glu27 diplotype over the obese phenotype that was dominantly mediated by the Arg16 variant. In contrast, the Gly16Gln27/Gly16Glu27 diplotype was associated with increased adiposity. In addition, the Arg16Gln27/Arg16Gln27 and Gly16Gln27/Gly16Glu27 diplotypes associated with lower and higher, respectively, levels of adiposity but this association was detected primarily in the group of active males. It must be noted, that these findings may differ between populations of different gender, age and environment. As such, the present results make clear that selecting a cohort of the "wrong" gender, age or activity could easily mask the modest effects of the ADRB2 gene on phenotype. Such effects, if present, may have contributed, at least in part, to the discrepancies in the findings of the present relative to some previous investigations.

6.5 Conclusion

This study is among the first few to investigate associations of polymorphic variants within the ADRB genes in adolescent children using both genotype and haplotype–based methods while considering interactions with physical activity. The ADRB2 gene was associated with modest influences on several obesity-related phenotypes in young males. The present results were consistent with a previously unreported, protective effect of the Arg16Gln27/Gly16Glu27 diplotype against increased adiposity that was dominantly mediated by the Arg16 allele and was influenced by participation in organised physical activity. In addition, the findings from haplotype-analysis indicated that the penetrance of the ADRB2 haplotype effects on obesity may be further mediated by the presence of an, as yet, undetermined factor(s). A significant association of the ADRB1 Ser49Gly genotype with BMI was also observed however this finding must be interpreted with caution. Further

studies are required to promote the current understanding of the genetic and molecular mechanisms mediating the influence of ADRB genetic variants on human obesity.

CHAPTER 7

EFFECTS OF INTERACTION BETWEEN ADRB POLYMORPHISMS AND LIFESTYLE ON PHYSIOLOGICAL, PHYSICAL AND SKILL-RELATED PHENOTYPES IN ADOLESCENT GREEKS

7.1 Introduction

All three ADRB genes code for 7-transmembrane GPCRs that are expressed in a variety of tissues (for reviews (Small et al. 2003),(Leineweber and Brodde 2004)). The primary function of these receptors is to mediate the activation by catecholamines of adenylyl cyclase, a key enzyme in intracellular signalling pathways. GPCRs are thought to play a central role in local tissue metabolism and function; in adipose tissue, for example, the ADRBs are involved in stimulating lipolysis and thermogenesis (for example (Carpene et al. 1998),(Bachman et al. 2002)). Studies have previously sought to associate polymorphisms of these genes with fitness-related phenotypes with some degree of success, albeit mainly in adults (for review (Rankinen et al. 2004)); for example, the ADRB1 Arg389 allele, conferring increased levels of adenylyl cyclase activity (Mason et al. 1999), has been associated with higher $\dot{V}o_{2\,max}$ in patients with cardiomyopathy (Wagoner et al. 2002). Genotyping of the two commonest ADRB1 variants Ser49Gly (rs1801252) and Arg389Gly (rs1801253) allows the inference of 3 haplotypes that represent the majority of variation along the complete length of the gene. In the ADRB2 gene, (Drysdale et al. 2000) has established 12 haplotypes; typing of codons 16 and 27 allows the inference of three of these haplotypes that account for 95% of the observed ADRB2 genetic variation in Caucasians (Drysdale et al. 2000).

Given the associations with fitness-related parameters in selected adult populations, the present study aimed to assess whether variation in the ADRB genes had an effect on physical, physiological and skill-related phenotypes in a large, representative sample of young individuals using both genotype and haplotype-based approaches. Furthermore, genotype-environment interactions with gender and participation in organised physical activity were also considered.

Table 7.1: Analysis of associations between genotypes/diplotypes and physical, physiological and skill-related phenotypes in total males. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05). Note that basketball throw has been abbreviated to (Basket Throw).

		Total Males									
		$\dot{V}O_{2\ max}$	**Sitting Height**	**Arm Span**	**Sit & Reach**	**Basket Throw**	**Throw & Catch**	**Hand-grip**	**Vertical Jump**	**40m Sprint**	**Agility Run**
Polymorphism	**Ser49Gly**	P=0.102 V=0.9% n=488	P=0.732 V=0.1% n=502	P=0.418 V=0.4% n=502	P=0.807 V=0.1% n=499	*P=0.068* *V=1.1%* *n=503*	P=0.815 V=0.1% n=503	P=0.904 V<0.1% n=503	P=0.363 V=0.4% n=502	*P=0.058* *V=1.2%* *n=492*	P=0.485 V=0.3% n=497
	Arg389Gly	P=0.179 V=0.7% n=490	P=0.426 V=0.3% n=504	**P=0.033** **V=1.4%** **n=504**	P=0.751 V=0.1% n=500	P=0.267 V=0.5% n=505	P=0.537 V=0.2% n=505	P=0.849 V=0.1% n= 505	P=0.110 V=0.9% n= 504	P=0.130 V=0.8% n= 491	**P=0.041** **V=1.3%** **n=498**
	ADRB1 diplotypes	P=0.199 V=1.5% n=484	P=0.757 V=0.5% n=497	*P=0.098* *V=1.9%* *n=497*	P=0.923 V=0.3% n=493	*P=0.087* *V=1.9%* *n=498*	P=0.890 V=0.3% n=498	P=0.832 V=0.4% n=498	P=0.218 V=1.4% n=497	*P=0.064* *V=2.1%* *n=486*	P=0.158 V=1.6% n=492
	Gly16Arg	P=0.491 V=0.3% n=473	P=0.159 V=0.8% n=485	P=0.418 V=0.4% n=485	P=0.951 V<0.1% n=482	*P=0.089* *V=1.0%* *n=486*	P=0.425 V=0.4% n=486	**P=0.002** **V=2.6%** **n=486**	P=0.621 V=0.2% n= 485	P=0.563 V=0.2% n= 474	P=0.264 V=0.6% n=480
	Gln27Glu	P=0.554 V=0.3% n=475	*P=0.082* *V=1.0%* *n=486*	P=0.674 V=0.2% n=487	P=0.229 V=0.6% n=484	P=0.422 V=0.4% n=488	P=0.510 V=0.3% n=488	P=0.137 V=0.8% n=488	P=0.612 V=0.2% n=487	P=0.998 V<0.1% n=476	P=0.823 V=0.1% n=482
	ADRB2 diplotypes	P=0.434 V=1.0% n=468	P=0.129 V=1.8% n=480	P=0.654 V=0.7% n=480	P=0.421 V=1.0% n=477	P=0.401 V=1.1% n=481	P=0.728 V=0.6% n=481	**P=0.013** **V=3.0%** **n=481**	P=0.858 V=0.4% n=480	P=0.812 V=0.5% n=469	P=0.407 V=1.1% n=475
	Trp64Arg	*P=0.085* *V=0.1%* *n=477*	P=0.829 V=0.1% n=491	P=0.280 V=0.5% n=491	P=0.994 V<0.1% n=487	P=0.468 V=0.3% n=492	P=0.622 V=0.2% n=492	P=0.526 V=0.3% n=492	P=0.982 V<0.1% n=491	P=0.957 V<0.1% n=478	P=0.673 V=0.2% n=485

7.2 Methods

7.2.1 Subjects and measurements For the present analysis, all information regarding subjects and measurements of physical, physiological and skill-related parameters in the Greek cohort were as described in General Methods (p. 33-37). The parameters assessed here included: $\dot{V}o_{2\,max}$, sitting height, arm span, sit and reach, basketball throw, throw and catch, hand-grip strength, vertical jump, agility run and the 40 m sprint.

7.2.2 DNA extraction, genotype determination and haplotype inference DNA extraction, quantification and storage as well as ADRB genotype determination and haplotype inference were performed as described in General Methods (p. 37-38 and 42-45).

7.2.3 Data Analysis All data and statistical analyses were carried out as described in General Methods (p. 45-54).

Table 7.2: Analysis of associations between genotypes/diplotypes and physical, physiological and skill-related phenotypes in total females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05).

		Total Females									
		$\dot{V}_{O_2\,max}$	Sitting Height	Arm Span	Sit & Reach	Basket Throw	Throw & Catch	Hand-grip	Vertical Jump	40m Sprint	Agility Run
Polymorphism	**Ser49Gly**	P=0.865 V=0.1% n=422	P=0.368 V=0.4% n=447	P=0.448 V=0.4% n=449	P=0.126 V=0.9% n=448	P=0.194 V=0.7% n=448	P=0.428 V=0.4% n=447	P=0.627 V=0.2% n=447	P=0.926 V<0.1% n=449	P=0.268 V=0.6% n=435	P=0.225 V=0.7% n=444
	Arg389Gly	P=0.849 V=0.1% n=428	P=0.873 V=0.1% n=452	P=0.342 V=0.5% n=454	P=0.876 V=0.1% n=453	P=0.139 V=0.9% n=453	P=0.156 V=0.8% n=452	P=0.983 V<0.1% n= 452	P=0.335 V=0.5% n= 454	P=0.505 V=0.3% n= 437	P=0.701 V=0.2% n=448
	ADRB1 diplotypes	P=0.693 V=0.7% n=414	P=0.403 V=1.2% n=439	P=0.566 V=0.9% n=441	P=0.403 V=1.2% n=440	P=0.156 V=1.8% n=440	P=0.260 V=1.5% n=439	P=0.740 V=0.6% n=439	P=0.748 V=0.6% n=441	P=0.411 V=1.2% n=427	P=0.371 V=1.2% n=436
	Gly16Arg	P=0.688 V=0.2% n=415	P=0.502 V=0.3% n=438	P=0.212 V=0.7% n=440	P=0.730 V=0.1% n=439	P=0.248 V=0.6% n=440	P=0.440 V=0.4% n=438	P=0.856 V=0.1% n=438	P=0.407 V=0.4% n= 440	P=0.969 V<0.1% n= 426	P=0.726 V=0.1% n=435
	Gln27Glu	P=0.160 V=0.9% n=417	P=0.588 V=0.2% n=441	**P=0.048** **V=1.4%** **n=443**	P=0.351 V=0.5% n=442	*P=0.090* *V=1.1%* *n=443*	*P=0.099* *V=1.1%* *n=441*	P=0.126 V=0.9% n=441	P=0.682 V=0.2% n=443	P=0.953 V<0.1% n=428	P=0.908 V<0.1% n=437
	ADRB2 diplotypes	P=0.244 V=1.6% n=412	P=0.890 V=0.4% n=435	P=0.228 V=1.6% n=437	P=0.900 V=0.4% n=436	P=0.358 V=1.3% n=437	P=0.278 V=1.5% n=435	P=0.517 V=1.0% n=435	P=0.831 V=0.5% n=437	P=0.968 V=0.2% n=423	P=0.877 V=0.4% n=432
	Trp64Arg	P=0.986 V<0.1% n=426	P=0.199 V=0.4% n=450	*P=0.079* *V=0.7%* *n=452*	P=0.512 V=0.1% n=451	P=0.164 V=0.4% n=451	P=0.507 V=0.1% n=450	P=0.289 V=0.3% n=450	P=0.713 V<0.1% n=452	P=0.516 V=0.1% n=435	P=0.482 V=0.1% n=447

7.3 Results

7.3.1 Genotyping All populations and subpopulations were in Hardy-Weinberg equilibrium (HWE) for all polymorphic loci tested; no significant differences were found in allele or haplotype frequencies. Genotypes for 953 individuals (504 males and 449 females) were successfully obtained for the ADRB1 Ser49Gly (rs1801252) polymorphism and for 960 individuals (506 males and 454 females) for the ADRB1 Arg398Gly polymorphism. Allele frequencies for the ADRB1 Ser49Gly (rs1801252) variant were, in both genders, $f_{(Ser49)}$=0.91 and $f_{(Gly49)}$=0.09 and for the ADRB1 Arg398Gly variant were $f_{(Arg389)}$=0.66 and $f_{(Gly389)}$=0.34 for males and $f_{(Arg389)}$=0.63 and $f_{(Gly389)}$=0.37 for females. Haplotypes were inferred for 940 individuals (499 males and 441 females); haplotype frequencies were $f_{(Ser49Arg389)}$=0.57, $f_{(Ser49Gly389)}$=0.34 and $f_{(Gly49Arg389)}$=0.09 for males and $f_{(Ser49Arg389)}$=0.54, $f_{(Ser49Gly389)}$=0.37 and $f_{(Gly49Arg389)}$= 0.09 for females. For the ADRB2 gene, 927 individuals (487 males and 440 females) were genotyped for the Gly16Arg (rs1042713) polymorphism and 932 individuals (489 males and 443 females) for the Gln27Glu (rs1042714) polymorphism. Allelic frequencies for Gly16Arg (rs1042713) were $f_{(Gly16)}$=0.59 and $f_{(Arg16)}$=0.41 for males and $f_{(Gly)}$=0.61 and $f_{(Arg16)}$=0.39 for females. For the Gln27Glu (rs1042714) variant, allelic frequencies were $f_{(Gln27)}$=0.66 and $f_{(Glu27)}$=0.34 for males and $f_{(Gln27)}$=0.65 and $f_{(Glu27)}$=0.35 for females. Based on published literature (Drysdale et al. 2000) diplotypes (haplotype allelic combinations) for 919 individuals (482 males and 437 females) were inferred; haplotype frequencies for males were $f_{(Arg16Gln27)}$=0.41, $f_{(Gly16Gln27)}$=0.25 and $f_{(Gly16Glu27)}$=0.34, and for females were $f_{(Arg16Gln27)}$=0.39, $f_{(Gly16Gln2)}$=0.27 and $f_{(Gly16Glu27)}$=0.34. For the ADRB*3* Trp64Arg (rs4994) polymorphism, 945 genotypes were established (493 males and 452 females); allelic frequencies were $f_{(Trp64)}$=0.94 and $f_{(Arg64)}$=0.06 for males and $f_{(Trp64)}$=0.95 and $f_{(Arg64)}$=0.05 for females. Thirty individuals were re-sampled giving identical genotypes on each occasion. Together with the Hardy-

Table 7.3: Analysis of associations between genotypes/diplotypes and physical, physiological and skill-related phenotypes in inactive (7.3a) and active (7.3b) males. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance ($0.05<P<0.1$) and where they are given in bold the tests were significant ($P<0.05$).

(7.3a)

		Inactive Males									
		$\dot{V}O_{2\ max}$	**Sitting Height**	**Arm Span**	**Sit & Reach**	**Basket Throw**	**Throw & Catch**	**Hand-grip**	**Vertical Jump**	**40m Sprint**	**Agility Run**
Polymorphism	**Ser49Gly**	**P=0.043** **V=6.6%** **n=95**	P=0.215 V=3.2% n=98	P=0.872 V=0.3% n=98	**P=0.239** V=3.0% n=98	P=0.377 V=2.0% n=99	P=0.657 V=0.9% n=99	P=0.739 V=0.6% n=99	*P=0.090* *V=5.0%* *n=97*	P=0.267 V=2.9% n=94	P=0.389 V=2.0% n=98
	Arg389Gly	P=0.153 V=4.0% n=94	P=0.890 V=0.2% n=97	P=0.738 V=0.6% n=97	P=0.864 V=0.3% n=97	*P=0.085* *V=5.1%* *n=98*	P=0.722 V=0.7% n=98	P=0.516 V=1.4% n=98	**P=0.042** **V=6.6%** **n=96**	**P=0.011** **V=9.5%** **n=93**	**P=0.015** **V=8.6%** **n=97**
	ADRB1 diplotypes	*P=0.083* *V=10.3%* *n=94*	P=0.677 V=3.3% n=97	P=0.972 V=1.0% n=97	P=0.702 V=3.2% n=97	P=0.170 V=8.0% n=98	P=0.974 V=1.3% n=98	P=0.821 V=2.3% n=98	**P=0.043** **V=11.8%** **n=96**	*P=0.066* *V=11.0%* *n=93*	P=0.102 V=9.5% n=97
	Gly16Arg	P=0.929 V=0.2% n=90	P=0.359 V=2.2% n=93	P=0.860 V=0.3% n=93	P=0.439 V=1.8% n=93	P=0.200 V=3.5% n=94	*P=0.069* *V=5.7%* *n=94*	P=0.161 V=3.9% n=94	P=0.978 V=0.1% n=92	P=0.617 V=1.1% n=89	P=0.833 V=0.4% n=93
	Gln27Glu	P=0.133 V=4.5% n=91	P=0.272 V=2.8% n=94	P=0.909 V=0.2% n=94	P=0.508 V=1.5% n=94	P=0.857 V=0.3% n=95	P=0.115 V=4.6% n=95	P=0.197 V=3.5% n=95	P=0.370 V=2.2% n=93	P=0.480 V=1.7% n=90	P=0.463 V=1.7% n=94
	ADRB2 diplotypes	P=0.108 V=10.2% n=88	P=0.301 V=6.8% n=91	P=0.970 V=1.0% n=91	P=0.661 V=3.7% n=91	P=0.429 V=5.4% n=92	*P=0.057* *V=11.5%* *n=92*	P=0.278 V=6.9% n=92	P=0.608 V=4.1% n=90	*P=0.077* *V=11.4%* *n=87*	P=0.572 V=4.4% n=91
	Trp64Arg	P=0.894 V<0.1% n=94	P=0.905 V<0.1% n=97	P=0.989 V<0.1% n=97	P=0.824 V=0.1% n=97	P=0.311 V=1.1% n=98	P=0.610 V=0.3% n=98	P=0.354 V=0.9% n=98	P=0.278 V=1.3% n=96	P=0.428 V=0.7% n=93	P=0.575 V=0.3% n=97

Weinberg equilibrium of the population these provided strong validation of the genotyping methods used.

7.3.2 Genotype associations with phenotypes For the ADRB1 gene, in the total group of males, significant differences were observed between the means of individual Arg389Gly (rs1801253) genotypes for arm span and agility run (table 7.1). As indicated by their mean scores (data not shown) Gly389 homozygotes had the lowest mean agility run times compared to Arg389 homozygotes and heterozygotes while Arg389 homozygotes had the longest mean arm span values. No significant associations were detected for ADRB1 variants in females (table 7.2).

For the ADRB2 gene, in the total group of males, significant differences were observed between the Gly16Arg (rs1042713) genotypes and ADRB2 diplotypes for hand-grip strength (table 7.1); as indicated by their mean values (data not shown), Gly16 and Gly16Gln27 homozygotes were stronger relative to their counterparts. In females, significant differences were detected between the means of individual Gln27Glu (rs1042714) genotypes for arm span (table 7.2); Gln27Glu heterozygotes had the longest mean arm span (data not shown).

No significant differences were observed between the ADRB3 Trp64Arg (rs4994) genotypes for any of the performance-related physical fitness phenotypes for either gender (tables 7.1 and 7.2).

7.3.3 Analysis by level of regular activity In inactive males, considering variants within the ADRB1 gene, significant differences were observed between the mean values of individual

(7.3b)

Polymorphism		Active Males									
		$\dot{V}O_{2\,max}$	Sitting Height	Arm Span	Sit & Reach	Basket Throw	Throw & Catch	Hand-grip	Vertical Jump	40m Sprint	Agility Run
	Ser49Gly	P=0.243 V=0.7% n=393	P=0.771 V=0.1% n=404	P=0.454 V=0.4% n=404	P=0.208 V=0.8% n=401	P=0.187 V=0.8% n=404	P=0.892 V=0.1% n=404	P=0.541 V=0.3% n=404	P=0.778 V=0.1% n=405	P=0.206 V=0.8% n=398	P=0.280 V=0.6% n=399
	Arg389Gly	P=0.191 V=0.8% n=396	P=0.225 V=0.7% n=407	**P=0.025** **V=1.8%** **n=407**	P=0.676 V=0.2% n=403	P=0.454 V=0.3% n=407	P=0.576 V=0.6% n=407	P=0.629 V=0.2% n=407	P=0.383 V=0.5% n=408	P=0.742 V=0.2% n=398	P=0.398 V=0.5% n=401
	ADRB1 diplotypes	P=0.241 V=1.7% n=390	P=0.577 V=1.0% n=400	*P=0.062* *V=2.6%* *n=400*	P=0.477 V=1.1% n=397	P=0.408 V=1.5% n=400	P=0.909 V=0.4% n=400	P=0.650 V=0.8% n=400	P=0.680 V=0.8% n=401	P=0.373 V=1.4% n=394	P=0.345 V=1.4% n=395
	Gly16Arg	P=0.593 V=0.3% n=383	P=0.250 V=0.7% n=392	P=0.457 V=0.4% n=392	P=0.745 V=0.2% n=389	P=0.189 V=0.9% n=392	P=0.225 V=0.8% n=392	**P=0.013** **V=2.5%** **n=392**	P=0.588 V=0.3% n=393	P=0.566 V=0.3% n=385	P=0.167 V=0.9% n=387
	Gln27Glu	P=0.793 V=0.1% n=384	P=0.100 V=1.2% n=393	P=0.659 V=0.2% n=393	P=0.294 V=0.6% n=390	P=0.362 V=0.5% n=393	P=0.789 V=0.1% n=393	P=0.429 V=0.4% n=393	P=0.339 V=0.6% n=394	P=0.679 V=0.2% n=386	P=0.557 V=0.3% n=388
	ADRB2 diplotypes	P=0.852 V=0.5% n=380	P=0.300 V=1.6% n=389	P=0.770 V=0.7% n=389	P=0.388 V=1.2% n=386	P=0.512 V=1.1% n=389	P=0.658 V=0.8% n=389	*P=0.074* *V=2.6%* *n=389*	P=0.681 V=0.8% n=390	P=0.705 V=0.8% n=382	P=0.551 V=1.0% n=384
	Trp64Arg	P=0.129 V=1.1% n=383	P=0.873 V=0.1% n=394	P=0.181 V=0.9% n=394	P=0.898 V=0.1% n=390	P=0.615 V=0.2% n=394	P=0.554 V=0.3% n=394	P=0.207 V=0.8% n=394	P=0.732 V=0.2% n=395	P=0.497 V=0.4% n=385	P=0.987 V<0.1% n=388

Ser49Gly (rs1801252) genotypes for $\dot{V}o_{2\,max}$ (table 7.3a); Gly49 homozygotes had the lowest $\dot{V}o_{2\,max}$ values (data not shown). In inactive males, the Arg389Gly (rs1801253) genotype significantly influenced vertical jump, 40m sprint times and agility run times (table 7.3a). Inactive Gly389 homozygous males jumped higher (figure 7.1), run faster and were more agile compared to their counterparts (mean value data not shown). For ADRB1 diplotypes in inactive males, significant differences were observed for vertical jump (table 7.3b) with Ser49Gly389 homozygotes jumping higher (mean value data not shown). No significant associations were detected for ADRB1 variants in inactive females (table 7.4a). In active males, significant differences were observed only between the Arg389Gly (rs1801253) genotypes with arm span (table 7.3b); as indicated by their mean values (data not shown), Arg389 homozygotes had the longest arm span. In active females, significant differences were detected between individual diplotypes for sitting height (table 7.4b) with Ser49Gly389/Gly49Arg389 individuals having the shortest trunk length (data not shown).

For the ADRB2 gene, and irrespective of gender, no significant genotype or diplotype differences were detected for inactive individuals (tables 7.3a and 7.4a). In contrast, in active males, significant Gly16Arg (rs1042713) genotype differences were observed for hand-grip strength (table 7.3b) with Gly16 homozygotes having the highest mean hand-grip (data not shown). In active females, significant Gln27Glu (rs1042714) genotype differences were observed for hand-grip strength (table 7.4b) with active Gln27Glu heterozygotes being stronger (data not shown).

For the ADRB3 gene no significant Trp64Arg (rs4994) genotype differences were observed, irrespective of gender and participation in organised activity (tables 7.3a&b and 7.4a&b).

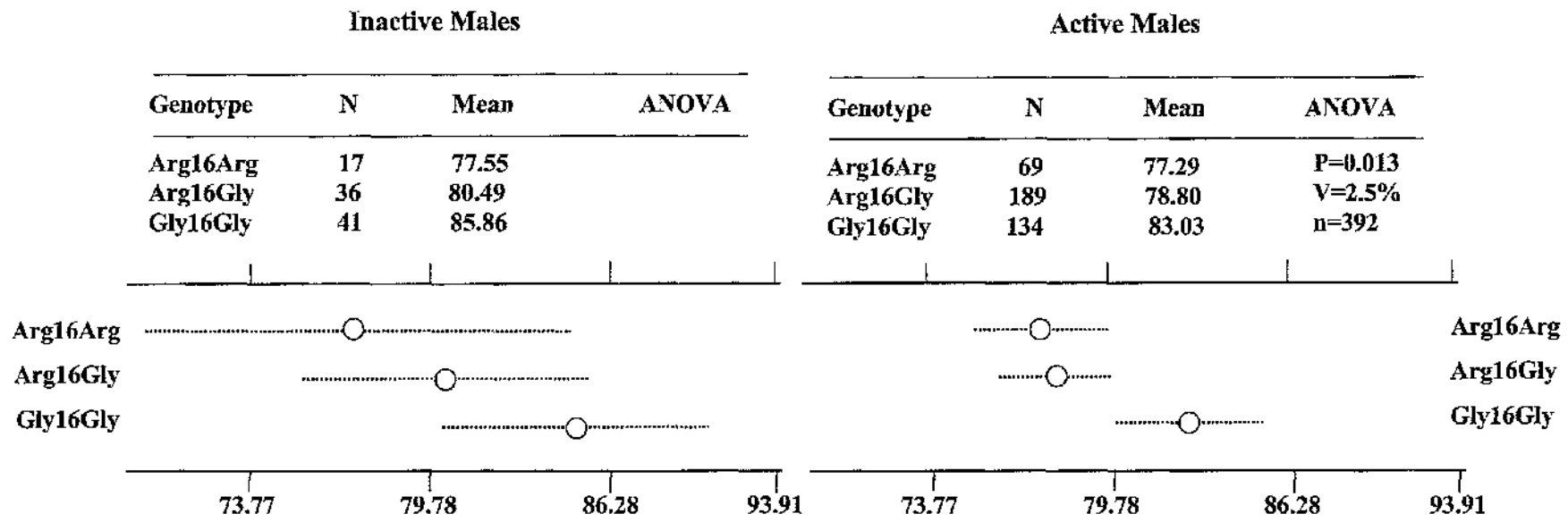


Figure 7.1: Gly16Arg genotype differences in hand-grip strength of active and inactive males; active Gly16Gly homozygotes have significantly higher mean hand-grip strength while no significant differences by genotype were observed for inactive males. Hand-grip strength is measured in (kg). (P)=probability, (V)=observed variance explained, (N)=number within group.

7.3.4 Determination of the type of genetic effect observed for associations with the ADRB polymorphisms Considering the phenotypic effects of the Arg389Gly (rs1801253) variant, additive genetic models explained 96% and 95% of the variance for arm span and agility run in total males, respectively, 96%, 99% and 100% of the variance for vertical jump, 40m sprinting times and agility run in inactive males, respectively, and 100% of the variance for arm span in active males. A genetic model where the phenotypic effects of one of the alleles were completely dominant over those of the other allele explained 87% of the variance for $\dot{V}o_{2\,max}$ in inactive males (Ser49 allele dominant), 95% and 83% of the variance for hand-grip strength in total and active males, respectively (Arg16 allele dominant), 79% of the variance for arm span in total females (Glu27 allele dominant) and 43% of the variance for hand-grip strength in active females (Gln27 allele dominant). For each parameter, the remaining variance could be accounted for by appropriately considered partial dominance models.

7.3.5 Associations of ADRB genetic variants with extreme phenotypes The odds ratios were broadly consistent with the phenotypic means reported in the ANOVAs and the genetic models identified by the correlation analyses. For the ADRB1 gene, no significant ORs were observed for arm span and agility run in total males. In the low $\dot{V}o_{2\,max}$ group of inactive males, ADRB1 Ser49 homozygotes were significantly under-represented in contrast to Gly49 homozygotes that were significantly over-represented (table 7.5); however, as no Gly49 homozygotes were present in the low $\dot{V}o_{2\,max}$ group, the calculation of an OR for Gly49 homozygotes was not possible in this case. In inactive males, ADRB1 Gly389 homozygotes were significantly over-represented in the high group for vertical jump (figure 7.2) and the low group (faster) for 40m sprinting times while Arg389

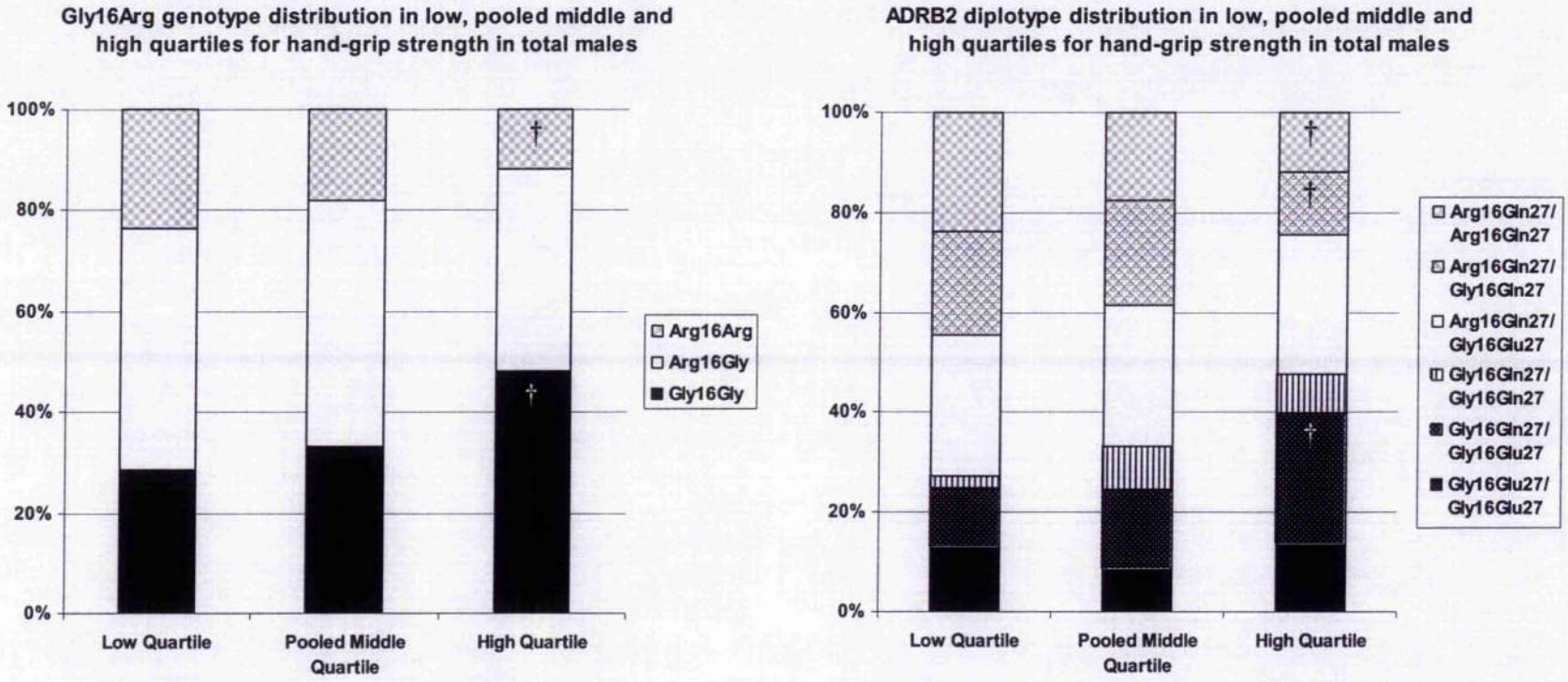


Figure 7.2: ADRB2 Gly16Arg and diplotype distributions in the low, pooled middle and high quartiles for hand-grip strength in the group of total males. Within quartiles significantly over and under-represented genotypes (tables 7.5 and 7.6) are indicated by (†). Gly16Gly and Gly16Gln27/Gly16Glu27 individuals were significantly over-represented in the high quartile for hand-grip strength. In contrast, Arg16Arg, Arg16Gln27/Arg16Gln27 and Arg16Gln27/Gly16Gln27 individuals were significantly under-represented in the high quartile for hand-grip strength.

homozygotes were significantly over-represented in the high group (less agile) for agility run times (table 7.5).

For the ADRB2 gene, in total males, Gly16 homozygotes were significantly over-represented in the high group for hand-grip strength while Arg16 homozygotes were significantly under-represented in the same group (table 7.5). In the high group for hand-grip strength in total males, Gly16Gln27/Gly16Glu27 heterozygotes were significantly over-represented in contrast to Arg16Gln27 homozygotes and Arg16Gln27/Gly16Gln27 heterozygotes that were significantly under-represented (table 7.6). On the other hand, in the low group for hand-grip strength in total males, Gly16Gln27 homozygotes were significantly under-represented while Arg16Gln27/Arg16Gln27 homozygotes were significantly over-represented (table 7.6). In total females, Gln27Glu heterozygotes were significantly under-represented in the low group for arm span (P=0.045, OR: 0.65, 95% CIs: 0.42-0.99).

Considering the significant findings for the analysis by levels of physical activity, inactive ADRB1 Ser49Gly389 homozygous males were significantly over-represented in the high group for vertical jump (table 7.6); active Ser49Gly389/Gly49Arg389 females were significantly over-represented in the low group for sitting height (P=0.001, OR: 4.17, 95% CIs: 1.67-10.40). For the ADRB2 gene, active Gly16 homozygous males were significantly over-represented in the high group for hand-grip strength (table 7.5). In active females, Gln27 homozygotes were significantly over-represented in the low group for hand-grip strength (P=0.041, OR: 0.83, 95% CIs: 1.02-3.30) in contrast to Gln27Glu heterozygotes that were significantly under-represented in the same group (P=0.002, OR: 0.40, 95% CIs: 0.22-0.73).

Table 7.4: Analysis of associations between genotypes/diplotypes and physical, physiological and skill-related phenotypes in inactive (7.4a) and active (7.4b) females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance (0.05<P<0.1) and where they are given in bold the tests were significant (P<0.05).

(7.4a)

		Inactive Females									
		$\dot{V}o_{2\ max}$	**Sitting Height**	**Arm Span**	**Sit & Reach**	**Basket Throw**	**Throw & Catch**	**Hand-grip**	**Vertical Jump**	**40m Sprint**	**Agility Run**
Polymorphism	**Ser49Gly**	P=0.579 V=0.6% n=180	P=0.251 V=1.5% n=191	P=0.208 V=1.6% n=192	P=0.344 V=1.1% n=191	P=0.462 V=0.8% n=192	P=0.766 V=0.3% n=191	P=0.644 V=0.5% n=191	P=0.505 V=0.7% n=192	P=0.871 V=0.2% n=185	P=0.379 V=1.0% n=188
	Arg389Gly	P=0.682 V=0.4% n=184	P=0.311 V=1.2% n=195	P=0.492 V=0.7% n=196	P=0.299 V=1.3% n=195	P=0.273 V=1.3% n=196	P=0.146 V=2.0% n=195	P=0.993 V=1.0% n=196	P=0.367 V=1.0% n=196	P=0.853 V=0.2% n=186	P=0.663 V=0.4% n=192
	ADRB1 diplotypes	P=0.726 V=1.6% n=177	*P=0.098* *V=4.9%* *n=188*	P=0.274 V=3.4% n=189	P=0.373 V=2.9% n=188	P=0.494 V=2.4% n=189	P=0.203 V=2.9% n=188	P=0.924 V=0.8% n=188	P=0.710 V=1.6% n=189	P=0.984 V=181% n=0.4	P=0.626 V=1.9% n=185
	Gly16Arg	P=0.888 V=0.1% n=176	P=0.689 V=0.4% n=185	P=0.400 V=1.0% n=186	*P=0.078* *V=2.8%* *n=185*	P=0.626 V=0.5% n=186	P=0.941 V=0.1% n=185	P=0.829 V=0.2% n=185	P=0.367 V=1.1% n=186	P=0.613 V=0.6% n=180	P=0.685 V=0.4% n=183
	Gln27Glu	P=0.565 V=0.7% n=175	P=0.686 V=0.4% n=185	P=0.440 V=0.9% n=186	P=0.164 V=2.0% n=185	P=0.492 V=0.8% n=186	P=0.275 V=1.4% n=185	P=0.781 V=0.3% n=185	P=0.794 V=0.3% n=186	P=0.872 V=0.2% n=179	P=0.901 V<0.1% n=182
	ADRB2 diplotypes	P=0.881 V=1.0% n=174	P=0.953 V=0.6% n=183	P=0.770 V=1.4% n=184	P=0.336 V=3.1% n=183	P=0.649 V=1.8% n=184	P=0.684 V=1.7% n=183	P=0.738 V=1.5% n=183	P=0.834 V=1.2% n=184	P=0.803 V=1.3% n=178	P=0.779 V=1.4% n=181
	Trp64Arg	P=0.720 V=0.1% n=184	P=0.200 V=0.9% n=195	P=0.576 V=0.2% n=196	P=0.786 V<0.1% n=195	P=0.220 V=0.8% n=196	P=0.904 V<0.1% n=195	P=0.374 V=0.4% n=195	P=0.148 V=1.1% n=196	P=0.268 V=0.7% n=186	P=0.457 V=0.3% n=192

7.4 Discussion

Polymorphisms within the ADRB1 and ADRB2 genes associated with physical and physiological phenotypes measured in the present cohort of adolescent children. These associations were predominantly found in males and were influenced by participation in organised physical activity. For the ADRB1 gene, the Ser49 (rs1801252) and Gly389 alleles and the corresponding Ser49Gly389 haplotype associated with enhanced physical and physiological parameter scores. For the ADRB2 gene, the Gly16 allele, in the homozygous state, and the corresponding Gly16Gln27/Gly16Gln27 genotype associated with enhanced hand-grip strength. Nevertheless, the present results must be interpreted with caution.

Multiple testing increases the risk of type I (false positive) errors. The Bonferroni and related corrections would allow for this but, as discussed in General Methods (p. 52-54), relative to the large number of tests performed here such corrections are likely to lower the α value to unrealistic levels (Perneger TV 1998),(Sokal and Rohlf 2001),(Sabatti et al. 2003). Considering as an example the group of total males, the number of statistically significant associations ($P<0.05$) expected by chance would be approximately four (0.05 x 70 tests per gender=3.5); this is the same as the number of tests actually found to be statistically significant and, as such, the findings in young males may have been partial to type I errors. Taking into account the nature, relative strength and the consistency of the observed effects across activity groups did not help substantially to improve confidence in these findings. For males, the effects on arm span, agility run and hand-grip strength were observed across activity-divided (sub)populations; however, as the lack of significant ORs indicated, a consistent genotype influence across the whole of the phenotypic distributions for arm span and agility run in total males and for arm span in active males was not

(7.4b)

Polymorphisms	Active Females									
	$\dot{V}O_{2\ max}$	Sitting Height	Arm Span	Sit & Reach	Basket Throw	Throw & Catch	Hand-grip	Vertical Jump	40m Sprint	Agility Run
Ser49Gly	P=0.528 V=0.5% n=242	P=0.271 V=1.0% n=256	P=0.267 V=1.0% n=257	P=0.314 V=0.9% n=257	P=0.449 V=256% n=0.6	P=0.306 V=0.9% n=256	P=0.422 V=0.7% n=256	P=0.752 V=0.2% n=257	*P=0.059* *V=2.3%* *n=250*	P=0.507 V=0.5% n=456
Arg389Gly	P=0.985 V<0.1% n=244	P=0.368 V=0.8% n=257	P=0.526 V=0.5% n=258	P=0.744 V=0.2% n=258	P=0.350 V=0.8% n=257	P=0.323 V=0.9% n=257	P=0.855 V=0.1% n=257	P=0.372 V=0.8% n=258	P=0.468 V=0.6% n=251	P=0.806 V=0.2% n=257
ADRB1 diplotypes	P=0.625 V=1.5% n=238	**P=0.024** **V=5.1%** **n=251**	P=0.467 V=1.8% n=252	P=0.575 V=1.5% n=252	P=0.430 V=2.0% n=251	P=0.159 V=3.2% n=251	P=0.332 V=2.3% n=251	P=0.689 V=1.2% n=252	P=0.167 V=3.2% n=245	P=0.507 V=1.7% n=251
Gly16Arg	P=0.568 V=0.5% n=239	P=0.493 V=0.6% n=253	P=0.362 V=0.8% n=254	P=0.337 V=0.9% n=254	P=0.109 V=1.7% n=254	P=0.225 V=1.2% n=253	P=0.716 V=0.3% n=252	P=0.851 V=0.1% n=254	P=0.514 V=0.5% n=246	P=0.954 V<0.1% n=252
Gln27Glu	P=0.173 V=1.5% n=242	P=0.840 V=0.1% n=256	*P=0.093* *V=1.9%* *n=257*	P=0.229 V=1.2% n=257	P=0.145 V=1.5% n=257	P=0.291 V=1.0% n=256	**P=0.018** **V=3.1%** **n=256**	P=0.923 V=0.1% n=257	P=0.726 V=0.3% n=249	P=0.903 V=0.1% n=255
ADRB2 diplotypes	P=0.202 V=3.1% n=238	P=0.946 V=0.5% n=252	P=0.264 V=2.6% n=253	P=0.561 V=1.6% n=253	P=0.210 V=2.8% n=253	P=0.374 V=2.1% n=252	*P=0.082* *V=3.9%* *n=252*	P=0.974 V=0.3% n=253	P=0.622 V=1.4% n=245	P=0.987 V=0.2% n=251
Trp64Arg	P=0.748 V<0.1% n=242	P=0.575 V=0.1% n=255	*P=0.063* *V=1.4%* *n=256*	P=0.544 V=0.1% n=256	P=0.411 V=0.3% n=255	P=0.329 V=0.4% n=255	P=0.548 V=0.3% n=254	P=0.386 V=0.3% n=256	P=0.941 V<0.1% n=249	P=0.775 V<0.1% n=255

detected. Furthermore, the significant effects on arm span and agility run would be difficult to account for based on the known functional role of the ADRBs (for reviews (Leineweber and Brodde 2004)). As indicated by the % of the observed variance explained, the strongest associations between genotype and phenotype were detected for arm span, $\dot{V}o_{2\,max}$, vertical jump and 40m sprinting times in inactive males; to a certain extent, this finding reflected the smaller group size of inactive males and the fact that activity status was considered when assessing the genetic influences on phenotype. For young females, significant differences were observed in only two of the tests hence these associations were most likely the result of a type I error. In light of these findings it is clear that the effects of polymorphic variants of the ADRB1 and ADRB2 genes on performance-related physical fitness in males were modest and caution must be exercised when interpreting these results.

Several previous studies (for examples (Drysdale et al. 2000),(Akey et al. 2001)) have advocated the use of haplotype-based methods to assess genetic effects mediating susceptibility to disease or complex traits as they offer greater analytic ability. Considering the *P*-values, haplotype analysis, where significant, did not produce markedly stronger associations compared to individual genotypes. Haplotypes explained a larger % of the observed variance compared to individual genotypes; however, the increase in the % of variance explained by haplotype analysis was either no larger than that expected by random sampling effects or reflective of the smaller sample size and the fact that activity status was considered when assessing genetic influences on phenotype. Nevertheless, the results of the genetic analysis for the influence of polymorphisms within the ADRB2 gene on hand-grip strength were consistent with previous reports (McGraw et al. 1998) of a dominant influence of the Gly16Arg (rs1042713) variant on receptor function. The findings from the present and previous studies cannot adequately account

Table 7.5: ADRB genotype odds ratios for phenotypes significant by ANOVA in males (tables 7.1 and 7.3). 95% confidence intervals are given in parentheses. Probability (P) <0.05 for all tests shown, * indicates P≤0.01. Low means lower shuttle score ($\dot{V}o_{2\ max}$), smaller arm span (arm span), longer agility run time (agility run), smaller jump (vertical jump), longer sprint time (40m sprint) and less strong (hand-grip strength); High indicates the converse. (n.s.) indicates X^2 tests that were not statistically significant. Note that for the Gly49Gly individuals in the low quartile of $\dot{V}o_{2\ max}$ in inactive males no meaningful odds ratio or confidence interval could be calculated as no Gly49 homozygotes were observed in that group [indicated by (-)].

ADRB Gene	ADRB Genotype	Phenotype	Males					
			Total		Inactive		Active	
			Low Quartile	High Quartile	Low Quartile	High Quartile	Low Quartile	High Quartile
ADRB1	Ser49Ser	$\dot{V}o_{2\ max}$	ANOVA not significant		0.36 (0.13-0.99)	2.44 (1.09-5.46)	ANOVA not significant	
	Gly49Gly	$\dot{V}o_{2\ max}$			-	n.s.		
	Arg389Arg	Arm Span	n.s.	n.s.	ANOVA not significant		n.s.	n.s.
	Arg389Arg	Agility Run	n.s.	n.s.	n.s.	2.81 (1.19-6.65)	ANOVA not significant	
	Gly389Gly	Vertical Jump	ANOVA not significant		n.s.	5.8 (1.73-19.63)	ANOVA not significant	
		40m Sprint			4.64 (1.40-15.36)	n.s.		
ADRB2	Arg16Arg	Hand Grip	n.s.	0.54 (0.20-0.99)	ANOVA not significant		n.s.	n.s.
	Gly16Gly	Hand Grip	n.s.	1.99* (1.31-3.00)			n.s.	2.16* (1.34-3.47)

for the lack of similar functional effects by the remaining diplotypes; for example, the Gly16Gln27 and Gly16Glu27 haplotypes have been associated with similar levels of agonist-promoted down-regulation. Therefore corresponding diplotypes must also be associated with concomitant differences in receptor density and agonist promoted down-regulation (for a review (Small et al. 2003)) although here a significant genetic effect on hand-grip phenotypic distribution was observed only for the Gly16Gln27/Gly16Glu27. These findings suggest that additional factors might contribute in defining the penetrance of the ADRB2 haplotype effects on hand-grip strength.

Differences between the Arg389Gly (rs1801253) genotypes in males were best explained by additive genetic models in which the heterozygote mean was intermediate between the two homozygote means. The phenotypic effects of an additive genetic model can be reconciled with molecular mechanisms that act via a linear dosage effect. In contrast, the phenotypic associations with the Ser49Arg (rs1801252) and Arg16Gly (rs1042713) variants were best explained by dominant genetic models; Ser49 and Arg16 alleles were dominant over their counterparts and in each case the heterozygote mean was nearly identical to the mean of the dominant homozygote. Dominant genetic effects on phenotype are most commonly mediated by alterations, beyond threshold values, in protein expression/activity and/or effects on other multimeric proteins. For total and active females, the tested genetic models did not adequately summarise the observed genetic variance; these findings suggested the presence of under or overdominant genetic effects where the heterozygote mean would be more extreme in its phenotypic expression than that of either homozygote. Although genetic models for under or overdominance can be formulated, they are difficult to reconcile with molecular mechanisms. As such, the

Table 7.6: ADRB diplotype odds ratios for phenotypes significant by ANOVA in males (tables 7.1 and 7.3). 95% confidence intervals are given in parentheses. Probability (P) <0.05 for all tests shown, * indicates P≤0.01. Low means smaller jump (vertical jump) and less strong (hand-grip strength); High indicates the converse. (n.s.) indicates X^2 tests that were not statistically significant.

ADRB Gene	ADRB Diplotype	Phenotype	Males			
			Total		Inactive	
			Low Quartile	High Quartile	Low Quartile	High Quartile
ADRB1	Ser49Gly389/ Ser49Gly389	Vertical Jump	ANOVA not significant		n.s.	5.8* (1.79-19.63)
ADRB2	Arg16Gln27/ Gly16Gln27	Hand Grip	1.69 (1.02-2.82).	0.55 (0.30-1.00)	ANOVA not significant	
	Arg16Gln27/ Gly16Gln27	Hand Grip	n.s.	0.55 (0.31-0.99).		
	Gly16Gln27/ Gly16Gln27	Hand Grip	0.28 (0.08-0.94)	2.07* (1.26-3.39)		

detected overdominance was most likely caused by outliers shifting the mean of the heterozygotes higher than the mean of either homozygote.

Most previous studies have concentrated on selected adult populations in which polymorphisms within the ADRB genes have been associated with obesity (for examples (Spielman and Ewens 1996),(Meirhaeghe et al. 2000)) and heart failure-related fitness (for examples (Wagoner et al. 2000),(Wagoner et al. 2002)). For example, the ADRB1 Gly49 (rs1801252) and Arg389 (rs1801253) variants and the ADRB2 Glu27 allele and Arg16Glu27 haplotype have been associated with improved $\dot{V}o_{2\,max}$ values in adult patients with heart disease (for example (Wagoner et al. 2000),(Wagoner et al. 2002),(Small et al. 2003)) and postmenopausal women (Moore et al. 2001). The present findings may be suggestive of a broader, albeit modest, role for the ADRB genes in human fitness than previously described. Although $\dot{V}o_{2\,max}$ results may not be in the same direction with previous findings, the present study was not performed on a selected adult population such as heart failure patients or postmenopausal women but rather on a large representative sample of young individuals. It is also possible that the findings of the present study could be partly explained by other aspects of ADRB biology such as sites of expression. Considering the ADRB3 Trp64Arg (rs4994) variant for example, several studies have reported positive associations and gender-specific effects with indices of obesity and type II diabetes (Ukkola et al. 2000) while the present study did not detect any significant influences of this variant on performance-related physical fitness phenotypes (Strazzullo et al. 2001),(Hao et al. 2004). It is possible that the differential expression of the ADRB3 receptor (primarily expressed in the adipose tissue; for review (Leineweber and Brodde 2004)) can explain the previously observed lack of association with obesity

indices and account for, at least in part, for the lack of a significant association with the performance-related phenotypes reported here.

7.5 Conclusion

In young males, a modest influence of polymorphisms within the ADRB1 and ADRB2 genes on performance-related physical fitness was detected that was influenced by participation in organised physical activity. Nevertheless, these results must be interpreted with caution. The ADRB1 Ser49 allele and Gly389 alleles and the corresponding Ser49Gly389 haplotype associated with improved scores for some of the physical fitness phenotypes while the ADRB2 Gly16 allele, in the homozygous state, and the corresponding Gly16Gln27/Gly16Gln27 diplotype associated with enhanced hand-grip strength. The significant findings for hand-grip strength may indicate the presence of pleiotropic effects of the ADRB2 Gly16Arg influence on phenotype. It is clear that further studies are required to confirm the present findings and promote the current understanding with regards to prospective functional consequences of ADRB variants in human performance-related physical fitness.

CHAPTER 8

GENERAL DISCUSSION

The primary objective of the present series of experiments was to assess whether interactions between lifestyle and polymorphic variants of selected candidate genes had an effect on a number of obesity and performance-related physical fitness phenotypes. An increasing body of work, summarised in annual reviews (Rankinen et al. 2004),(Snyder et al. 2004), has recognised more than 530 genes, chromosomal locations and QTL that are implicated in the genetics of obesity and performance-related fitness. Much of this research has concentrated on adult populations, highly selected or otherwise, despite evidence suggesting that younger age groups may be a better subject population to investigate the genetics of multifactorial traits as the environment had less time to take effect (Maes et al. 1996),(Maes et al. 1997). The series of gene association studies described in Chapters 4-7 were among the first few (for example (Ellsworth et al. 2002)) to investigate such associations using both genotype and haplotype-based methods in a sufficiently large, representative and genetically homogeneous (by ethnicity) population of adolescent children while considering genotype-environment interactions with gender and participation in organised physical activity. In particular, the effects of interactions between gender, participation in organised physical activity and polymorphic variants of the ACE, ADRB1, ADRB2 and ADRB3 genetic loci on a number of physiological, physical and skill-related physical fitness phenotypes were investigated. Prior to this analysis, an assessment of the general health and physical fitness of the present population of adolescent children, as indicated by their mean physiological, physical and skill-related fitness scores, was performed (Chapter 3). In the present chapter, the general findings and conclusions of these series of studies are discussed along with suggestions for future research.

8.1 The health and performance related-fitness of the Greek adolescent cohort: a comparison with Australian norms

Overweight and obesity rates in Greek adolescents were high; on average, 23.6% of children were overweight and 6.9% were obese. Relative to other populations the present cohort of adolescent children had among the highest prevalences of overweight (tables 3.4, 3.5 and 3.6); these results were in agreement with previous reports of the high prevalence of overweight in Greek children (Krassas et al. 2001b),(Lissau et al. 2004). Furthermore, matched to other similarly aged Greek and representative Australian populations (Krassas et al. 2001),(Magarey et al. 2001),(Karayiannis et al. 2003), the participants of the present cohort had, at large, a higher prevalence of obesity. Relative to normative data from Australian adolescents of similar age, Greek adolescents, irrespective of gender, had a higher mean body mass, were less aerobically fit and less agile (tables 3.2 and 3.3). In addition, Greek female and male adolescents were, on average, outperformed in upper-body strength and skill-related trials; however, the lower-body strength-related performance of Greek adolescents was comparable, and on occasion higher, than the one of their Australian counterparts (tables 3.2 and 3.3).

There is no clear explanation with regards to the results for the high overweight and obesity rates in Greek adolescents as the published literature on the specific age group is limited. Previous studies have indicated that Greek families tend to overfeed their children and that young Greeks increasingly adopt diets based on high energy foods (Mamalakis and Kafatos 1996),(Hassapidou and Fotiadou 2001). In addition, a limited number of studies have suggested that Greek adolescents adopt sedentary lifestyles as these are indicated by proxies of physical inactivity such as television viewing (Krassas et al. 2001a). The negative influence on health of such behaviours early in life has been previously reported in the published literature (for a review (Ebbeling et al. 2002)). As such, the results of the aforementioned studies could help explain the present findings with

regards to the lower health and performance-related physical-fitness of Greek adolescents. In light of the high overweight and obesity rates of the participants of the present cohort of Greek children (tables 3.4, 3.5 and 3.6), the differences in mean body mass observed relative to the Australian normative data reflected, most likely, the higher levels of adiposity in Greek children. Previous studies have suggested that body composition, in contrast to physical activity, is inversely related to motor-related physical fitness and $\dot{V}o_{2\,max}$ (Johnson et al. 2000),(Minck et al. 2000),(Janz et al. 2002),(Deforche et al. 2003). As such, the present findings are in agreement with previous reports suggesting an inverse relationship between increased adiposity and $\dot{V}o_{2\,max}$ (Johnson et al. 2000) and furthermore suggest a role for $\dot{V}o_{2\,max}$ as a predictor of obesity. Assuming that the lower $\dot{V}o_{2\,max}$ of Greek adolescents reflected lower levels of physical activity then this may explain, in part, the higher mean body mass and the lower performance-related physical fitness of Greek children. Furthermore, the findings regarding the lower performance-related physical fitness of Greek adolescents were most likely reflective of the negative influence that high body mass and body fat have on motor-related physical fitness.

The observed significant differences in the age-specific mean values for the physiological, physical and skill-related fitness phenotypes between Greek and Australian children could be the result of the Australian group being very fit or the Greek adolescents having poor physical fitness. As already described (for example table 3.6), the participants of the present cohort of Greek children had a high prevalence of overweight relative to other populations, significantly including Australians. For the same comparison, data from representative Australian populations (Magarey et al. 2001) indicated that their overweight rates were average relative to Europeans. In addition, the mean $\dot{V}o_{2\,max}$ values of

Australian children were comparable with the age and gender-specific normative $\dot{V}o_{2\,max}$ scores for other populations (Council of Europe 1988) (table 3.7) while, for the same comparison, the mean $\dot{V}o_{2\,max}$ scores of the Greek adolescents were considerably lower (table 3.7). These findings would suggest that the higher mean body mass and lower aerobic fitness of the Greek adolescents (tables 3.2 and 3.3) was not the result of the Australian children being an overly fit group. It is more likely, that the health and physical fitness of the present cohort of Greek adolescents was below the average of other populations. As such, these findings would account for the observed differences between the mean scores of physiological, physical and skill-related phenotypes of the Greek relative to Australian children.

8.2 Effects of interactions between polymorphisms in the selected candidate genes and lifestyle on obesity and performance-related physical fitness phenotypes

8.2.1 ACE polymorphisms, lifestyle and physiological, physical and skill-related phenotypes Considering the findings from Chapter 4, the ACE I-allele, conferring lower levels of ACE expression and activity, was associated with lower scores in the obesity-related measures. In contrast, the ACE D-allele, conferring higher levels of ACE expression and activity, was associated, at large, with higher scores for the obesity-related parameters. These significant effects were influenced by the participation of subjects in organised physical activity (table 4.2). The genetic effect on the obesity-related phenotypes was additive, a genetic effect where increasing the dosage of a particular allele directly increases the size of its effect. A good example of the phenotypic expression of additive genetic effects is presented in figure 4.1 where the total skinfolds thickness of ID heterozygotes is approximately half-way between those for each homozygote. The present

results were in agreement with the direction of the findings of a recent study that reported a significant interaction of the ACE DD genotype with increased BMI in adult males (Strazzullo et al. 2001). However, in the present series of experiments (Chapter 4) significant associations were only observed in young females (tables 4.1 and 4.2).

The results of the experiments described in Chapter 5 associated polymorphisms of the ACE gene with several of the performance-related physical fitness phenotypes in young females that were influenced by the activity status of the subjects (table 5.3). In particular, homozygotes for the I-allele and the corresponding H6 haplotype, linked to lower levels of ACE expression and activity, were associated with enhanced physical, physiological and skill parameter scores (for example figure 5.1). In contrast, the D-allele, conferring higher levels of ACE expression and activity, was associated, in homozygous or heterozygous form, with low or moderate physiological, physical and skill-related fitness. The significant phenotypic differences between genotypes were, at large, explained by a dominant genetic model with the D-allele being dominant to the I-allele. The results of haplotype analysis indicated that the majority of the genetic effects were accounted for by the I/D polymorphism or a variant in strong linkage disequilibrium with it such as the A22982G (rs4363) (Zhu et al. 2000). Previous studies have associated the ACE I-allele with elite endurance performance (Myerson et al. 1999), greater $\dot{V}o_{2\,max}$ in postmenopausal women (Hagberg et al. 1998) and with improved responses to physical training in adult males (Montgomery et al. 1998) and females undergoing HRT (Woods et al. 2001). Correspondingly, the D-allele has been associated with elite power-oriented performance (Nazarov et al. 2001), quadriceps strength in patients with CLD (Hopkinson et al. 2004) and with responses to physical training in male subjects (Folland et al. 2000). The present findings (Chapter 5) advocated a broader role for the ACE gene in human fitness and

physical performance than previously described. A part of the present findings, linking the I-allele with enhanced strength-related performance in inactive females, may conflict with some of the previous studies suggesting an association of power-oriented performance with the D-allele. However, the present study was not performed in a highly selected population, nor did it assess intra-individual responses to a highly structured training program in adults. Nevertheless, some of the present results were in the same direction with findings from previous studies; for example, the significant association of the I-allele with $\dot{V}o_{2\,max}$ observed in inactive females (table 5.3) has been previously reported by (Hagberg et al. 1998) in adult females.

The I/D polymorphism shows strong association with levels of circulating (Rigat et al. 1990) and tissue bound (Danser et al. 1992) ACE in Caucasians although this association is less strong in populations of African descent (Bloem et al. 1996),(Cox et al. 2002). Also, the I–allele has been previously associated with lower levels of ACE expression (Davis et al. 2000),(Mizuiri et al. 2001) and with improved endothelial cell survival (Hamdi and Castellon 2003). The present results suggested that reducing the levels of ACE expression and activity (linked with the I-allele) resulted in lower levels of adiposity and enhanced performance-related physical fitness. It is important to note however that these effects may differ between populations of different gender, age and environment. Through its key role in vascular homeostasis (Crisan and Carr 2000) ACE is a plausible candidate for influence on cardiovascular phenotypes. In addition, RAS systems are present, with all their functional components, in localised tissues such as the adipose and skeletal muscle tissues where ACE has been suggested to influence growth, differentiation and metabolism (Negrel et al. 1989),(Hennes et al. 1996),(Engeli et al. 1999),(Jones and Woods 2003). Localised RAS systems have also been described in the central nervous system

(Bunnemann et al. 1992) and the gut where ACE activity has been associated with increased nutrient absorption (Levens 1986). Currently, the molecular mechanisms that mediate ACE influences on obesity and performance-related physical fitness are unknown. The previous findings with regards to the functional implications of the ACE gene would suggest that the effects on these diverse physiological, physical and skill-related phenotypes may be mediated by variation in the levels or the activity of the ACE peptidase in the systemic circulation and/or local tissues to influence whole-body energy balance.

Reducing circulating ACE levels (I-allele) would radically alter local and systemic blood flow and lead to differential usage of fat stores in different parts of the body. Lower ACE expression and activity may reduce nutrient absorption in the gut while variation in the ACE activity of the central nervous system might, theoretically, affect satiety mechanisms and modify food intake. The significance of the ACE I/D genotype on obesity-related phenotypes may be further mediated by the tissue-specific availability of angiotensin I for conversion to angiotensin II that is the effector of this mechanism. The adipose RAS through its influence on adipose tissue metabolism (Hennes et al. 1996) may contribute in altering substrate mobilisation from fat stores where angiotensin II activity has been associated with lipogenesis and fat storage in the adipocytes (Jones et al. 1997). Such an effect would be consistent with the present findings as the lower ACE activity of the I-allele would lower the levels of angiotensin II in the adipose tissue and, in turn, lower the levels of lipid synthesis and storage. On the other hand, the effects of the ACE gene on performance-related physical fitness may be strongly associated with the influences of the local RAS in muscle. It has been previously shown that the increased levels of plasma and tissue angiotensin II are inversely associated with skeletal muscle perfusion (Harridge et al. 1996). These effects have a variety of functional consequences for performance-related

phenotypes, significantly including $\dot{V}o_{2\,max}$. ACE inhibition, hence lower ACE activity, induces improvements in skeletal muscle perfusion in patients with chronic heart failure and results in increases of $\dot{V}o_{2\,max}$ (Drexler et al. 1989). These findings are in agreement with the present results associating the I allele, hence lower ACE activity, with higher $\dot{V}o_{2\,max}$ values in young females. Also, alterations in systemic and tissue blood flow could, in part, account for the enhanced strength-related performance observed in young females. Increased vasodilation is associated with lower angiotensin II production and a decrease in bradykinin degradation that are mediated by lower ACE activity (Crisan and Carr 2000). Furthermore, decreases in bradykinin degradation have been previously associated with increases of capillary density in muscle tissue (Schieffer et al. 1995). It is possible therefore that increased blood flow, mediated by lower levels of ACE activity (I-allele) and increased vasodilation and capillary density, may facilitate substrate delivery and positively influence nutrient uptake. Such effects would result in an increased metabolic efficiency of the muscle tissue and lead to improved strength-related performance.

8.2.2 ADRB polymorphisms, lifestyle and physiological, physical and skill-related phenotypes The results of the experiments described in Chapter 6 indicated that, in young males, the ADRB2 Gly16 allele and the corresponding Gly16Glu27/Gly16Glu27 and Gly16Gln27/Gly16Glu27 diplotypes associated with high adiposity while the ADRB2 Arg16 allele and the corresponding Arg16Gln27/Gly16Glu27 and Arg16Gln27/ Arg16Gln27 diplotypes associated with low and moderate adiposity (for example figures 6.1 and 6.2). Significant associations were observed only in young males and were influenced by organised activity participation (tables 6.1 and 6.2). The data for young males were consistent with a previously unreported, protective effect of allelic combinations of the ADRB2 Arg16Gln27 haplotype over the obese phenotype that was

dominantly mediated by the Arg16 variant. In addition, a significant association was observed for the ADRB1 Ser49Gly (rs1801252) polymorphism with BMI however this effect was not reflected on other obesity-related phenotypes and, as such, must be interpreted with caution. In agreement with the findings of previous reports (McGraw et al. 1998b) haplotype analysis indicated that the Gly16Arg (rs1042713) variant dominantly influenced ADRB2 receptor function. Previous studies have linked all four alleles of the Gly16Arg (rs1042713) and Gln27Glu (rs1042714) polymorphisms with an increased risk for obesity. For example, (Large et al. 1997) indicated that Glu27 homozygous females were associated with increased body fat and that the Gly16Arg variant (rs1042713) did not influence the obese phenotype. In contrast, (Meirhaeghe et al. 2000) has suggested that the Gln27Gln genotype is associated with higher scores for obesity-related phenotypes in men but not in women and that if Gln27 homozygotes carried the Arg16 allele the aforementioned associations were stronger. Furthermore, (Hellstrom et al. 1999) indicated that the Glu27 variant positively associates with obesity in adult females but it is negatively associated with obesity in adult males. In the present series of experiments (Chapter 6) no significant associations were observed between the Gln27Glu variant and obesity related phenotypes in either males or females. However, the present results were in agreement with previous reports (for example (Ellsworth et al. 2002)) of a Gly16 allele association with increased adiposity and obesity in males but not in females. In addition, the present findings were consistent with previous results suggesting an influence of physical activity in the functional implications of the ADRB2 polymorphisms (Meirhaeghe et al. 1999).

The findings from the experiments described in Chapter 7 indicated that polymorphisms within the ADRB1 and ADRB2 genes associated with physiological, physical and skill-

related phenotypes in male adolescent children (for example table 7.1). Participation in organised physical activity influenced the observed significant associations (table 7.3). For the ADRB1 gene, the Ser49 and the Gly389 alleles, linked to reduced agonist-promoted down-regulation and stimulation, respectively, as well as the corresponding Ser49Gly389 haplotype associated with enhanced performance-related parameter scores. For the ADRB2 gene, the Gly16 allele and the corresponding Gly16Gln27 haplotype, linked to low levels of receptor density and high agonist-promoted down-regulation, were associated with enhanced hand-grip strength (figures 7.1 and 7.2). The influence of the Arg389Gly (rs1801253) variant on phenotype was best explained by an additive genetic model in which the heterozygote mean was intermediate between the two homozygote means. In contrast, the variance of the genetic effects on phenotype of the Ser49 and Arg16 alleles was best explained by dominant models. The significant associations for the ADRB2 haplotypes were consistent with previous reports of a dominant influence of the Gly16Arg variant in receptor function (McGraw et al. 1998) and as such were consistent with the aforementioned findings in Chapter 6. Nevertheless, the present results, at least for the ADRB1 variants, must be interpreted with caution; considering the nature, the relative strength and the consistency of the observed significant effects for the ADRB1 variants across activity-divided groups did not substantially improve confidence in these results. Most previous studies have concentrated on selected adult populations and have associated polymorphisms within the ADRB genes with obesity and heart failure-related fitness phenotypes (for a review (Rankinen et al. 2004)). In particular, the Gly49 and Arg389 variants of the ADRB1 gene, the ADRB2 Glu27 allele and the ADRB2 Arg16Glu27 haplotype have been previously associated with improved $\dot{V}o_{2\,max}$ values in adult patients with heart disease (for example (Wagoner et al. 2000),(Wagoner et al. 2002),(Small et al. 2003)) and postmenopausal women (Moore et al. 2001). As such, the present findings

advocated a broader role for the ADRB genes in human fitness than previously described. The present results for $\dot{V}o_{2\,max}$ (Chapter 7), linking only the ADRB1 Ser49 allele with improved $\dot{V}o_{2\,max}$ values, were not in the same direction as seen in previous studies, which have reported Gly49 allele-specific influences on $\dot{V}o_{2\,max}$ only in combination with the Arg389Gly variant. Nevertheless, it must be noted that the present series of experiments were not performed on a selected adult population such as heart failure patients or postmenopausal women but on a large representative sample of young individuals. As such, the present results may not be directly comparable with those of previous investigations.

The biological mechanisms through which variants of the ADRB genes influence obesity or performance-related physical fitness are, as yet, not clear. ADRBs are GPCRs, stimulated by endogenous catecholamines to trigger the intracellular activation of adenylyl cyclase and mediate a variety of cellular responses (for a review (Small et al. 2003b)). Considering the adipose tissue, ADRBs are involved in stimulating lipolysis and thermogenesis (for examples (Carpene et al. 1998),(Ravussin and Gautier 1999),(Bachman et al. 2002)). Genetic polymorphisms altering receptor function could, therefore, influence obesity-related phenotypes. The ADRB2 Gly16 variant has been associated with higher agonist promoted down-regulation, lower receptor density, and hence, reduced receptor efficiency and lower lipolysis (McGraw et al. 1998). The aforementioned effects could promote increased fat accumulation and obesity and, as such, this model of ADRB2 function would be consistent with the present findings linking the Gly16 allele with high adiposity. Other than the Gly16Arg (rs1042713) variant, a number of SNPs have been reported in the ADRB2 gene some of which are functional (Drysdale et al. 2000). Considering haplotypes, *in vitro* studies have suggested that agonist-promoted down-

regulation is similar between the Gly16Glu27 and the Gly16Gln27 haplotypes and, for both, higher compared to the Arg16Gln27 haplotype (for a review (Small et al. 2003)). As such, the functional implications of diplotypes for obesity-related phenotypes were dominantly mediated by the influence of the Gly16Arg (rs1042713) variant. Previous reports in adult males have indicated that physical activity can counterbalance the obesity-promoting effects of certain ADRB2 variants (Meirhaeghe et al. 1999). It is well established that participation in physical activity promotes adrenaline release from the adrenal medulla (for a review (Kjaer 1998)). Coupled to the increased receptor density and lower agonist promoted down-regulation of the Arg16 relative to the Gly16 allele (McGraw et al. 1998), the increased release of adrenaline in response to physical activity would promote lipolysis and decreased adiposity. These findings would be consistent with the present results indicating lower levels of adiposity in active Arg16 male carriers.

Considering the results for performance-related physical fitness, *in vivo* studies of the Gly16Arg (rs1042713) polymorphisms have shown that Gly16 homozygotes have higher vasodilatory responses; however, this effect is reversed for lower limb blood flow (for review (Leineweber and Brodde 2004)). It is possible that long term vasodilatory responses in the upper limbs, associated with the Gly16 allele and hence lower receptor density, influence beneficially hand-grip strength. The reversal of this effect in the lower limbs (for review (Leineweber and Brodde 2004)) may account, in part, for the lack of an association of the Gly16Arg (rs1042713) genotype, and corresponding haplotypes, with lower body strength-related performance. Alternatively, increased adiposity has been previously suggested to associate with increased hand-grip strength in children (Deforche et al. 2003). Gly16 allele-specific effects favouring increased adiposity have been reported here (tables 6.1 and 6.2) and in previous investigations (Ellsworth et al. 2002). It is possible therefore,

that the beneficial effect of the Gly16 allele, and the Gly16Gln27/Gly16Glu27 diplotype, observed in the present study were mediated, at least in part, through the same type of molecular mechanism that accounts for increased adiposity. Considering the results from individual genotypes suggested that the significant association of ADRB2 haplotypes with hand-grip strength in young males was dominantly mediated by the Arg16 allele; a similar finding has been shown for the influence off the Gly16Arg variant on obesity-related phenotypes (Chapter 6). These findings may go somewhat towards increasing confidence in the observed associations of the Gly16Gly genotype and the Gly16Gln27/Gly16Glu27 diplotype with increased hand-grip strength. Furthermore, these results suggest the presence of potential pleiotropic effects of the Gly16Arg variant in mediating the functional effects of the ADRB2 receptor on phenotype.

The lack of functional studies assessing the potential influence of polymorphic variants of the ADRB1 gene with performance-related physical fitness makes the interpretation of the present results troublesome. The ADRB1 Gly49 variant has been associated with increased agonist-promoted down-regulation due, primarily, to decreases in mRNA and positive influences on exercise capacity in patients with heart failure (Wagoner et al. 2002). In contrast, the Ser49 allele has been associated with increased receptor expression (for review (Small et al. 2003)) and higher mean heart rate (Ranade et al. 2002). On the other hand, the ADRB1 Arg389 variant has been suggested to associate with increased adenylyl cyclase activity and intracellular concentration of cAMP relative to the Gly389 receptor (Mason et al. 1999). Furthermore, previous studies in discordant sibling pairs have linked the Arg389 variant with increased diastolic blood pressure and hypertension (Wagoner et al. 2002). The present results indicated that the increased receptor expression, linked to the Ser49 allele, and the linear decrease in adenylyl-cyclase activity, coupled to the Gly389 allele, may be

beneficial for $\dot{V}o_{2\,max}$, lower body strength-related performance and agility scores in young males. The beneficial effects of the ADRB1 variants for performance-related physical fitness will, most likely, reflect the effects of these variants in cardiovascular fitness. For example, Arg389Gly variant-mediated changes in systemic and local tissue blood flow could facilitate the metabolic efficiency of the muscle tissue and result in improved lower-body strength related performance. Considering the Ser49 variant, it is possible that it acts as a cofactor mediating the functional consequences of other genes or polymorphisms; such suggestions have been previously made (Wagoner et al. 2002) and would be consistent with the weak nature of the observed effect. It is clear that further studies are required to promote the current understanding of the influence that the ADRB1 gene has on performance-related physical fitness.

8.2.3 Common findings for the effects of interactions between polymorphisms in the selected candidate genes and lifestyle on obesity and performance-related physical fitness phenotypes The series of experiments described in Chapters 4-7 assessed the impact of the interaction between polymorphic variants in selected candidate genes, gender and lifestyle on a number of health and physical fitness phenotypes. The findings from these studies indicated the presence of a number of common points between them. A large number of twin of twin and familial studies (for example (Maes et al. 1996),(Bouchard et al. 1998)) has indicated that obesity and performance-related physical fitness have a strong genetic component that is likely to implicate a number of minor genes (Arner 2001),(Rankinen et al. 2004); each of these genes will have a small but significant contribution to the observed phenotype. As indicated by the % of variance explained for significant gene associations (for examples tables 4.1, 5.1, 6.1 and 7.1) the effects of the selected candidate genes on phenotype were modest. Irrespective of considerations for organised physical activity

participation, significant associations, on average, explained 2% to 4% of the observed phenotypic variance and, as such, the present findings were in line with what would be expected for multi-factorial traits.

The aforementioned significant findings demonstrated gender-specificity; the influence of ACE genetic variants was limited to young females in contrast to the effects of the ADRB genes that were observed in males alone. Accounting for gender-specific differences in the function of genetic determinants is troublesome and largely speculative. Considering the ACE gene, it has been shown that the ACE enzymic activity is responsive to oestrogen and that higher oestrogen levels concomitantly lower ACE activity (Proudler et al. 1995). It is possible that the observed beneficial effects for obesity and performance of the I-allele, linked with lower serum and tissue-bound ACE activity and expression (Rigat et al. 1990),(Danser et al. 1992),(Davis et al. 2000), are more pronounced in young females as they are strengthened by the presence of hormones such as oestrogen that lower ACE activity even further. 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 that the influence of these cofactors becomes detectable only later in life. Age-related variations in hormonal levels (Feldman et al. 2002) and gene expression (Casselmann et al. 2004) have been previously described. For example, it is possible that the beneficial influences of the ACE gene were masked in young males by the age-dependent effects of another co-factor like androgens (Feldman et al. 2002) or other obesity-related genes. Alternatively, the gender-specific effects of certain variants may be explained by increased functionality of a gene product in certain tissues. For example, it is known that men primarily accumulate fat in their abdominal region whereas females accumulate fat in the gluteal area (Kissebah and Krakower 1994). ADRBs in the

abdominal region are many times more sensitive compared to the glutcal region, while the inhibition of lipolysis by ADRA2 receptors is greater in the abdominal region of men (Leibel and Hirsch 1987). In males, the presence of allelic variants associated with increased agonist-promoted down-regulation and/or reduced receptor density, such as the Gly16 allele (Small et al. 2003), coupled to the higher sensitivity of abdominal ADRBs would compliment the greater inhibitory effect of ADRA2 receptors on lipolysis and chronically promote increased adiposity. In contrast, allelic combinations associated with lower levels of agonist-promoted down-regulation and higher receptor density such as the Arg16 allele (Small et al. 2003) would counter-balance the inhibitory effect of ADRA2 receptors and promote lipolysis.

The significant associations observed were influenced by participation in organised physical activity. It is well established that physical activity is positively associated with health and physical fitness (for example (Philippaerts et al. 1999)). Evidence from epidemiological studies have suggested that the lack of physical activity (or the increased inactivity) is one of the major factors contributing to the global rise of the obesity epidemic (Prentice and Jebb 1995). Physical activity participation positively influences a number of performance and physical fitness-related phenotypes such as $\dot{V}o_{2\,max}$ and physical skill (Minck et al. 2000),(Volver et al. 2000),(Michaud et al. 2002). In addition, physical activity has a profound impact on body function as it stimulates the production of hormones like adrenaline that mediate a variety of cellular and systemic responses. Previous investigations have indicated that the contribution of genetic determinants in obesity may change in response to lifestyle modifications such as when individuals partake physical activity (for example (Meirhaeghe et al. 1999)). These findings were consistent with the results reported by the series of gene association studies presented here (Chapters

4-7); for example, the ACE D-allele was significantly associated with higher obesity related scores in inactive but not in active males (for example figure 4.1). Furthermore, the significant associations of the Gly16Arg variant with obesity-related phenotypes in total and active males (tables 6.1 and 6.2) indicated that physical activity can synergistically interact with the genotype to "protect" Arg16 carriers against increased adiposity. These findings would suggest that although multi-factorial traits such as obesity and performance-related physical fitness have a strong genetic component (Bouchard 1997), genes are not deterministic but only predispose individuals to particular traits. Nevertheless, it must be noted that the dependency of the significant effects described in previous Chapters on gender and environmental influences would suggest that these effects may differ between populations of different gender, age or environment. In light of the shortcomings of previous studies, it was clear that selection of cohorts of the "wrong" gender, age or environment would most likely mask the subtle genetic effects of the aforementioned candidate genes on phenotype.

As indicated by the *P*-values, haplotype analysis did not produce markedly stronger associations compared to individual genotypes. Nevertheless, for significant associations, diplotypes, at large, explained a higher % of the observed variance relative to individual SNPs (for example table 4.1) but the increase in the % of variance explained by haplotype analysis was no larger than what would be expected by random sampling effects. Furthermore, the mechanics of haplotype analysis requiring the splitting of the data in many genetic sub-groupings may have, on occasion, underpowered these studies to detect significant effects (for example BMI in active males; table 6.2). Nevertheless, the present results did not suggest evidence against the greater analytical power of haplotypes or their use in the investigation of complex traits; if, for example, SNPs at positions 16

(rs1042713) and 27 (rs1042714) of the ADRB2 gene had been independently assessed, the protective effect over the obese phenotype of a genetic subdivision of the Gly16Arg (rs1042713) variant would have been most likely missed. In addition, haplotype results, significant or otherwise, did not contradict findings from multiple individual SNPs. Haplotypes do have a greater analytical power as they simultaneously assess the influence of a number of loci on phenotype (Akey et al. 2001),(Pritchard and Cox 2002) 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.

The present results offered support to previous suggestions that study populations of younger individuals may be a more powerful approach to investigate complex traits relative to adult populations as the environment had less time to have an effect (Maes et al. 1996),(Maes et al. 1997). In addition, the present results indicated that the cohort under investigation should be correctly subdivided by gender and participation in organised physical activity in order for a study to detect subtle genetic influences. Nevertheless, employing adolescent populations for the investigation of complex traits poses significant challenges for an investigation. It is well established that children normally increase their weight and body fat content through puberty (Hergenroeder and Klish 1990). $\dot{V}o_{2\,max}$ and skeletal growth patterns are associated with sexual maturation (Meredith and Dwyer 1991),(Gasser et al. 2001) while exercise ability and physical skill are partial to factors such as development (Volver et al. 2000). These findings would suggest that considering phenotypic differences in children and adolescents without adjusting for developmental effects will make the conclusive identification of genetic determinants very difficult. Criteria describing the stages of sexual maturity indicators in boys and girls have been previously established (Marshall and Tanner 1969),(Marshall and Tanner 1970) and have

been used extensively in the literature (for examples (Goldstein et al. 2001),(Papadimitriou et al. 2002)). However, as indicated by others (Sun et al. 2002), sexual maturity assessments are meaningful when the age of a child already in a stage can be compared with suitable national reference data for sexual maturity stages and growth in normal healthy peers. Few published studies (for example (Papadimitriou et al. 2002)) have assessed sexual maturity stages and growth in Greece; however, these studies were not performed at a national scale. Furthermore, the aforementioned criteria have been previously criticised as difficult to use in defining puberty because of age-related variations in the onset of the indicators used to differentiate between stages (Siervogel et al. 2003). Such approaches may not be practical for the purposes of population-based research (Rockett et al. 2004) as they require individual, nude visual examination. To assess genetic influences on phenotype, prospective gene association studies require, by design, large sample sizes (Clayton and McKeigue 2001) and involve large numbers of phenotypic tests hence individual subject assessments would place great strain on association studies. Self-assessment methods have been devised to counter the problems from visual examination (Duke et al. 1980),(Morris NM and Udry JR 1980) but the validity of these methods has been repeatedly questioned especially for use in adolescents (Schlossberger et al. 1992),(Hergenroeder et al. 1999),(Bonat et al. 2002). An assessment of the stages of sexual maturation by the criteria established from (Marshall and Tanner 1969),(Marshall and Tanner 1970) was not available for the present cohort. In light of the aforementioned findings, such data, even if available, may not have been informative for the present population of adolescents from Greece.

Age, gender and height are associated with sexual maturation (for examples (Malina and Bielicki 1996),(Siervogel et al. 2003)). As such, age, gender and height have been used

previously as proxies to accommodate differences in growth and development from childhood through young adulthood. For example, (Ellsworth et al. 2002) have used gender and age-stratified analysis to account for growth and developmental effects in their investigation of the effects of the ADRB2 gene on childhood obesity. The present study attempted to account for differences in growth and development of the participants of the present cohort by considering both age and height-stratification approaches through Z-scores conversion in each gender separately. Z-scores conversion prior to parametric analysis has been previously described in studies investigating genetic influences on physiological and physical fitness-related phenotypes in children and adolescents (for example (Ferrari et al. 1998)). In the absence of a gold standard it was not possible, and was out of the scope of this investigation, to assess the value or applicability of age and height as proxies for sexual maturation; these issues have been described elsewhere (for examples (Tanner 1992),(Malina and Bielicki 1996),(Siervogel et al. 2003),(Ellsworth et al. 2002)). Nevertheless, the present findings did indicate that age and height, for the present cohort at least, had similar capabilities as proxies for growth and development (Appendix 3).

As discussed in General Methods (p. 50-54), association studies are at risk of type I errors as a result of multiple testing. Adjusting the probability of type I errors, α, to a level where it will not exceed the selected probability of statistical significance is an acceptable way of dealing with this important issue (Sankoh et al. 1997),(Goodman 1998). The present findings indicated that where the number of the tests performed was low multiple testing corrections helped differentiate between valid and biased results. However, as the number of tests increases so does the probability of type I errors that, in turn, will result in a concomitant reduction of α to unrealistically low levels (Appendix 4). As data analysis and

interpretation depend on factors such as the functional relevance and biological plausibility of the results (Royall RM 1997) practical alternatives to account for the observed significant effects could be considered (Perneger TV 1998),(Bacchetti 2002). Accounting for the present results based on the criteria described in General Methods (p. 54) helped differentiate between valid and biased results. Using this set of criteria in Chapters 4 and 6 produced conclusions that, on average, matched those made by the Šidak correction. Adjusting for multiple testing reduces the probability of type I errors however it simultaneously increases the likelihood of type II errors so that real associations are being missed (Sabatti et al. 2003). In addition, it is possible that even after multiple correction findings significant by the α value may be biased. In light of all the above, it might be a better practice to discuss all significant findings based on their strength, consistency and biological plausibility irrespective of whether multiple testing adjustments have been performed or not. Such approaches are in keeping with good scientific practice (Royall RM 1997) and would serve to improve confidence in significant findings.

8.3 General conclusions

From the experimental findings of these investigations, a number of conclusions can be made. These conclusions reflect key points generated by the discussion of the present findings relative to the existing knowledge for each topic. It must be noted that the conclusions regarding the findings for the genetic effects described in Chapters 4-7 may vary between populations of different gender, age and environment.

a. The present cohort was based on a large, representative population of adolescent children from Central Greece and it included a wide range of data describing their physiological, physical and skill-related physical fitness. As such, at the date of the

writing of this report, and relative to the published literature, this was the sole cohort with such a wide range of physiological, physical and skill-related physical fitness data available on Greek adolescents. From the results of the study assessing the general health and performance-related physical fitness of adolescent Greek children described in Chapter 3 the following conclusions can be made:

i. Greek adolescents demonstrated high rates of overwight and obesity with males having a two-fold higher prevalence of obesity relative to females. The present population of adolescent Greek children had, on average, higher rates rates of overweight and obesity when compared to adolescent children from other similar populations from Greece.

ii. Relative to normative data from Australian children of similar ages the Greek cohort consisted of individuals that had higher mean body mass, lower aerobic fitness, and levels of physical activity, were less agile and were, on average, outperformed in upper-body strength-related trials. The age and gender-specific differences observed between the mean values for the physiological, physical and skill-related phenotypes of Greek and Australian adolescents were, most likely, the outcome of the higher mean body mass and lower aerobic fitness of Greek children. Nevertheless, the aforementioned findings were not the outcome of a higher than average health and physical fitness of the Australian children but were, most likely, reflective of the negative influence that the high adiposity and the lower than average aerobic fitness of the Greek adolescents had on their health and performance-related physical fitness.

iii. Considering overweight rates, the present population of adolescent Greeks had among the highest prevalences of overweight relative to other European and representative Australian populations and, in addition, higher obesity rates when compared to the findings of other studies in similarly aged populations from Australia. Furthermore, Greek adolescents had lower aerobic fitness, as indicated by their $\dot{V}o_{2\ max}$, relative to international references.

b. From the experimental chapters assessing the influence of genetic variants of the ACE and ADRB genes on physiological, physical and skill-related phenotypes in the present cohort of adolescent children (Chapters 4-7) the following conclusions can be made.

i. The present findings advocated a broader role for the ACE gene in obesity and performance-related physical fitness phenotypes than previously described in selected adult populations. Furthermore, the present findings were consistent with previous reports indicating that the ACE gene exhibits pleotropic effects. The ACE I-allele and, where significant, the corresponding H6 haplotype associated with lower adiposity and enhanced physiological, physical and skill parameter scores. The observed effects, although significant, were modest and in line with what would be expected for multi-factorial traits. Significant associations were gender-specific and suggested the existence of a gene-environment interaction capable of modifying the impact that variants in the ACE gene have on the phenotypes under investigation. The results for haplotypes, where significant, suggested

that the majority of the genetic effects were accounted for by the I/D polymorphism or a variant in strong LD with it. Polymorphic variants of the ADRB genes had a modest role in influencing obesity and performance-related physical fitness phenotypes. Considering obesity-related phenotypes, the data were consistent with a previously unreported, protective effect of allelic combinations of the ADRB2 Arg16Gln27 haplotype over the obese phenotype that was dominantly mediated by the Arg16 variant. For performance-related physical fitness phenotypes, the ADRB1 Ser49 and Gly389 alleles as well as the corresponding Ser49Gly389 haplotype associated with enhanced physical and physiological parameter scores; nevertheless, caution must be exercised when interpreting these results. The ADRB2 Gly16 allele and the corresponding Gly16Gln27 haplotype associated with enhanced hand-grip strength scores. These findings indicated that the majority of the genetic effects on phenotype were mediated by the Gly16Arg variant. In addition, the implication of the Gly16 allele with increased adiposity and hand-grip strength would suggest pleiotropic effects of the Gly16Arg variant influencing the receptor function. Significant associations for both ADRB1 and ADRB2 genes were gender-specific and influenced by participation in organised physical activity.

ii. Relative to individual genotypes, the use of haplotypes, on average, did not produce markedly stronger associations and although it did explain more of the observed variance that increase was no larger than what would be expected by random sampling effects. The mechanics of haplotype analysis, requiring an increase in the number of degrees of freedom considered, may have, on occasion, underpowered the present series of candidate gene

association studies to detect significant effects. Nevertheless, the present results do not suggest evidence against the greater analytical power of haplotypes or their use in the investigation of complex traits and do not contradict findings from multiple individual SNPs. They do however indicate the possibility that the present study population was not large enough to support the investigation of certain small gene effects when so many different genetic groupings were called for and suggest a dependency of haplotype-based approaches on sample size that is proportional to the strength of the effect under investigation.

iii. The observed gender-specificity of significant effects reflects, most likely, the many factors, genetic and environmental, that result in differences of adipose tissue metabolism and performance-related trials between males and females as well as differing stages of physical maturity between genders. The interaction of genetic polymorphisms with physical activity provides further evidence that genes are not deterministic, only predisposing individuals to particular traits but subject to be overridden by lifestyle choices. The present results offered support to previous suggestions indicating that younger individuals may be a better subject population relative to adults to investigate genetic influences of complex traits despite sexual maturation and development that take place at this stage of life. It is also clear that selecting or subdividing a cohort by the "wrong" gender, age or activity could easily mask the small effects of the ACE and ADRB genes on phenotype. The presence of such effects may have contributed to the shortcomings of previous investigations.

iv. Association studies may be subjected to type I errors caused by multiple testing. Accounting for the risk of type I errors by Bonferroni and related corrections, although possible, is very conservative when a large number of tests is involved and will concomitantly increase the likelihood of type II errors. A practical alternative is to discuss significant findings with respect to their biological plausibility, number and relative strength over a number of phenotypes and across phenotypic groups. This approach is in keeping with good scientific practice and can help assess the validity or bias of significant findings; as such, this approach should be exercised irrespective of considerations for corrections for multiple testing.

8.4 Suggestions for future research

As outlined in the General Introduction (p. 7), the phenotypic heterogeneity (V_P) of multi-factorial traits is the collective outcome of genetic (V_G), non-genetic (V_E) and interactive gene-environment (V_{GE}) variance. Genetic variance (V_G) is further subdivided into additive (V_A), dominant (V_D) and gene-gene interaction (V_I) variance. The design of the present investigations did consider non-genetic sources of variation (V_E) in the form of gender, age and organised physical activity participation and their interaction with inter-individual genetic differences (V_{GE}). Furthermore, unlike most previous reports, the present study has attempted to discern between additive (V_A) and dominant (V_D) genetic influences on the phenotypes under investigation. Nevertheless, effects arising from gene-gene interaction (V_I) variance were not assessed here. Given the recent development of DNA arrays, the future of gene association studies may well lie in simultaneously assessing, other than additive and dominant effects, the interactive influence on phenotype of a large number of polymorphic variants. The natural follow-on of the present investigation would be to

consider genetic influences on phenotype arising from the interaction between polymorphisms in all three ADRB genes and subsequently attempt to consolidate these findings with the effects of the ACE variants.

Considering the suggestions with regards to younger individuals being a better subject population relative to adults to investigate genetic influences of complex traits would indicate that a population of individuals younger than the present cohort of adolescents may be an even better subject population to assess such effects. This type of approach would help validate the present results and possibly offer a greater insight on the genetic basis of complex traits such as obesity or performance-related physical fitness. Currently, a novel study involving 2033 preschool children, aged 0-6 years, from urban and rural areas of Greece is being performed. Preliminary genetic analysis for the ACE gene indicated gender and age specific effects in the influence of the I/D polymorphism on a number of obesity-related phenotypes. Significant associations were observed predominantly in young females where the D-allele was significantly associated with increased BMI and adiposity. As such, the results from preschool Greek children support the present findings in adolescent Greeks with regards for the influence of the ACE gene on obesity-related phenotypes (unpublished data).

APPENDICES

List of appendices

Appendix 1A: Glasgow University Ethics Committee Application

UNIVERSITY OF GLASGOW

ETHICS COMMITTEE FOR NON CLINICAL RESEARCH INVOLVING HUMAN SUBJECTS

RESEARCH SUBMISSION

Name of person(s) submitting research proposal **Dr Yannis Pitsiladis; Professor Susan Ward**

Position held **Lecturer in IBLS; Director of CESAME and Professor of Exercise Science and Medicine**

Department/Group/Institute/Centre - **Centre for Exercise Science and Medicine (CESAME), IBLS**

Date of submission **February, 2000**

Project Title: **Genetic influences on exercise capacity, body composition and habitual physical activity levels**

1. Describe the basic purposes of the research proposed.

Background of investigation

An ever increasing number of phenotypes are being attributed to unique gene polymorphisms (e.g. obesity, hypertension, physical performance, social behaviour). The inherent integrated response of a physiological system, however, means that discrete associations are difficult to prove. Thus, to demonstrate a direct association between a polymorphism and an alteration in a specific physiological function will necessitate that other related systems are not involved. In this proposal we seek to explore correlations between genotype and phenotype in various exercise-related traits in a large cohort of normal healthy children. We intend to achieve this by performing association tests using polymorphisms in candidate genes selected on the basis of their known physiological functions in humans and other mammals (e.g. angiotensin converting enzyme, muscle specific creatine kinase, beta-2 adrenoceptor). The aim of this research is, therefore, to determine if the previously described relationships are 'unique' as has been suggested.

2. Outline the design and methodology of the project. Please include in this section details of the proposed sample size.

Plan of Investigation

We propose to:

1. select polymorphic candidate genes known to, or with a strong potential to, influence particular components of exercise capacity (e.g. aerobic and anaerobic capacities) and habitual physical activity levels;
2. design allele-specific polymerase chain reaction detection tests for each polymorphism (large majority of which will pre-exist), where no such test exists currently to develop assays for them;
3. generate the set of phenotypic traits to be studied, using a cohort of 1,400 healthy male and female subjects (10-18 yr) by:
   (a) obtaining DNA samples non-invasively;
   (b) characterizing aerobic exercise capacity (as pulmonary, cardiovascular and muscle contributions to maximal oxygen utilization) and anaerobic exercise capacity (as "sprint" power-generating parameters);
   (c) profiling habitual physical activity;
4. obtain genotypes at all markers for all individuals; and
5. assess association, correlation and covariance between alleles at each marker and the measured, exercise-related phenotypic variables.
6.

Methods/Design of investigation

Ten schools selected to include urban and rural areas in Greece will be invited to participate in this project. We propose to study 1400 healthy children and adolescents aged between 11-18 years. Subjects will be asked to complete a series of fifteen tasks designed to measure physical, physiological and skill attributes during normal Physical Education (PE) classes. The majority of the proposed tasks are currently being utilised, to a greater or lesser degree, by schools typically as part of the normal PE syllabus; i.e. 40-metre sprint, Basketball throw, "Throw and Catch", Multi-stage Shuttle Run, "Eurofit" Agility test, "Sit and Reach", Hand Grip Strength and Vertical Jump. The participating PE staff will be trained to administer a subset of these tasks (1-5, below) during normal PE classes. Specifically, they will be advised on the precise testing protocols to be employed to ensure that there is standardisation of testing throughout every school involved in the project and that the data collected is not compromised through local variations. In this way, much of the testing procedures will become an integral part of the PE programme and can be spread out throughout a longer time period. A second subset of tasks (6-12) will be administered by our research team, who will attend the school to conduct the remainder of the tests at a mutually convenient time.

The tasks to be conducted by the PE staff are the following:

(1) Basketball throw
(2) 40 meter sprint
(3) Agility test
(4) Throw and Catch test
(5) Multi-Stage Shuttle Run test

The tasks the research team will conduct are:

(6) Basic body measurements (height, sitting height, arm span, weight)
(7) Body composition
(8) Hand grip strength
(9) Vertical Jump
(10) Sit and Reach
(11) DNA Sampling

All subjects will be in good health at the time of testing. Subject eligibility will be assessed by subjects completing a medical questionnaire. Parental and subject consent will be sought prior to testing *Statistical analysis:* Statistical analysis of the data will be carried out using appropriate parametric and/or non-parametric tests.

3. Describe the research procedures as they affect the research subject and any other parties involved.

All testing will take place at the respective schools (e.g. school common room, multi-purpose area, stage space and/or games hall).

The testing will contain no more risk than any other PE activity. All subjects are routinely instructed to cease exercising if they experience any discomfort or have any concern for their well being.

All subjects will be healthy individuals without a history of any significant medical problem(s). The good health of each subject will be established prior to the study by taking the subject's medical history, which is supported by a written assurance from the subject and parent(s) or guardian(s). Subjects with a history of cardiorespiratory or neurological disease will be excluded from participation, as will those having an acute upper respiratory tract infection at the time of study.

Close supervision of the subject is ensured at all times by the supervising investigator and/or PE staff.

The risks associated with performing maximal exercise are minimal as long as the subject is appropriately instructed and familiarised with the testing procedures and is appropriately supervised. All exercise tests will be preceded by a 10-15 min "warm-up" and "warm-down".

All activities will conform to local education authorities health and safety procedures and will comply with any specific health and safety requirements identified by individual schools.

4. What in your opinion are the ethical considerations involved in this proposal? (You may wish for example to comment on issues to do with consent, confidentiality, risk to subjects, etc.)

The ethical concerns are very minor. The risks during testing are small and comparable to those encountered during normal PE classes. Delayed-onset muscle soreness may result after testing in some subjects; however, this typically subsides within 24-48 hours quickly and is not debilitating in any way.

Volunteers will be fully informed of the testing procedures and free to withdraw from the study at any time.

Saliva will be handled, stored and disposed of according to standard health and safety procedures.

5. Outline the reasons which lead you to be satisfied that the possible benefits to be gained from the project justify any risks or discomforts involved.

The research undertaken will contribute towards the identification of physiological and behavioural functions of the selected candidate genes. It will also shed light on possible selection pressures operating on these genes during human evolution. The minimal risk and discomfort associated with the above procedures are considered to be worthwhile to gain the information required.

7. Are arrangements for the provision of clinical facilities to handle emergencies necessary? If so, briefly describe the arrangements made.

During all activities, a qualified first aider will be available. All testing will take place within immediate access to first aid facilities, including ice. All members of the research team are trained in CPR.

8. In cases where subjects are identified from information held by another party (for example, a doctor or hospital) describe the arrangements whereby you gain access to this information.

N/A

9. Specify whether subjects will include students or others in a dependent relationship.

All subjects will be pupils at the respective schools. However, no subjects will be under pressure from staff to participate in the project.

10. Specify whether the research will include children or those with mental illness, disability or handicap. If so, please explain the necessity of using these subjects.

All subjects will be children and adolescents aged between 11-18 years. It is necessary to use this population in order to minimise the effects of environmental factors on the measured physiological outcomes.

11. Will payment be made to any research subject? If so, please state the level of payment to be made, and the source of the funds to be used to make the payment.

NO

12. Describe the procedures to be used in obtaining a valid consent from the subject. Please supply a copy of the information sheet provided to the individual subject.

Each participant and parent(s) will receive an information sheet outlining the tests to be performed prior to providing their written consent. A verbal explanation will also be given and any queries answered.

13. Comment on any cultural, social or gender-based characteristics of the subject which have affected the design of the project or which may affect its conduct.

None

14. Give details of the measures which will be adopted to maintain the confidentiality of the research subject.

The information obtained will be anonymised and individual information will not be passed on to anyone outside the study group. The results of the tests will not be used for selection purposes.

15. Will the information gained be anonymized? If not, please justify.

Yes

16. Will the intended group of research subjects, to your knowledge, be involved in other research? If so, please justify.

No

18. Please state location(s) where the project will be carried out.

Existing School or Educational institution's sports facilities.

Appendix 1B: Consent form

Ευρεκα Sport

Fitness Testing Research Programme

I _____(PRINT) being the parent/guardian of

_____

in class _____

Name of School _____

give permission to have him/her included in the Eureka Sport fitness testing programme.

Signed _____

Date _____

Please sign and complete this form and return it to your child's physical education teacher.

Thank you for supporting this project

Appendix 1C: Parental information letter

Dr Yannis P. Pitsiladis
Eureka Sport School-age Sports Counselling Programme

Dear Parent or Guardian,
The University of Glasgow has embarked on a project to establish a range of up to date fitness parameters for school children from a range of European countries. It is hoped that from the results of the research undertaken we will be able to establish an unique Health Related Fitness, Sports Talent Identification and Sports Counselling programme for school age children. This project is funded by the European Commission and by Mars Incorporated.

We are delighted that your school has agreed to support and contribute to the initial research phase of the project and it is to this end that I am now writing to you.

The initial phase of this research will require a team of University researchers to conduct a number of fitness and skill based tests on school pupils in Greece. This process is necessary in order to obtain data on the physical and physiological characteristics of Greek school-children between the ages of 11 and 18 years. As such, children covering a complete cross-section of Greek society will be assessed, which, in turn, will give us a more comprehensive picture of fitness of Scottish School children.

The tests included in the programme encompass tests for speed, hand-eye co-ordination, strength, power, agility, cardio-vascular endurance and lung function. Basic body measurements such as height, weight and body fat will also be obtained. In addition, we wish to collect a small amount of saliva for subsequent DNA analysis. This will enable us to investigate a number of aspects of performance that may be related to the genetic make up of the individual.

The test results will be matched against similar profiles derived from a comprehensive list of sports and physical recreation activities and used to develop an interactive computer programme. This software programme will enable young people to match their physical attributes and sporting preferences to potential sport suitability. The predictions that the software programme will provide may change the outlook of many school pupils who might be motivated to try different sports and activities than they might not otherwise have previously considered. We anticipate that the software package, when completed, will be an invaluable tool for school Physical Education Departments in their day to day work and in the interest they have for all their pupils health and well being.

We appreciate that many parents will have concerns relating to their children being subjected to tests that require a degree of physical effort and others that they may find awkward. All the tests that have been included are currently being used to a greater or lesser degree by PE departments and the PE staff will be contributing to the collection of test information throughout the year. However, the skinfold test for measuring of body fat using sites at the back of the arm and just below the shoulder blade could be disconcerting for certain individuals. All the tests are

important to the final outcome of the research and are strictly monitored. All attempts are taken to motivate and reassure participants and avoid any awkwardness through the professionalism of the testers.

All information collected will be dealt with in the strictest confidence and will be subject to the Data Protection Act. The research group will not pass any details on to any other organisation.

We would respect your concerns and your decision should you not wish your child to participate if they are selected by the researchers and PE staff. If you are willing to allow your child to take part in the research then I would be grateful if you could complete the return slip and return it to your child's PE teacher.

I would like to thank you in advance for your help in our research programme.

Yours sincerely

Dr. Yannis Pitsiladis
Eureka Sport Project Manager

Appendix 1D: Participant information form

Ευρεκα Sport

Participant Information Form

Please complete the following general questionnaire prior to completing and recording any of the activity test results.

General Information **School Class No** _____

Name: | **First name:** | **Initials**

Nationality: | **Region:** | **School:**

Ethnic Origin (circle)	**Asian**	**Afro-Caribbean**	**Caucasian**	**Arabian**	**Chinese**	**African**	**Other (please specify)**

			Day	Month	Year
Address:		**Test Date:**			
		Date of Birth:			
		Mother's Occup:			
Post		**Father's Occup:**			

Gender	**Female** ☐ **Male** ☐	**Right-handed :** ☐ **Left-handed:** ☐

Do you take part in any physical activities in your free time? (e.g. walking, cycling, football, swimming etc.)	YES ☐			NO ☐	
If **YES**, how many hours per week?	0 to 2hrs ☐	2 to 4hrs ☐	4 to 6hrs ☐	6 to 8hrs ☐	more ☐
Are you a member of any sports clubs?	YES ☐			NO ☐	
If **YES**, what is your preferred sport?					
How many hours per week do you practice?	0 to 2hrs ☐	2 to 4hrs ☐	4 to 6hrs ☐	6 to 8hrs ☐	more ☐
Number of hours of physical education practiced at school per week:	0 to 2hrs ☐	2 to 4hrs ☐	4 to 6hrs ☐	6 to 8hrs ☐	more ☐

Do you smoke cigarettes?	**YES** ☐		**NO** ☐
If **YES**, how many cigarettes per day?	Less than 10 cigarettes:		☐
	Between 10 and 20		☐
	More than 20 cigarettes:		☐
Do you consume alcoholic drinks?	Never ☐	Occasionally ☐ (e.g. on special occasions)	Regularly ☐ (e.g. most weekends)
Do you suffer from any particular medical conditions?	**YES** ☐		**NO** ☐
If **YES**, and you know what it is, Please specify			
Are you required to take any form of medication?	**YES** ☐		**NO** ☐
If **YES,** and you know what it is, Please specify			

Appendix 2: Testing protocols

Warm Up and Cool Down

Participants should be given the opportunity to conduct a pre session warm up routine particularly where maximum effort or prolonged effort is required. Light aerobic activity combined with appropriate stretching of the principal joints and muscle groups should be undertaken.

Participants should also be encouraged to keep warm throughout the session maintaining a state of physical readiness. At the conclusion of the session the participants should be taken through a cool down procedure.

Pre Test Practice

The Catch and Throw test is the only test in which any form of practice will be allowed. This is only to reinforce the requirements of the particular test.

Once the participant has demonstrated sufficiently to the Tester that he or she understands the tests' requirements then the Tester is at liberty to commence the actual test.

In all other practical situations the participants will have two attempts to achieve a maximum performance. Only in the case of the Multi-Stage Shuttle test will there be only one attempt.

MULTI-STAGE SHUTTLE RUN

Purpose To assess aerobic fitness (endurance ability).

Equipment Cadence compact disc for the shuttle run. Masking Tape. Compact Disc player. Marker cones (8). 20 m marked distance on a surface that is flat, even and slip resistant. Stop watch. Shuttle run data collection forms.

Procedure Check the speed of the compact disc player using the one minute calibration period. Measure the 20 m distance and mark with tape (if available) and marker cones. Instruct the participant to run to the opposite end and place one foot behind the line by the time the next beep sounds. If the participant arrives before the whistle (s)he should turn (pivot) and wait for the signal, then run to the opposite line to reach it in time for the next signal. At the end of each minute the time interval between whistle is decreased and the running speed becomes progressively faster. Ensure that the participant reaches the end line each time and does not turn short. Emphasize to the participant to turn and pivot rather than run in an arc which takes more time. Each participant continues running for as long as possible until (s)he can no longer keep up with the indicated tempo. The criterion for eliminating a participant is two lengths in a row where (s)he is more than two steps from the end when the whistle blows.

Scoring Record the last level and shuttle the participant successfully completed.

SITTING HEIGHT

Purpose To determine the vertical distance from the sitting surface to the top of the head (vertex). It is the total of trunk, neck and head length.

Equipment Stadiometer or steel measuring tape firmly mounted on a wall, accurate to 0.1 cm. If a measuring tape is used, a set square will also be required. Ensure that the floor surface is even and firm. Small bench or box of known height (approximately 40 cm).

Procedure Place the bench or box centrally at the base of the stadiometer or measuring tape. The participant sits on the bench with knees forward, legs hanging over the bench and hands resting on the thighs which are parallel with the floor. The buttocks and shoulders rest lightly against the stadiometer/tape, which is positioned vertically in the mid-line behind the participant. The participant is instructed to look straight ahead, take a deep breath and sit as tall as possible (ensure the head is not tilted backwards). If using a stadiometer, lower the platform until it makes firm contact with the top of the head.

Scoring Height is recorded to the nearest 0.1 cm. To obtain the participant's sitting height, subtract the height of the bench from the recorded measurement.

ARM SPAN

Purpose To determine the horizontal distance between the tips of the middle (longest) finger of each hand (excluding the fingernails) when both arms are extended to the sides and maximally to the level of shoulders.

Equipment A ruler of at least 3 m long. A flat surface (usually a wall) and an adjustable block that is fixed to the wall. A ruler.

Procedure The participant stands erect with the back against the wall, feet together, and heels, buttocks and shoulders touching the wall. The arms are outstretched to the sides and maximally at shoulder level in contact with the wall, and with the palms facing forward. The tip of the middle finger of the one hand is kept in contact with the block. When making this measurement, it is imperative that the participant's arms are outstretched maximally and that they are held in this position until the reading is taken. If the participant is tall/short and their arms are above/below the ruler, ensure the arms are held in a horizontal position and use a ruler held vertically to line up the end of the finger tip with the measure.

Scoring Record arm span to the nearest 0.5 cm.

SIT & REACH

Purpose To determine the flexibility of the participants, trunk and hamstrings.

Equipment Sit and reach box placed on an even piece of floor.

Procedure The participant should be barefoot and sit with his or her feet together and flat against the upright base of the sit and reach box. He or she should rest the fingers on the starting scale on top of the box with the metal sliding pointer at the tips of their fingers.The

participant should then stretch forward sliding his or her hands and the pointer down the length of the box until he or she can reach no further. Participants should be instructed to slowly stretch forward, reach as far as possible (maintain this position for 2 sec). Participants should not jerk into the stretch or stretch too fast. Allow two trials for each participant.

Scoring Record the participants furthest attempt to the nearest 0.5 cm

THROW & CATCH

Purpose To measure the participant's ability to accurately throw a tennis ball underarm against a target and catch it with one hand.

Equipment A basket with at least ten tennis balls. Masking tape. Circular target (cardboard and black in colour) 30 cm in diameter (preferably laminated). Mount to wall with masking tape. Tape measure (3 m accurate to 1 cm).

Procedure The target should be placed on the wall with the bottom edge level with the participant's shoulder height. Mark a line on the ground 2.5 m from the target using the masking tape. The participant stands behind this line. The participant is instructed to throw the ball with the preferred hand towards the target and attempt to catch the ball with the same hand. Practice trials are allowed until the participant demonstrates the understanding of the task and have got the 'feel for it'. The ball must be thrown underarm and not allowed to bounce before being caught. Each trial is only successful if the ball hits the target (any part of the ball on the target is acceptable) and the participant catches the ball. A catch is only considered successful if the ball is caught cleanly and not trapped to the body. The participant is not allowed in front of the line to catch the ball. The participant is given 10 attempts throwing and catching with the preferred hand. This is followed by 10 attempts throwing with the preferred hand and catching with the non-preferred hand.

Scoring One point for each successful hit on target and catch. To score a point: The ball must be thrown underarm. The ball must hit the target. The ball must be caught cleanly without being trapped to the body. The participant must not move in front of the line to catch the ball. Add together the score for catching with the preferred and non-preferred hand. The total possible score is 20.

BASKETBALL THROW

Purpose To measure upper body power by determining the distance the participant can project the basketball in a forward direction.

Equipment Standard size and weight senior basketball. 15 m tape measure accurate to 5 cm.

Procedure The participant sits with their buttocks, back and head resting against a wall. The legs rest on the floor horizontally in front of the body. The participant uses a two handed chest pass to push the ball in the horizontal direction as far as possible. A one arm or shoulder pass is not allowed. Ensure that the participant keeps the head, shoulders and

buttocks in contact with the wall and the ball is thrown only using the arm and shoulder muscles. Allow two trials for each participant.

Scoring Record the longest distance thrown to the nearest 5 cm (measure from the base of the ball where it makes contact with the ground on the first bounce).

HAND-GRIP STRENGTH

Purpose To measure static forearm strength.

Equipment Takei Grip-D Hand Dynamometer.

Procedure Have the participant adjust the instruments grip range until the second joint of the forefinger is bent through 90 degrees. The participant should stand upright and relaxed extending his or her arms downwards. The participant will grip the instrument and exert full force without letting their arms touch their body. During the measurement the instrument should not be waved about. The participant is allowed two trials with each hand. The first trial should be with the right hand with the next trial using the left hand. (This will be counted as the first trial for the left hand.). The second trial for each hand should then be administered.

Scoring After each attempt the score should be recorded in kilograms. Record the best score for each hand. Add the two best scores together to give the final recorded score for the test.

VERTICAL JUMP

Purpose To measure the power of the legs by assessing the vertical distance that the participant can jump.

Equipment Takei Jump-MD

Procedure Insert the clasp of the rope on the rubber mat. Have the participant stand on the centre rubber mat and wrap the belt around his or her waist tightly. Turn the pulley gently in the direction of the arrow to take any slack out of the rope. The participant's arms remain at his or her side as they go into a crouch position. The participant can choose the depth of crouch they feel comfortable with. The participants may bounce if desired in final preparation of their jump. The participant then jumps straight up. Record the height of the jump displayed in centimetres. Reset the display by pressing the ON/C button and again turn the pulley to remove any slack out of the rope ready for the second trial. Allow two trials for each participant.

Scoring Record each height that is identified on the display panel in centimetres. Record the greatest height as the best score. Note: If the Jump-MD is not reset by pressing the ON/C button, it will display the higher of the two attempts automatically.

40 m SPRINT

Purpose To measure the ability to run as fast as possible from a stationary position to a point 40 m further.

Equipment Stopwatch. 10 marker cones. 40 m running track that is straight, level and placed cross wind (if a grass surface is used ensure that it is dry).

Procedure Mark a 40 m running track with marker cones placed at 10 m intervals. The participant starts in a standing position, one foot in front of the other -the front foot placed exactly on the line. The tester should stand at the finish line, call ready and sweep down their arm quickly to start the participant (do not call 'go'). As the arm sweeps down the tester should simultaneously start the stopwatch which is held in the descending hand. Stop the stopwatch when the participant's chest crosses the line. Emphasise to the participant to run as quickly as possible. Allow two trials for each participant, with a short rest between the trials.

Scoring Record the time taken to run the fastest trial to the nearest 0.1 of a second

AGILITY RUN

Purpose To measure the ability to change direction of the body quickly whilst moving (i.e. agility).

Equipment Stopwatch. Two parallel lines (1.2 m in length) marked on the ground 5 m apart (measured between the two inside edges of the line). Marker cones (4). The floor surface should be flat, even and slip resistant. A gymnasium floor is often dusty and slippery. If this is the case it is better to conduct the test outside on a concrete or bitumen level surface.

Procedure The subject starts from behind one line with their front foot exactly on the line. On the command 'go' the participant runs forward as quickly as possible to the other line, pivots and returns to the start line. This constitutes one cycle, with five cycles required in total. The participant must touch both feet beyond the lines and between the marker cones except at the end of the 5th cycle when they should run past the finish line without slowing. Start the stopwatch on the command 'go' and stop it when the subject's chest crosses the line. Allow two trials for each participant. If the participant slips, do not include this result but conduct another trial.

Scoring Record the time taken to the nearest 0.1 of a second for the fastest trial.

Appendix 3: Comparison of the analysis of significant differences between ACE genotypes/diplotypes and obesity-related phenotypes for age and height standardised data in young females

Table A: Analysis of associations between genotypes/diplotypes and obesity-related phenotypes for age and height standardised data in total females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance ($0.05<P<0.1$) and where they are given in bold the tests were significant ($P<0.05$).

Table B: Analysis of associations between genotypes/diplotypes and obesity-related phenotypes for age and height standardised data in inactive females. For each test the probability (P), the percent variance explained in ANOVA (V) and the number of individuals (N) is given. Where (P), (V) and (N) are given in *italics* the tests showed tendencies to significance ($0.05<P<0.1$) and where they are given in bold the tests were significant ($P<0.05$).

Appendix 3 continued…

Table A

Total Females

Polymorphism		Age Standardised Data				Height Standardised Data			
		BMI	**Triceps Skinfolds**	**Subscapular Skinfolds**	**Total Skinfolds**	**BMI**	**Triceps Skinfolds**	**Subscapular Skinfolds**	**Total Skinfolds**
	I/D	P=0.169 V=0.7% N=480	**P=0.019** **V=1.7%** **N=481**	**P=0.028** **V=1.5%** **N=481**	**P=0.010** **V=1.9%** **N=481**	P=0.161 V=0.8% N=480	**P=0.015** **V=1.7%** **N=481**	**P=0.058** **V=1.2%** **N=481**	**P=0.016** **V=1.7%** **N=481**
	G7831A	P=0.309 V=0.5% N=461	**P=0.025** **V=1.6%** **N=462**	P=0.186 V=0.7% N=462	**P=0.036** **V=1.4%** **N=462**	P=0.253 V=0.6% N=461	**P=0.011** **V=1.9%** **N=462**	P=0.220 V=0.7% N=462	**P=0.032** **V=1.5%** **N=462**
	C8968T	P=0.431 V=0.4% N=461	**P=0.004** **V=2.4%** **N=462**	*P=0.053* *V=1.3%* *N=462*	**P=0.009** **V=2.0%** **N=462**	P=0.405 V=0.4% N=461	**P=0.003** **V=2.5%** **N=462**	*P=0.088* *V=1.0%* *N=462*	**P=0.012** **V=1.9%** **N=462**
	Diplotypes	P=0.921 V=0.6% N=448	*P=0.083* *V=2.8%* *N=449*	P=0.392 V=1.6% N=449	P=0.187 V=2.2% N=449	P=0.829 V=0.7% N=448	**P=0.036** **V=3.1%** **N=449**	P=0.392 V=1.4% N=449	P=0.143 V=2.2% N=449

Appendix 3 continued…

Table B

Inactive Females

Polymorphism	Age Standardised Data: BMI	Age Standardised Data: Triceps Skinfolds	Age Standardised Data: Subscapular Skinfolds	Age Standardised Data: Total Skinfolds	Height Standardised Data: BMI	Height Standardised Data: Triceps Skinfolds	Height Standardised Data: Subscapular Skinfolds	Height Standardised Data: Total Skinfolds
I/D	**P=0.041** **V=3.1%** **N=206**	**P=0.012** **V=4.3%** **N=206**	**P=0.001** **V=6.6%** **N=206**	**P=0.001** **V=6.6%** **N=206**	**P=0.045** **V=3.3%** **N=206**	**P=0.010** **V=4.4%** **N=206**	**P=0.002** **V=5.8%** **N=206**	**P=0.002** **V=6.0%** **N=206**
G7831A	P=0.197 V=1.7% N=196	P=0.110 V=2.3% N=196	*P=0.071* *V=2.7%* *N=196*	**P=0.042** **V=3.2%** **N=196**	P=0.248 V=1.4% N=196	P=0.118 V=2.2% N=196	P=0.136 V=2.0% N=196	*P=0.085* *V=2.5%* *N=196*
C8968T	P=0.321 V=1.2% N=196	**P=0.015** **V=4.3%** **N=196**	**P=0.013** **V=4.4%** **N=196**	**P=0.006** **V=5.2%** **N=196**	P=0.368 V=1.0% N=196	**P=0.009** **V=4.7%** **N=196**	**P=0.026** **V=3.7%** **N=196**	**P=0.009** **V=4.7%** **N=196**
Diplotypes	P=0.624 V=2.8% N=190	P=0.325 V=4.3% N=190	*P=0.067* *V=6.9%* *N=190*	P=0.111 V=6.1% N=190	P=0.509 V=2.9% N=190	P=0.168 V=4.9% N=190	*P=0.058* *V=6.5%* *N=190*	*P=0.081* *V=6.0%* *N=190*

Appendix 4: Šidak correction for multiple testing in the presence of partial correlations between the tested phenotypes

Summary table for the Šidak correction performed in the presence of partially correlated phenotypes (General Methods: Multiple Testing: p. 52-54). The obesity-related phenotypes considered for the correction were BMI, triceps and subscapular skinfolds. None of the performance-related physical fitness phenotypes tested in Chapters 5 and 7 was excluded from the correlation. The Šidak correction was performed using the SISA website (Uitenbroek 2005). (r_{Obes}) is the mean correlation coefficient for the correlation between BMI and skinfolds at the triceps and subscapular sites and (r_{Perf}) is the mean correlation coefficient for the performance-related physical fitness phenotypes. (N) number of tests performed, (α) significance threshold for making a type I error adjusted for multiple testing. The identifying number for the Chapters where each group of phenotypes and genetic loci was considered is given in parenthesis.

		Obesity-related phenotypes (Chapters 4 & 6)	**Performance-related physical fitness phenotypes (Chapters 5 & 7)**
		r_{Obes}=0.776	**r_{Perf}=0,045**
ACE (Chapter 4 & 6)	**N**	72	240
	α	0.019	0.0002
ADRB (Chapter 6 & 7)	**N**	126	420
	α	0.017	0.0001
All loci	**N**	198	660
	α	0.016	0.0001

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