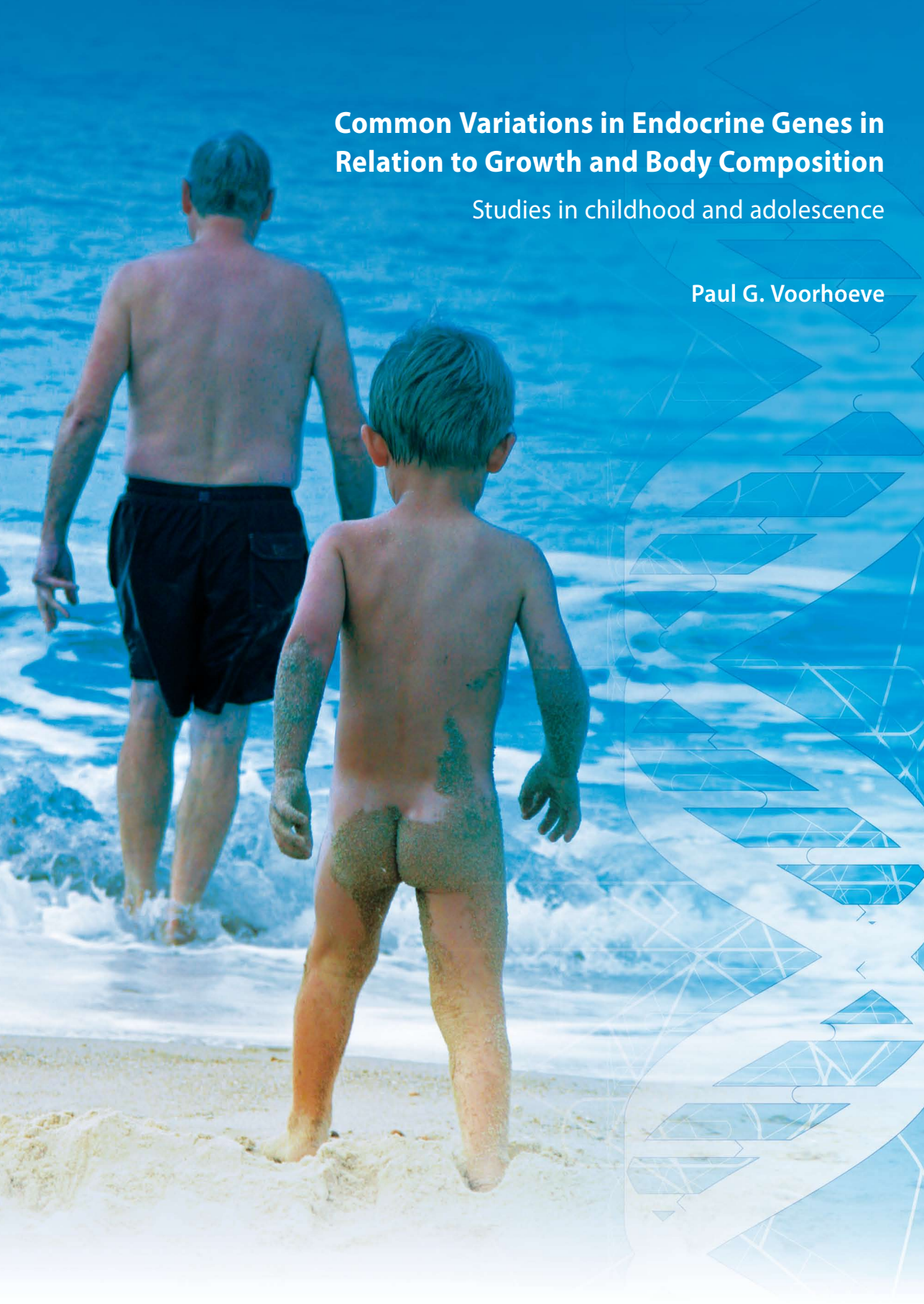

Common Variations in Endocrine Genes in Relation to Growth and Body Composition

Studies in childhood and adolescence

Paul G. Voorhoeve

The studies presented in this thesis were performed at The Department of Pediatric Endocrinology and the Department of Public & Occupational Health and EMGO+ Institute, VU University Medical Center in Amsterdam and at the Department of Internal Medicine, Erasmus Medical Center in Rotterdam, The Netherlands.

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Variaties in endocriene genen in relatie tot groei en lichaamssamenstelling

Studies bij kinderen en adolescenten

Proefschrift

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Ales liekt simpel as t kloar is.

(Gronings gezegde)

List of abbreviations

ACTH	adrenocorticotrophic hormone
AD	autosomal dominant
AGA	appropriate for gestational age
AGAHLS	Amsterdam Growth And Health Longitudinal Study
ALS	acid-labile subunit
AR	autosomal recessive
AR	androgen receptor
AVP	arginine vasopressin
BMI	body mass index
Bonestaak	Bone Study Around Amsterdam in Kids
CA-repeat	cytosine adenine repeat
CAG-repeat	cytosine adenine guanine repeat
CNV	copy number variant
CRH	corticotrophin-releasing hormone
CVD	cardiovascular disease
DBD	DNA-binding domain
DST	dexamethasone suppression test
E2	estradiol
ER	estrogen receptor
ERE	estrogen-response elements
FM	fat mass
FFM	fat free mass
GA	gestational age
GC	glucocorticoid
GR	glucocorticoid receptor
GH	growth hormone
GHRH	growth hormone releasing hormone
GHBP	growth hormone binding protein
GWAS	genome wide association study
HPA	hypothalamic-pituitary-adrenal
IGF-I	insulin-like growth factor I
IGF1R	insulin-like growth factor I receptor
IGF-II	insulin-like growth factor II
IGFBP	IGF binding protein
IUGR	intra uterine growth retardation
LBD	ligand binding domain
LD	linkage disequilibrium
NA	not available
ND	not determined
OMIM	online Mendelian inheritance in men
QTL	quantitative trait locus

RFLP	restriction fragment length polymorphism
SDS	standard deviation score
SEM	standard error of the mean
SGA	small for gestational age
SNP	single nucleotide polymorphism
TAD	transactivation domain
T2DM	type 2 diabetes mellitus
VNTR	variable number of tandem repeats
WC	waist circumference
HC	hip circumference
WHR	waist-to-hip ratio

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Introduction

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Introduction

Introduction

1.1 General introduction

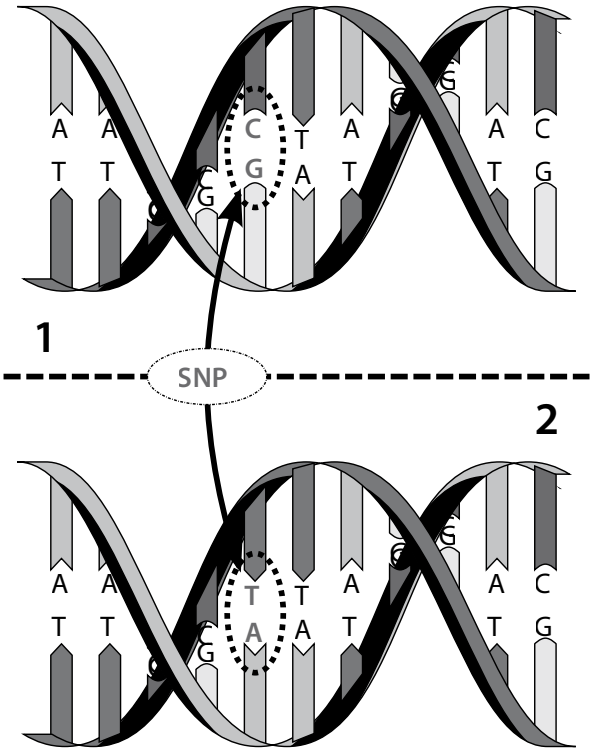
Growth and changes in body composition during childhood and adolescence are regulated and influenced by multiple factors. Among the well-known important hormonal systems are the growth hormone – insulin-like growth factor I (IGF-1) axis, sex steroids and glucocorticoids. Environmental factors like a safe environment, the absence of disease and sufficient nutritional intake are equally important. Another important factor associated with postnatal growth and body composition is size at birth.

The non-environmental factors are more or less inheritable. Variation in genetic background among individuals determines to a large extent phenotypic outcome. Based on population frequency, genetic variations can be divided into genetic polymorphisms and mutations. A genetic polymorphism is a variant, which occurs in more than 1% of the normal population. A gene mutation is a change in genetic information, which occurs in less than 1% of the normal population.

Genetic polymorphisms are assumed to play an important role in gene expression and explaining heritability. The most common types of genetic polymorphisms are single nucleotide polymorphisms (SNPs) and variable number of tandem repeats (VNTRs). SNPs are single nucleotide substitutions of another nucleotide (figure 1). SNPs are found across the whole genome. VNTRs are short simple nucleotide sequences which are organized as tandem repeats (figure 2). Every gene holds several hundreds of SNPs (approximately 1 SNP per 100 BP). Most polymorphisms are localized in non-coding areas of the gene and many have not been properly analysed regarding their function, e.g., in regulation of gene expression itself. Many polymorphisms are not involved in gene expression, but flag other polymorphisms which are functionally involved in gene expression. In population genetics this can be investigated using linkage disequilibrium (LD). LD describes a situation in which some combinations of alleles or genetic markers occur more or less frequently in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies.

This thesis describes common variations, polymorphisms, in several endocrine genes in relation to growth and body composition. The studies focus on polymorphisms in the IGF-1 gene, the glucocorticoid receptor (GR) gene, the estrogen receptor α (ERα or ESR1) gene and the androgen receptor (AR) gene. Most studies were performed in two large cohorts of healthy children and adolescents, who grew up in the same area of the Netherlands, but were born with an interval of approximately 20 years. One study also describes the results of a study in a group of children born small for gestational age (SGA) without catch up growth.

Figure 1 Single nucleotide polymorphism (SNP).



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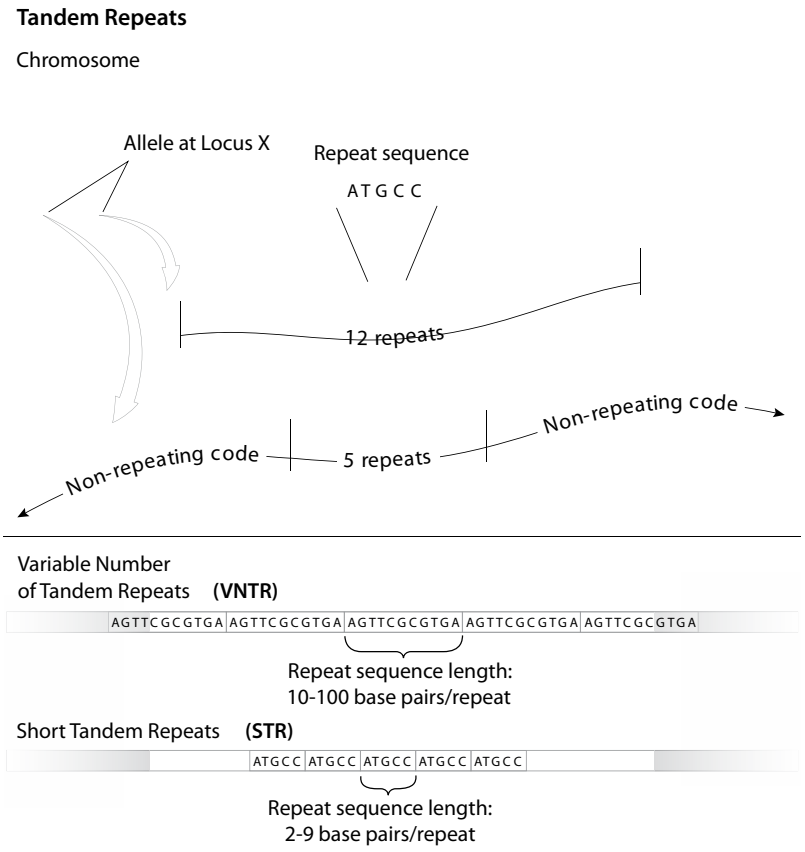
1.2 Growth and body composition in children and adolescents

Background

Longitudinal growth and body composition are important parameters in childhood and adolescence. They both give information on actual health and future risks for several diseases.

Figure 2 Diagram that shows the variable number of tandem repeats (VNTRs) at a specific locus on the chromosomal nuclear DNA.



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Four stages of growth can be considered: fetal, infant, childhood and puberty.
Fetal growth: in the first trimester the organ systems are formed which is coordinated by the expression of various developmental genes. In the second trimester further growth takes place by cellular hyperplasia and peak growth velocity is reached. The third trimester is dominated by maturation of the organs and further body growth. The intrauterine environment, which is determined by maternal factors and placental function, has a more dominant influence on fetal growth throughout gestation than

genotype, although several endocrine factors have been identified that play a role in intrauterine growth, such as IGF-I, IGF-II, and insulin.
Infancy: in the first year of life children grow rapidly (25 cm/year), but at a decelerating rate. Besides nutritional input the GH-IGF-1 system, as well as genetic factors play a role in this stage.
Childhood: in the second and third year the child establishes its own growth channel, which is highly correlated with parental height. By four years of age average growth velocity is 7 cm/year. At this stage GH and thyroid hormone are the major hormonal determinants of growth.
Puberty: this last growth phase is characterized by a growth spurt followed by a rapid decrease of growth velocity due to fusion of the growth plates. Besides GH and IGF-1, estrogen is the main determinant of pubertal growth and epiphysial fusion in boys and girls.[1]
Besides hormonal and genetic factors also general health and psychosocial well being are important factors influencing longitudinal growth.
Economic prosperity in the last decades has improved general health and life expectancy. This is for example reflected in an increase in longitudinal growth over the last decades. One of the major drawbacks of modern life is the wide availability of energy-dense foods with high fat and refined carbohydrate content, which, in combination with an increasingly sedentary life style, has led to an ever increasing overweight and obesity epidemic in children and adolescents.

Size at birth and later risks

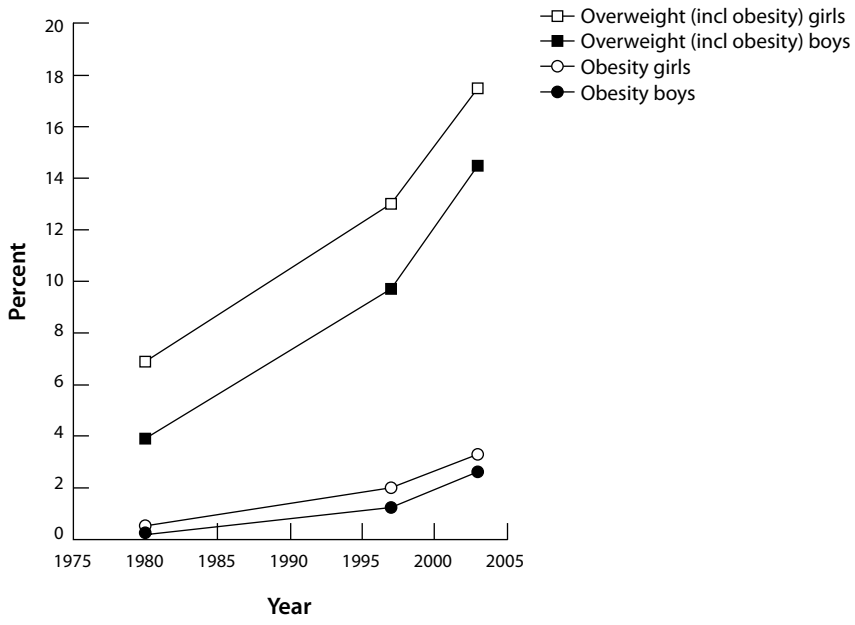
Since the first publication of Barker and colleagues[2] on the association between low birth weight and adult disease, an abundance of studies has confirmed the relation between low birth weight, and the development of several diseases in later life, like hypertension, obesity, dyslipidaemia, insulin resistance, glucose intolerance, type 2 diabetes, and cardiovascular disease. In addition, developmental sequelae affecting the GH-IGF axis, adrenal and gonadal function are seen, particularly in individuals with abnormal weight gain in infancy and childhood. The postnatal weight gain seems to be particularly important in the relation between birth weight and adult disease. For example excessive weight gain during childhood and adolescence in individuals who were born SGA, indicates a poor prognosis for the development of coronary heart disease in later life.[3] Moreover rapid weight gain in infancy, even within the first weeks of life, can lead to hypertension, obesity, and related morbidities before the third decade of life.[4-6] The exact mechanism underlying these associations is still unknown, but several hypotheses have been proposed: 1) *fetal origins of adult disease hypothesis:* this hypothesis states that fetal malnutrition leads to permanent endocrine and metabolic alterations in the fetus; the so-called re-programming. In utero the fetus benefits from these adaptations but in the long term this re-programming

results in an increased risk for adult disease. 2) *fetal insulin hypothesis:* this hypothesis states that the association between low birth weight and later insulin resistance is genetically mediated by genes which both affect fetal growth and insulin sensitivity in childhood and adulthood.[7] 3) *growth acceleration hypothesis:* this hypothesis states that not low birth weight per se, but rapid postnatal growth is responsible for the increased risk for cardiovascular disease in later life.[5] 4) *fat accumulation hypothesis:* this hypothesis states that especially fat accumulation, and not only growth in height and weight, is the potential risk factor.[8;9]

Body composition: genetics and environment.

Overweight is a rapidly growing global public health problem. Also in the Netherlands the prevalence of overweight and obesity has increased sharply over the last 20 to 30 years (figure 3). Overweight and obesity increase the risk of early mortality and severe illnesses, such as heart and vascular diseases, diabetes and psychosocial problems.[10-15]

Figure 3 Secular trend in 1980-2003 in the prevalence of overweight (including obesity) and obesity in 4-15-year-old children living in the Netherlands. Reprinted from Van den Hurk.[14]



Over the past 20 years substantial progress has been made in the molecular elucidation of monogenic forms of obesity.[16-18] These findings have had important implications for our understanding of childhood obesity, because most of these genetically determined forms typically become manifest in infancy and early childhood. These monogenic forms of obesity however, like for example mutations in the melanocortin-4 receptor gene (MC4R), are rare and form only a minority in the total group of children with obesity. Multiple other genes are considered to influence body composition to a much smaller amount. Although several twin studies have shown high heritability estimates in the range of 0.6-0.9 for body mass index,[19-21] it has been shown that the genes relevant for weight regulation in childhood presumably only partially overlap with those active in adulthood, thus partially explaining why intraindividual correlations of BMI during childhood and adulthood are considerably lower than between adolescence and adulthood.[22;23]

Although this heritability of obesity may be attributed largely to metabolic processes, it is widely assumed that also behavioural patterns are, to a large extent, genetically determined. Besides obesogenic genes, other genes are known to have a more beneficial role in body composition. On top of this genetic background, gene-gene and gene-environment interactions are supposed to play an important role in the development of overweight and obesity. Many factors in modern day living contribute to the obesogenic environment, which threatens especially those groups which are genetically vulnerable from a metabolic and/or behavioural perspective (figure 4).

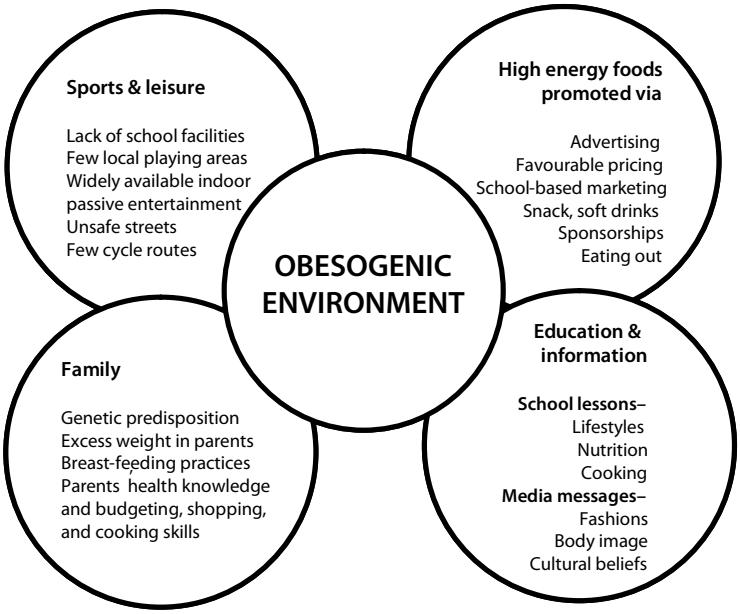
1.3 Insulin-like growth factor 1 (IGF-1)

Regulation of the growth hormone-IGF-1 axis.

Growth hormone (GH) secretion from the pituitary gland is regulated by the hypothalamic factors GH releasing hormone (GHRH) and somatostatin. Other neurotransmitters and neuropeptides also control the release of GH, of which ghrelin is the most potent GH secretagogue. GHRH and ghrelin stimulate GH secretion by binding to their respective receptors in the pituitary. Somatostatin inhibits GH release (figure 5). GH binds to the transmembrane GH receptor (GHR). Activation of the GHR results in transcription of target genes, like IGF-1, IGF-binding protein 3 (IGFBP-3) and acid-labile subunit (ALS). Most of the anabolic actions of GH are mediated by IGF-1, but GH has also many cellular effects that are independent of IGF-1.[24]

IGF-1 shows structural and functional similarities with insulin and has important anabolic and metabolic effects. It is produced mainly in the liver, but also in local tissues where it acts in an autocrine/paracrine manner. Most of the IGF-1 in the circulation is bound to IGFBP's of which IGFBP3 is the most important. Less than 1% of

Figure 4 Factors contributing to the obesogenic environment.

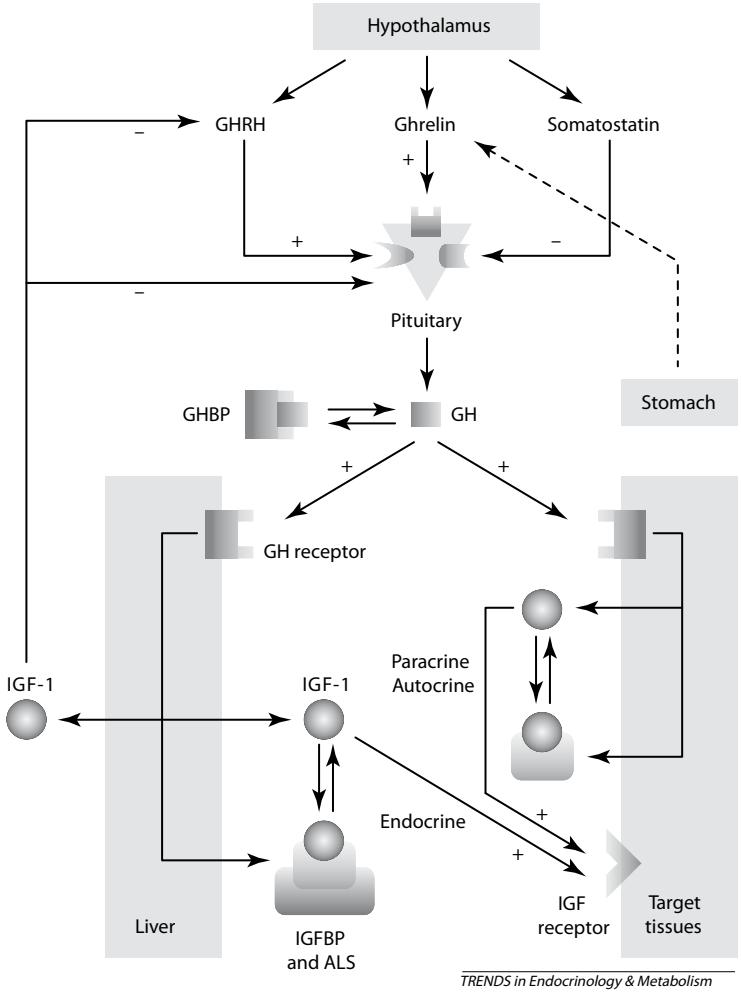


IGF-1 is not bound and circulates in a free form. Binding of IGF-1 to the IGF-1 receptor results in activation of this tyrosine kinase receptor leading to physiological actions.

The effects of IGF-1

IGF-1 is a peptide that plays an important stimulatory role in skeletal growth, cell differentiation and metabolism. It is also known to influence body composition.[26;27] Intrauterine growth is mainly dependent on placental function and supply of essential nutrients. GH seems not to be a major regulator of fetal growth. This in contrast to fetal IGF-1, IGF-II and insulin, which are the most important endocrine regulators of fetal growth. Their concentrations are mainly regulated by genetic factors and fetal nutrition. IGF-1 and IGF-II levels are reduced in infants born SGA compared to infants born appropriate for gestational age (AGA), suggesting an important role for low IGF-I levels in intrauterine growth retardation.[28-30]

Figure 5 The GH-IGF-IGFBP axis. Adapted from Holt.[25]

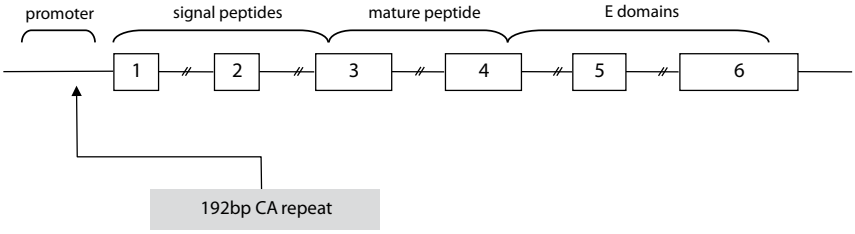


After birth IGF-1 levels decrease in the first six months with a subsequent increase in late infancy.[31;32]

In childhood IGF-1 concentrations slowly increase. They are not influenced by BMI in prepubertal normal-weight children.[33-35] This is probably because BMI is a very rough estimate of body composition and/or nutritional status, both of which influence IGF-1

in situations like obesity or malnutrition. In prepubertal girls IGF-1 is positively correlated with thigh muscle mass, peak oxygen uptake and bone mineral density.[36;37]
In puberty high peak levels of IGF-1 are reached under the influence of increased GH secretion, possibly in combination with increased sensitivity to GH. Pulsatile GH secretion is stimulated by gonadal steroids.

Figure 6 Schematic overview of the IGF-1 gene and its CA-repeat VNTR studied in this thesis.



In obese adult subjects, IGF-1 levels are negatively associated with BMI, waist-hip-ratio (WHR) and abdominal fat content.[38;39] However, in randomly selected healthy subjects, IGF-1 was not associated with BMI after adjustment for age.[40-51]

IGF-1 gene polymorphisms

The gene encoding IGF-1 (OMIM*147440, NM_000618) is located on the long arm of chromosome 12 (12q22-q24.1). The gene is about 83kb in size and consists of 6 exons. Exon 1 and 2 encode the signal peptide, exon 3 and 4 encode for the active protein and exon 5 and 6 encode the E domain (figure 6). Two functional promoters for IGF-1 have been characterized.

Serum IGF-1 levels, especially at young age, are highly inheritable as shown in several twin studies.[52-55]
Although IGF-1 genetic association studies have been performed widely, results on the functionality of these polymorphisms is limited. Several polymorphisms in the IGF-1 gene have been investigated of which the microsatellite 192bp CA-repeat in the promoter region, located 841 bp before the start of exon 1, is the most extensively

studied. This is also the studied polymorphism in this thesis. The number of CA repeats ranges between 10 and 24, with the most common allele containing 19 CA repeats in the Caucasian population.
Extensive association studies between several polymorphisms (mostly the 192 bp CA-repeat) and diverse outcomes such as IGF-1 levels, (prenatal) growth, birth size, postnatal anthropometrics, cardiovascular disease, diabetes mellitus and malignancies have been performed and reviewed.[56-75]

1.4 Glucocorticoids

Regulation of the hypothalamic-pituitary-adrenal axis
The secretion of glucocorticoids (GCs) is controlled by the hypothalamic-pituitary-adrenal (HPA)-axis by a negative feedback system (figure 7). In response to certain stimuli, like physical or psychological stress and the circadian rhythm, neurons in the paraventricular nucleus of the hypothalamus secrete corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH and AVP stimulate the pituitary to secrete adrenocorticotrophic hormone (ACTH), which is derived from the precursor pro-opiomelanocortin (POMC). ACTH binds to a G-protein coupled receptor on the membrane of cells in the zona fasciculata of the adrenal gland. This leads to the activation of a cascade of enzyme systems involved in the synthesis of steroids, eventually leading to the production of three important groups of steroids: mineralocorticoids, glucocorticoids and androgenic steroids (figure 8). In man the most important glucocorticoid is cortisol. Cortisol exerts a strong negative feedback, both at the level of the hypothalamus and the pituitary, completing the negative feedback loop.[76]
The HPA-axis is influenced by stressful events and a circadian rhythm.[77] During a 24-hour cycle ACTH, and cortisol in response, reaches peak levels prior or at the time of awakening and gradually decline during the day, reaching a nadir during the late evening. The circadian rhythm is not present at birth, but starts to develop from several weeks to 9 months after birth.[78]

The effects of glucocorticoids
Glucocorticoids have effects throughout the whole human body and act on several important systems (figure 9).
Glucocorticoids antagonize the effects of insulin by promoting gluconeogenesis and inducing lipolysis and proteolysis. This leads to increased blood glucose concentrations and subsequently to increased glycogen formation. Glucocorticoids negatively influence body composition by redistribution of body fat, with deposition of adipose tissue on the abdomen and trunk and in the viscera.[79] They also reduce muscle mass,

Figure 7 The hypothalamic-pituitary-adrenal axis. An overview of the regulation of glucocorticoids by the HPA-axis. Under influence of stress and the circadian rhythm, the hypothalamus secretes CRH which stimulates the production of ACTH in the pituitary gland. In response the adrenal gland secretes cortisol with subsequent metabolic effects. Cortisol inhibits its own production by a negative feedback loop at the hypothalamic and pituitary level. The metabolic effects modulate the stress response and the circadian rhythm.

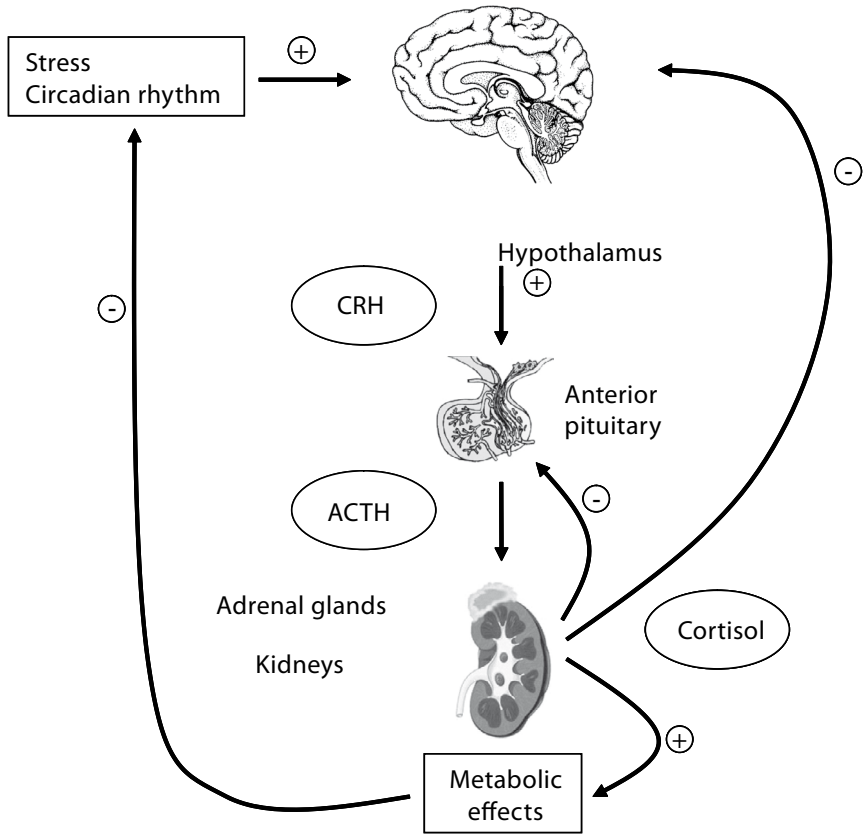
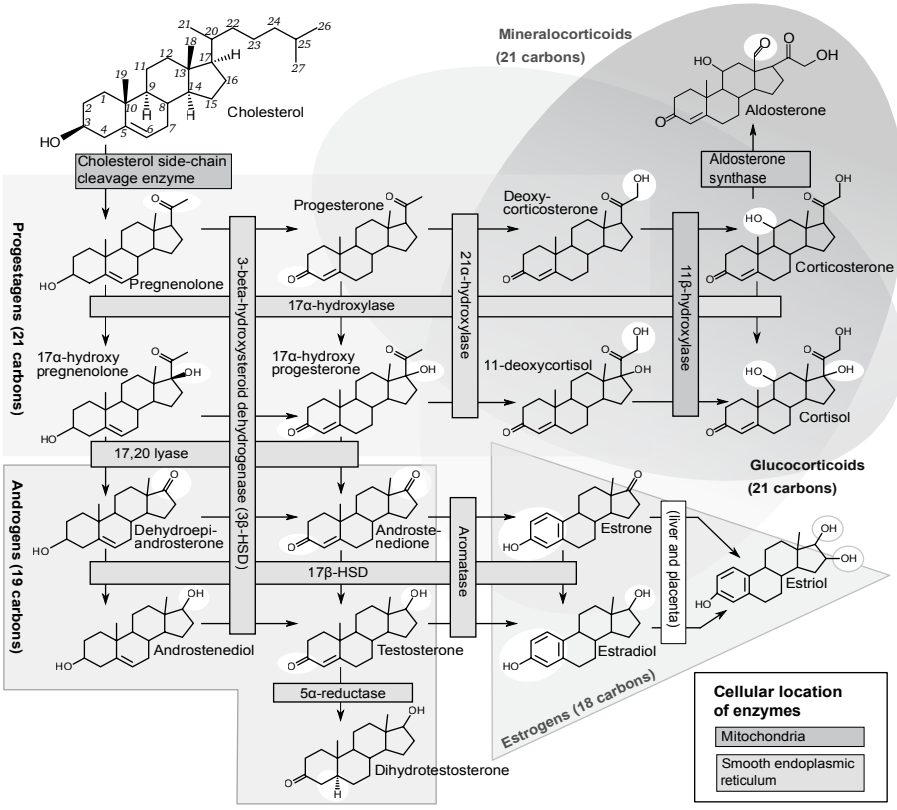


Figure 8 Enzymes, their cellular location, substrates and products in human steroidogenesis.



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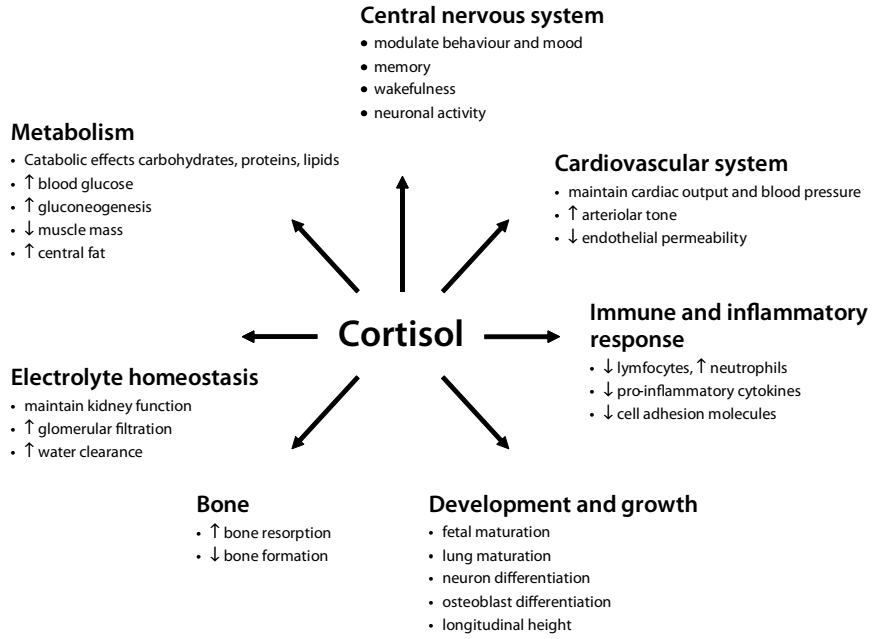
eventually leading to muscle atrophy.[80] Glucocorticoids are involved in embryonic growth and development.[81;82] Exogenous glucocorticoids inhibit fetal growth and result in lower weight at birth.[83] Glucocorticoids also influence the immune and inflammatory systems by decreasing the activity and production of immunomodulatory and inflammatory cells.[84] Furthermore glucocorticoids are important in maintaining cardiac output and vascular tone.[85;86] They influence bone formation and kidney function, and are known to modulate behaviour, mood and memory.[87-89]
Glucocorticoids are widely used as anti-inflammatory and immunosuppressive drugs. Prolonged use of these drugs in supraphysiological doses can lead to insulin resistance,

Figure 9 Overview of the various cortisol actions and physiological effects.



Adapted from thesis E. van de Akker 2008.

diabetes mellitus, hypertension and redistribution of body fat with an increase of visceral fat. They negatively influence bone formation, resulting in osteoporosis and growth retardation in children.[90] Long lasting exposure to glucocorticoids may lead to cerebral damage, especially in the hippocampus.

Glucocorticoid sensitivity

In the normal population a considerable interindividual variation exists in the sensitivity to GCs. Within individuals however, sensitivity to GCs is rather stable.[91;92] These findings indicate that the HPA-axis is set at a stable and reproducible set point for every individual. Among the normal population, the variation in glucocorticoid sensitivity seems to be normally distributed. Individuals in the extremes are relatively hypersensitive or resistant to GCs. Only few studies on these variables in the extremes have been published.[93] The variability in sensitivity is reflected in the wide range of responses to the same relative dose of GCs used in the treatment of various diseases.

While some patients develop severe side effects during treatment with GCs, such as osteoporosis, diabetes mellitus, hypertension, myopathy, impaired wound healing and fat redistribution, others do not develop side effects and/or show no response to treatment at all, even at higher dose.[94;95] These interindividual differences are thought to be the result of minor genetic variations (single nucleotide polymorphisms (SNPs) or other small changes in the DNA sequence) in genes involved in the bioavailability and the sensitivity to the effects of GCs. One of the most investigated and evident factors is the glucocorticoid receptor gene.

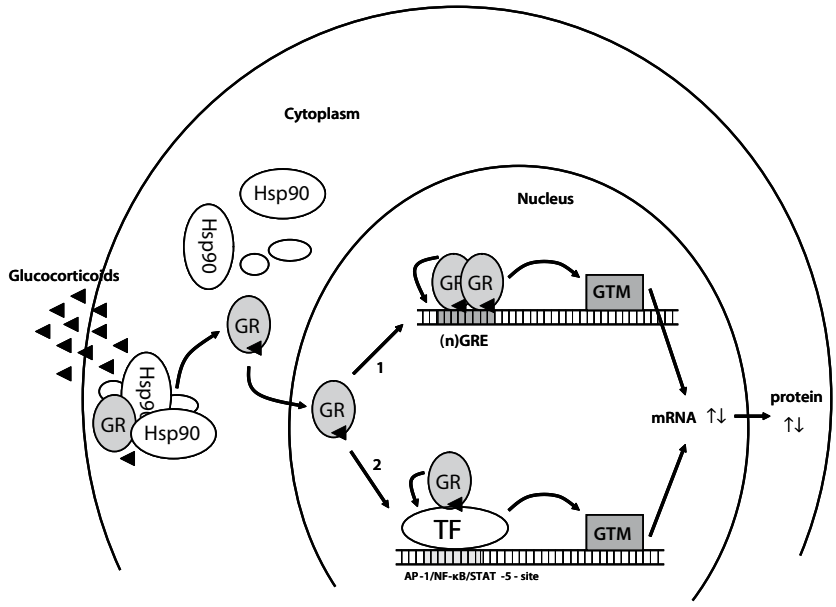
The glucocorticoid receptor

Glucocorticoids cross the cell membrane by passive diffusion because of their lipophilicity. In the cytoplasm, they bind to the GR, which mediates most, if not all, of the GC-induced actions (figure 10). The GR resides predominantly in the cytoplasm in a complex that includes heat-shock protein 90 (hsp90). Upon glucocorticoid binding, this complex dissociates and the GR moves into the nucleus and further regulates transcriptional activity of hormonally responsive genes. Further action is accomplished through three possible mechanisms: 1) interaction with specific DNA motifs, the hormone response elements, which are present in the promoter regions of steroid-responsive genes. In combination with transcriptional co activators further gene activation or gene repression is realised. 2) interaction with other transcription factors like activating protein-1 (AP-1) or nuclear factor-kappa B (NF-κB), leading to repression of their transcriptional activity.[96-99] 3) non-genomic actions, which are mostly faster than genomic effects.[100;101]

The glucocorticoid receptor (NR3C1) is a member of the nuclear receptor family of ligand-activated transcription factors. NR3C1 stands for "nuclear receptor subfamily 3, group C, member 1". Like other nuclear receptors, the GR has a modular structure of three domains: the amino-terminal transactivation domain (TAD), the central DNA-binding domain (DBD) and the carboxy-terminal ligand-binding domain (LBD). The TAD directs transactivation of target genes, the DBD interacts with glucocorticoid response elements in the DNA and the LBD binds to specific steroid and heat shock protein binding sites.[102;103]

The GR gene is located on chromosome 5 (5q31). The gene is about 158 kb in size and consists of 9 different exons (figure 11). Exon 1 and the first part of exon 2 form the 5'-untranslated region (5'-UTR). Several alternative exons 1 were identified and designated as exon 1A, 1B and 1C, which are each preceded by their own promoter.[99;104] Exon 1A contains three separate alternative splice sites, resulting in mRNA transcripts containing exons 1A1, 1A2 or 1A3. The regulation and downstream function of this promoter heterogeneity is still largely unclear, but it is suggested that these differences might indicate that GR expression is cell type specifically regulated.[105]

Figure 10 Simplified model of glucocorticoid receptor mediated transcriptional modulation.



Glucocorticoids enter the cell by passive diffusion. Upon glucocorticoid binding, the GR dissociates from an Hsp90 containing multi-protein complex and translocates to the nucleus. Once there, GR modulates target gene transcription via (1) direct interaction with (negative) glucocorticoid response elements (GRE) as a dimer and (2) cross-talk with other DNA-bound transcription factors such as AP-1, NF-κB. The resulting modulation of target gene transcription leads to altered protein expression. GTM= general transcription machinery; TF= other transcription factors. Reprinted from thesis H. Russcher 2006.

Alternative splicing of the ligand binding domain gives rise to three different 3'-splice variants of the glucocorticoid receptor: GR-α which binds ligand and is functionally active; GR-P, which is thought to activate the function of GR-α; and GR-β, which is a dominant negative inhibitor of GR-α action.[99;106] The regulation of the expression of different splice variants is thought to be important in the regulation of tissue specific glucocorticoid sensitivity.[107;108] Abnormal levels of GR-α, GR-β and GR-P have been found in GC-resistant patients with autoimmune diseases.[109-112]

Glucocorticoid receptor gene polymorphisms

A still increasing number of polymorphisms or SNPs is known in the GR gene. Most of

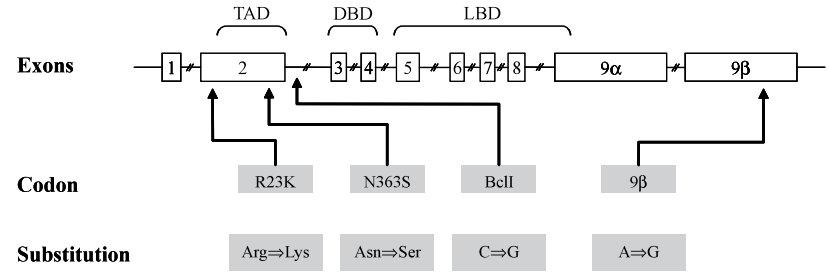
them can be found in the National Center for Biotechnology Information (NCBI) SNP database (http://www.ncbi.nlm.nih.gov/SNP). Only a few of them have been studied and shown to be functionally active. The SNPs described in this thesis have all been associated with changes in glucocorticoid sensitivity. These SNPs are the ER22/23EK, two SNPs which are always linked, the N363S polymorphism in the coding region of the GR, the BclI restriction fragment length polymorphism (RFLP) in intron 2, and the GR-9β polymorphism in the 3'-UTR of exon 9β.[113-115] Three of the four polymorphisms were found to be mutually exclusive, only the ER22/23EK is always found in combination with the 9β SNP, but not the other way around. Therefore haplotypes were constructed regarding the possible allelic combinations of these SNPs (figure 11).

ER22/23EK or R23K (rs6189 and rs6190): This polymorphism contains two SNPs in codons 22 and 23 in exon 2 of the GR gene. Both SNPs are always linked. The first one is silent, changing codon 22 from GAG to GAA, both coding for glutamic acid (E). The second, changing coding 23 from AGG to AAG results in a conservative amino acid change from arginine (R) to lysine (K). The ER22/23EK polymorphism is associated with a relative resistance to GCs as shown by higher cortisol levels after a 1 mg dexamethasone suppression test in carriers of the polymorphism. Carriers also have a healthier cardiovascular and metabolic profile as shown by lower total cholesterol and low-density lipoprotein cholesterol levels, lower fasting insulin concentrations, an increased insulin sensitivity and lower C-reactive protein (CRP) levels. Also other beneficial associations have been found in carriers of this polymorphism, like a better survival in the elderly and a lower risk on dementia and white matter lesions.[113]

The molecular mechanism through which this SNP influences translation is by altering the secondary structure of GR mRNA, forcing translation of the mRNA into protein from codon AUG-1(GR-A) at the expense of initiation from codon AUG-27 (GR-B). The relative GC resistance is caused by the fact that the transactivating capacity of GR-A is lower than that of GR-B. Transrepression seems to be unchanged because the GR-A and GR-B isoforms are equally potent at inhibiting the transactivating activity of NF-κB.[116;117]

N363S (rs6195): This SNP in exon 2 changes codon 363 from AAT to AGT, resulting in a serine (S) for asparagine (N) substitution. This polymorphism has been associated with increased GC sensitivity as shown by lower cortisol levels after a low dose dexamethasone suppression test in carriers of the polymorphism. An increased insulin response to dexamethasone and a tendency towards lower bone mineral density have been observed. An association with increased body mass index and abdominal obesity has been described, although this was not always confirmed in other studies.[118-121] The molecular mechanism through which this SNP exerts its effect is still unclear.

Figure 11 Schematic overview of the glucocorticoid receptor gene and its single nucleotide polymorphisms studied in this thesis. Also shown are the nucleotide and/or amino acid substitutions and the allele frequencies of the haplotypes in our cohorts.



Haplotype	polymorphism	allele frequency AGAHLS	allele frequency BS
1	wildtype G A C A	43.3 %	42.6 %
2	BclI G A (G) A	37.2 %	39.3 %
3	9β G A C (G)	12.1 %	12.4 %
4	N363S G (G) C A	3.7 %	2.7 %
5	R23K+9β (A) A C (G)	3.7 %	3.0 %

***Bcl*I (rs41423247):** This RFLP is a C to G nucleotide substitution in intron 2 of the GR gene. This polymorphism is also associated with increased GC sensitivity as shown by lower cortisol levels after a 1-mg dexamethasone suppression test in carriers of the polymorphism. The SNP has been associated with increased abdominal obesity at middle-aged subjects, while at older age, a lower BMI was found, in combination with a tendency towards lower lean body mass.[113] The molecular mechanism by which this polymorphism exerts its effect remains to be elucidated. Because the SNP is intronic, it might be linked to another SNP in the promoter region of the GR gene, which could result in enhanced expression, or a SNP in the 3'-UTR that could stabilize the mRNA.

9β (rs6198): This polymorphism is an A to G nucleotide substitution in the 3'-UTR of exon 9β. This substitution is located in an "ATTTA" motif, which is changed to "GTTTA". This change is known to lead to an increased expression and a more stable GR-β mRNA in vitro.[114] GR-β is produced by alternative splicing of the GR gene. This β

isoform of the GR resides in the nucleus of cells and does not bind to GCs or activate GC-response genes. It functions as a dominant negative inhibitor of the active GR-α isoform. It is suggested that increased expression and stability of the GR-β variant of the GR leads to a relative GC resistance,[122] although a DST in elderly persons did not show significant differences between carriers and non-carriers of the 9β polymorphism.[123] In this study also no differences were found in BMI, WHR, insulin sensitivity, total cholesterol, LDL-cholesterol and HDL-cholesterol concentrations. Another study though, found an association between the 9β polymorphism and a more favourable lipid profile in men and a decreased WHR in women.[124]

The GR-β isoform is not expressed evenly among all tissues. High levels of the β-variant of the GR are found in immune cells.[125] In carriers of the 9β polymorphism a more active immune system is seen due to reduced Transrepression.[123] In other studies the 9β polymorphism was found to be associated with an increased susceptibility to rheumatoid arthritis, reduced risk for Staphylococcus aureus nasal carriage and an increased cardiovascular risk profile, caused by increased inflammation parameters.[114;126;127] It seems that the effect of the 9β polymorphism on other tissues and cells than immune cells is rather limited.

1.5 Estrogens

Estrogens

The naturally occurring estrogens estrone (E1), 17β-estradiol (E2), and estriol (E3) are derived from cholesterol. The main active form of estrogen is 17β-estradiol which can be synthesized from estrone and testosterone. Other estrogen metabolites are less active and have less affinity for the estrogen receptors. Estradiol is one of the sex hormones responsible for gender dimorphism and reproduction. It is a pleitropic hormone with many biological actions. In puberty it promotes female secondary sex characteristics. Furthermore it promotes bone formation and ends longitudinal growth by closure of the growth plates in males and females. In the absence of estradiol signalling in bone, significant consequences in both men and woman have been described, resulting in osteoporosis as well as continued linear bone growth due to non-closure of the epiphyses.[128] In adulthood, withdrawal of the effects of estradiol at menopause from non-reproductive tissues like the skeleton, the cardiovascular system, and the brain, is a major risk factor for the development of osteoporosis, coronary heart disease, stroke, and possibly neurodegenerative diseases.

The estrogen receptor

Estradiol has some local autocrine or paracrine actions, but the majority of estradiol is released into the circulation and for 98% reversibly bound to sex-hormone-binding

globulin and with less affinity to albumin. Estradiol can freely diffuse across plasma and nuclear membranes, but is sequestered only within cells that contain estrogen receptors (ER). After freely diffusing into the cell, estradiol binds to the ligand-binding domain of the receptor; this complex of estradiol and ER then diffuses into the cell nucleus. These estradiol-ER complexes bind to specific sequences of DNA called estrogen-response elements (ERE) as homo- or heterodimers. These ERE DNA sequences function as enhancers, inducing signal translation to the genes.

Two estrogen receptors are known: ERα (ESR1) and ERβ (ESR2). The development of ERα and ERβ knockout mouse models (αERKO and βERKO) has lead to a better understanding of the different effects of these two receptors in a number of tissues.[129] ERα and ERβ are both very important in the central nervous system, bone, lung, urogenital tract, cardiovascular system, ovary, testis, kidney and colon.

The ERα gene is the most well studied of the two estrogen receptors. The ERα gene is located on chromosome 6q25. It is a large gene, spanning almost 300 kb of which only 1791 base pairs are transcribed to the actual ERα protein. The gene has a large promoter area of 150 kb (figure 12).

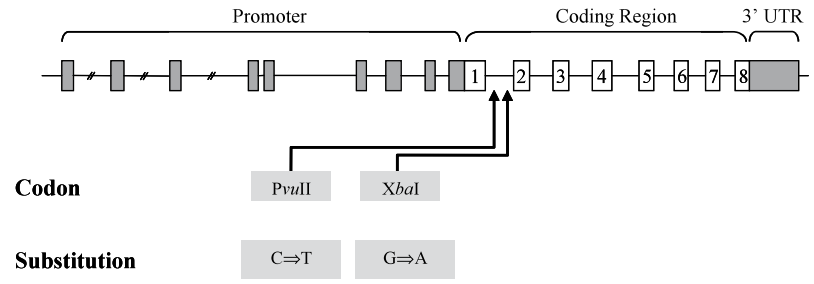
Estrogen receptor gene polymorphisms

The ERα gene contains numerous polymorphisms. Only some of them have been studied and found to be functional. In our studies we focused on two intronic SNP's, identified by the restriction enzymes PvuII and XbaI, located at -397 and -351 from the start of exon2. These polymorphisms have also been described at http://www.ncbi.nlm.nih.gov/projects/SNP under identification numbers rs2234693 (c.454-397T>C) and rs9340799 (c.454-351A>G). These polymorphisms were chosen because they are well known polymorphisms in literature and are common in the general population. They both have been associated with functionality. In most studies both SNP's are analyzed together and haplotypes are constructed to increase genetic resolution.[130;131]

The ERα PvuII-XbaI polymorphisms have been associated with shorter statue, BMD and increased risk of vertebral fractures in postmenopausal women, although findings are inconsistent among different studies.[130;132-140] Furthermore associations have been found with hypertension and ischemic heart disease,[141-143] body mass index (BMI),[144] fat mass,[145]waist circumference[146] and estradiol levels.[147] Studies in children or adolescents on such associations are scarce.[148-150] In general it is suggested that carriers (especially females) of PvuII-XbaI haplotype I are at increased risk of a number of diseases associated with reduced estradiol effects, possibly due to reduced expression of ERα and consequently a decreased end-organ estradiol sensitivity in combination with reduced estradiol levels.

The molecular mechanism through which these SNP's exert their effects is still unclear. Possibly altered ERα expression via altered binding of transcription factors plays a role. This mechanism was supported by findings of Herrington et al., who

Figure 12 Schematic overview of the estrogen receptor α (ERα) gene and its single nucleotide polymorphisms studied in this thesis. Also shown are the nucleotide substitutions and the allele frequencies of the haplotypes in our cohorts.



Haplotype	polymorphism			allele frequency AGAHLS	allele frequency BS
1	px	T	A	51.6 %	52.9 %
2	PX	C	G	36.3 %	35.5 %
3	Px	C	A	11.7 %	11.5 %
4	pX	T	G	0 %	0 %

showed that the T-allele of the PvuII RFLP eliminates a functional binding site for the transcription factor B-myb.[151] This suggests that that presence of this allele may result in lower ERα transcription.

Another plausible mechanism is that one or both of the polymorphisms may influence alternative splicing of the ERα gene, leading to an exon 1-truncated human ERα isoform (46-kDa ERα) with different properties compared to the 66-kDa ERα. This hypothesis still needs to be confirmed.

Still it can not be excluded that the observed associations are driven by linkage LD with another, so far unknown, functional polymorphism.

1.6 Androgens

Androgens

Androgens are derived from cholesterol. The primary and most well-known androgen is testosterone, which is mainly produced in the testis in males. It can be converted to, the more potent, dihydrotestosterone (DHT) by the enzyme 5α-reductase or to estradiol by the enzyme aromatase. Other well known androgens are dehydroepiandrosterone (DHEA), mainly produced in the adrenal cortex, and androstenedione, produced by the testis, adrenal cortex and ovaries (figure 8).

Androgens are steroid hormones that stimulate the development and maintenance of male characteristics by binding to androgen receptors. During gestation androgens are required for external virilisation of the developing male fetus. In puberty androgens are responsible for the development of secondary sexual characteristics, linear growth spurt and several anabolic effects like promotion of muscle growth[152] and inhibition of subcutaneous fat deposition,[153] increase in hematocrit and a fall in plasma high-density lipoprotein (HDL) levels. Also a variety of behavioural and psychological changes, including development of sexual potency and libido take place at puberty.[154;155]

The androgen receptor

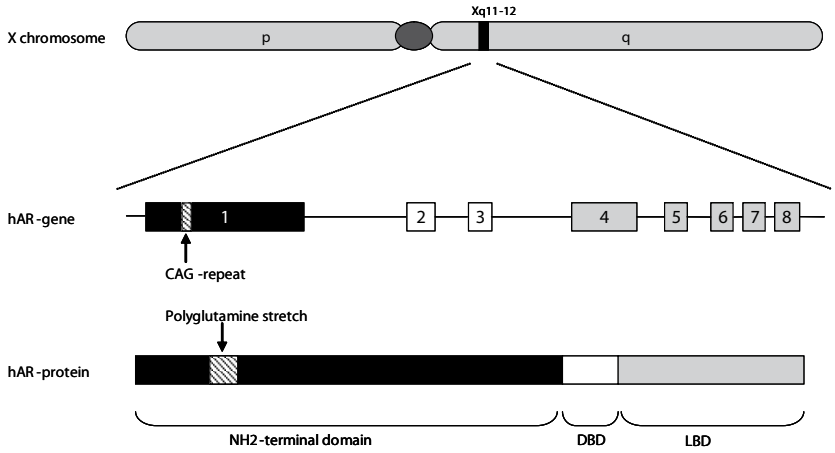
The androgen receptor (AR) belongs to the group of steroid receptors, which is a subgroup of the nuclear receptor family of transcription factors. The AR is essential in the development of the male phenotype. High AR levels are present in the tissues of the male urogenital tract, including the prostate. Low AR expression was found in many other tissues in both males and females, including mammary gland, kidney, liver, brain, genital skin fibroblasts and keratinocytes, hair follicles, cardiac and skeletal muscle, and salivary glands. The androgens, T and DHT are the main AR ligands. In target tissues testosterone is converted into the more active DHT by 5α-reductase.
The liganded AR translocates to the nucleus and binds to regulatory DNA elements in the promoter region of the target genes to influence the transcription rate through interaction with cofactors and transcription machinery.[156] Binding of the androgen-AR complex activates or represses the expression of androgen-regulated proteins.

The AR gene is localized on the X chromosome at Xq11-12. The gene is about 187 kb in size and consists of eight exons. It has, like other members of the steroid receptor superfamily, three main functional domains: the transactivation (TAD), the DNA-binding domain (DBD) and the ligand-binding domain (LBD).[157-159] (figure 13)

Androgen receptor gene polymorphism

The androgen receptor (AR) gene contains a highly polymorphic CAG microsatellite repeat sequence, which normally ranges from 8 to 31 repeats and averages 20 repeats

Figure 13 Schematic overview of the androgen receptor gene and the CAG repeat polymorphism studied in this thesis.



in length. The CAG repeat is located in exon 1 in the TAD and encodes for a polyglutamine chain in the NH2-terminal transcriptional activation domain of the AR gene (figure 13). Triple-repeat DNA sequences are known to be potential sites of genetic instability as is shown for example in the fragile-X syndrome and myotonic dystrophy.

The basal and ligand-induced transactivational activity of the AR is inversely associated with the length of this CAG repeat chain.[160] This modulatory effect on androgen-dependant gene transcription is probably mediated by causing a differential affinity of co activator proteins to the encoded polyglutamine stretch.[161;162] Associations between CAG repeat length and serum testosterone levels have been found, indicating that weaker AR activity is compensated for by higher androgen levels.[163-165]

Associations with phenotypic variations in androgen action have been studied extensively in adults.[166] A lot has been published on the inverse relationship between the repeat length and the androgen-dependent clinical endpoints: for example correlations of the repeat length has been reported with HDL-cholesterol, flow-mediated arterial dilatation, body fat, insulin and leptin levels and male infertility. Inverse correlations have also been reported with acne, male-type balding, sperm counts, BMD, prostate size, serum PSA levels and prostate cancer risk. However, also many reports have been published with equivocal or opposite associations.

Conflicting findings have been reported on the associations of the AR CAG repeat length and body composition. Previous studies have observed that CAG repeat length correlates negatively with BMI[167] or positively with fat free mass.[168] However, also positive correlations of CAG repeat length with BMI or body fat content were found.[169;170] Another study found unfavourable effects of low testosterone levels on body composition among elderly men with longer CAG repeats.[171] A recent study in young men (20 – 29 years) found that AR CAG repeat length correlated negatively with muscle mass of the thigh and lower trunk and total lean body mass, but positively with subcutaneous adipose tissue and total fat mass.[172]

1.7 Aims and outline of the thesis

Aims of the thesis

Longitudinal growth, pubertal development and change in body composition in childhood and adolescence are largely determined by genetic, environmental and hormonal factors. Also intra uterine growth, especially when resulting in a low birth weight, is associated with multiple adverse effects in later life, such as impaired longitudinal growth, altered body composition, increased risk for type 2 diabetes, hypertension, dyslipidaemia and cardiovascular disease. Among the hormonal factors which play a crucial role in growth and body composition are IGF-1, glucocorticoids, estrogens and androgens. Several polymorphisms in genes, encoding for these hormones or their receptors, have previously described associated or proven functional effects, related to the function of these hormones or their related effects, especially in adults. In children and adolescents studies are scarce. In this thesis we aimed to study associations between variations in these genes and parameters of growth and body composition in healthy children and adolescents and children born small for gestational age (SGA). The studies were performed in 2 large, well validated, cohorts of healthy Dutch Caucasian children and adolescents and in a smaller cohort of children born SGA.

The following research questions were formulated:

- Is the 192bp CA-repeat polymorphism in the IGF-1 gene promoter region associated with birth weight or with low birth weight associated outcomes?
- Are glucocorticoid receptor polymorphisms associated with size at birth?
- Is the 192bp CA-repeat polymorphism in the IGF-1 promoter region associated with parameters of body composition in children and adolescents?
- Are there associations between 4 well described glucocorticoid receptor polymorphisms and parameters of body composition and muscle strength in healthy children and adolescents?
- Are 2 well known polymorphisms in the estrogen receptor α gene associated with

growth and body composition in healthy children and adolescents?
- Is the CAG repeat polymorphism in the androgen receptor gene associated with growth and body composition in healthy children and adolescents?

Outline of the thesis

Chapter 1 gives an introduction on the topics described in this thesis

Chapter 2 describes the association of an IGF-1 promoter gene polymorphism with birth weight and certain risk factors for cardiovascular disease and diabetes.

Chapter 3 reports on the associations between glucocorticoid receptor gene polymorphisms and size at birth in a Dutch healthy (reference) cohort. The prevalence of these glucocorticoid receptor gene polymorphisms in a group of children born small for gestational age (SGA), without catch-up growth, is studied and compared with the reference cohort.

Chapter 4 presents the association of an IGF-1 promoter gene polymorphism with parameters of body composition in young healthy subjects in two cohorts of different generations.

Chapter 5 describes the associations of the ER22/23EK polymorphism of the glucocorticoid receptor gene with body composition and muscle strength in young adults.

Chapter 6 describes the associations of several glucocorticoid receptor gene polymorphisms with parameters of body composition in young healthy subjects in two cohorts of different generations.

Chapter 7 reports on the associations of two polymorphisms in the estrogen receptor α gene with parameters of body composition in young healthy subjects in two cohorts of different generations.

Chapter 8 presents a study on the associations of the CAG repeat polymorphism in the androgen receptor gene and longitudinal growth, puberty and body composition in healthy children and adolescents.

Chapter 9 discusses our main findings of this thesis and these findings are placed in perspective.

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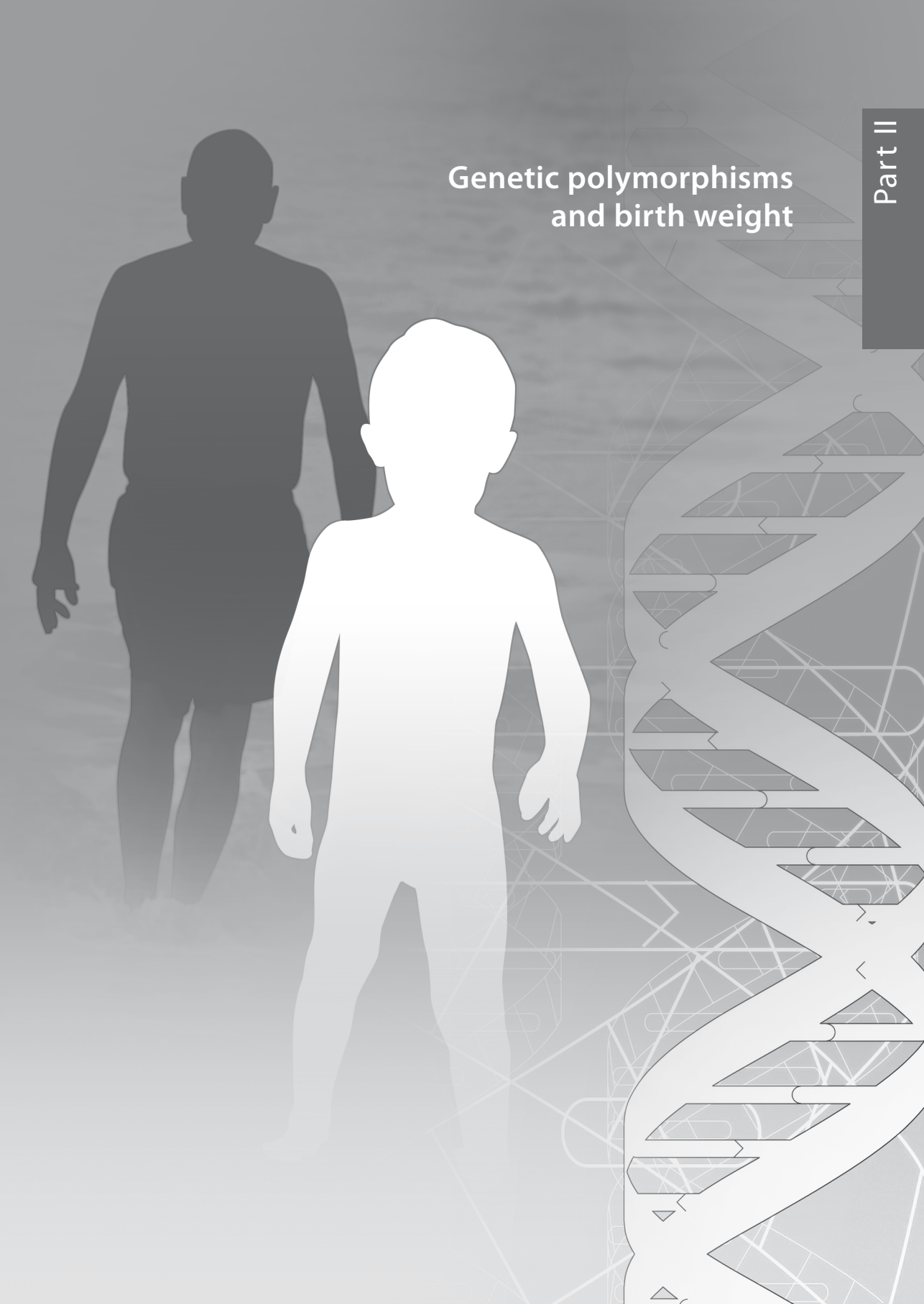
Part II

Genetic polymorphisms and birth weight



2

An IGF-1 promoter polymorphism modifies the relationship between birth weight and risk factors for cardiovascular disease and diabetes at age 36

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Abstract

Objective: To investigate whether IGF-I promoter polymorphism was associated with birth weight and risk factors for cardiovascular disease (CVD) and type 2 diabetes (T2DM), and whether the birth weight - risk factor relationship was the same for each genotype.

Design and participants: 264 subjects (mean age 36 years) had data available on birth weight, IGF-I promoter polymorphism genotype, CVD and T2DM risk factors. Student's t-test and regression analyses were applied to analyse differences in birth weight and differences in the birth weight – risk factors relationship between the genotypes.

Results: Male variant carriers (VCs) of the IGF-I promoter polymorphism had a 0.2 kg lower birth weight than men with the wild type allele (p=0.009). Of the risk factors for CVD and T2DM, solely LDL concentration was associated with the genotype for the polymorphism. Most birth weight – risk factor relationships were stronger in the VC subjects; among others the birth weight – systolic blood pressure relationship: 1 kg lower birth weight was related to an 8.0 mmHg higher systolic blood pressure

Conclusions: The polymorphism in the promoter region of the IGF-I gene is related to birth weight in men only, and to LDL concentration only. Furthermore, the genotype for this polymorphism modified the relationships between birth weight and the risk factors, especially for systolic and diastolic blood pressure.

Background

Insulin-like growth factor-1 (IGF-I) is a peptide that is involved in fetal growth and cell differentiation.[1;2] In addition, it has been suggested that this peptide plays a role in the regulation of glucose homeostasis and cardiovascular function.[3-6] Lower IGF-I levels are also associated with increased levels of serum low-density lipoprotein (LDL).[7] IGF-I also plays a role in plaque development.[8]

A genetic polymorphism comprising a variable length cytosine-adenine (CA) repeat sequence in the promoter region of the *IGF-I gene* has been identified, and is thought to influence the transcription rate of IGF-I, which in turn affects serum IGF-I levels.[9] Since IGF-levels are associated with fetal growth and adult risks for cardiovascular disease (CVD) and type 2 diabetes (T2DM), it has been suggested that the polymorphism in the promoter region of the *IGF-I gene* might be relevant to the fetal origins hypothesis. This hypothesis suggests that an adverse environment during the intra-uterine period negatively affects fetal growth (often estimated by birth weight), and results in adaptations that permanently change the structure and functions of the body, which leads to an increased risk for disease, such as CVD and T2DM, at adult age.[10] An alternative hypothesis is that impaired fetal growth and increased risk for CVD and T2DM share a common genetic factor.[11;12] A study performed in Rotterdam in the Netherlands has recently shown that the absence of the wild type allele (192 base pair (bp)) in the promoter region *IGF-I gene* was related to lower birth weight.[13] In addition, the intra-uterine environment may interact with genetic polymorphisms.[14] This has already been found in other studies, two of which concerned on birth weight and genetic factors in insulin metabolism.[15-17] These data raise the issue of whether or not birth weight also interacts with the *IGF-I gene*.

In the Amsterdam Growth and Health Longitudinal Study (AGAHLS), data on birth weight, and risk factors for CVD and T2DM have been collected. The *IGF-I gene* has now been analysed for the 192 bp polymorphism,[9;13] in order to address the following four research questions: 1) Is the *IGF-I promoter* polymorphism genotype associated with birth weight? 2) Is *IGF-I promoter* polymorphism genotype associated with risk factors for CVD and T2DM? 3) Is birth weight associated with risk factors for CVD and T2DM and finally 4) Do intra-uterine environment and the *IGF-I promoter* polymorphism genotype interact? Or, in other words, is the association between birth weight and risk factors for CVD and T2DM different for each genotype of this polymorphism?

Methods

Participants

The Dutch population described in this study are all participants of the AGAHLS. The AGAHLS is an observational study with repeated measurements, which started in 1976 to monitor boys and girls with a mean age of 13 years.[18] During the most recent measurement in which 433 subjects participated, subjects had than reached a mean age of 36 years, information was obtained concerning birth weight, *IGF-I promoter* polymorphism genotype, glycated hemoglobin (HbA_{1c}), carotid intima-media thickness (IMT), blood pressure, anthropometry and serum low density lipoprotein (LDL) cholesterol levels. Three-hundred-ninety-one subjects completed the birth-weight questionnaire of whom 380 had written information or information from parents. For the purpose of the present study, subjects who were born pre-term (before 37 weeks of gestation, 27 subjects), were one of a twin (11 subjects) or were of non-Caucasian ethnicity (9 subjects) were excluded. Another 69 subjects were not taken into account for the analyses because of errors or missing values in the genotyping or risk factor measurements. Finally, for 264 subjects (152 women) there were complete data sets on the *IGF-I promoter* polymorphism genotype, birth weight, and adult risk factors for CVD and T2DM. Subjects included for the analyses were smaller (1.76m vs. 1.77m, p=0.030) and had less fat-free mass (56.5 kg vs. 57.9 kg, p=0.056) compared to subjects not included for these analyses, but with data on the outcomes available (N = 109). On all other outcome variables the groups did not differ. (Some outcome measures had missing values, which were due to error in the specific measurement.) All subjects were apparently healthy at the time of the measurements, and none had been diagnosed with T2DM. All subjects gave written informed consent and the Medical Ethical Committee of the VU University Medical Center approved the protocol.

Birth weight

Data on birth weight were obtained by means of a questionnaire. The questions concerned birth weight, gestational age, being one of a twin, and ethnicity, and subjects were also asked about the source of the information. Only those who had received the information from their parents or had it in written documents were included, as this has been shown to be a valid method.[19;20] Subjects born preterm (gestational age < 37 weeks) were excluded, this may have independent effects on adult health or influence the relationship between birth weight and adult health.[21-23] Twins were excluded because they have different fetal growth patterns, which might cause error when analysing the relationship between birth weight and adult health outcomes. Subjects who retrieved the birth weight information from their parents' memory (n=112) had slightly higher mean birth weights (3.54 ± 0.53 kg) compared

with the subjects who retrieved the requested information from written documents (n=152; 3.44 ± 0.48 kg), but this difference was not significant (p=0.10). Furthermore, they did not differ significantly on any of the outcome measures.

Polymorphism in the promoter region of the IGF-I gene

IGF-I promoter polymorphism genotypes were determined as described earlier.[9] In brief, DNA was isolated using standard methods. PCR was performed in a final volume of 10 μL containing 10 ng DNA, 10* Gold (Au) buffer (Perkins and Elmer), 200 M dNTP, 30 pmol of each primer, 3 mM MgCl2, 0.5 U Ampli Tag Gold polymerase (Perkins and Elmer). The PCR program concisted of 30 cycli of 30 sec 95 °C, 30 sec 55 °C and 30 sec 72 °C and additionally 5 min of denaturation at 95 °C before the first cycle and an extension of 10 min. at 72 °C after the last cycle. Forward primers were labelled with FAM (Weber & May 1989) to determine the size of the PCR products by fragment analysis (ABI-Prism genetic analyser with Genescan 2.1 software). The Genescan 350/500 Tamra was used as internal size standard within the fragment analysis.

Rietveld et al.[24] recently demonstrated that subjects who were homozygous for the 192 bp or the 194 bp allele had comparable IGF-I blood levels, while individuals who were homozygous for either alleles shorter than 192 bp or longer than 194 bp had significantly lower serum IGF-I levels. Therefore, we decided to regard all subjects who were homozygous for 192 bp or 194 bp, or were carrier of a 192 bp allele and a 194 bp allele as wild types (WTs). Consequently, all subjects who were carrier of a variant allele, which is either shorter than 192 bp or longer than 194 bp, were grouped as variant carriers (VCs).

Risk factors for CVD and T2DM

The following risk factors for CVD and T2DM were measured: body mass index (BMI), waist circumference, waist-to-hip ratio (WHR), total fat mass (FM), total fat-free mass (FFM), carotid intima-media thickness (IMT), systolic and diastolic blood pressure (SBP, DBP), resting heart rate, LDL cholesterol levels and HbA_{1c} as an estimate of glucose metabolism (unfortunately no glucose measures were available). BMI was calculated as body weight (kg) divided by body height (m^2). Standing height was measured with a stadiometer to the nearest 0.001m. Body weight (kg) was measured to the nearest 0.1 kg using a spring balance scale (Van Vucht, Amsterdam, The Netherlands), with subjects dressed only in underwear. Waist (at the level of the umbilicus) and hip circumference were measured with a flexible steel tape to the nearest 0.1 cm. WHR was calculated as the ratio between waist circumference and hip circumference. Fat mass (FM) was estimated from four skinfolds (biceps, triceps, subscapular and supra iliacal) with the Durnin and Womersley equation.[25] The four skinfolds were measured according to standard procedures.[26] FFM was calculated by subtracting FM from body weight.

IMT of the right common carotid artery was obtained by an ultrasound scanner equipped with a 7.5 MHz linear array probe (Pie Medical, Maastricht, The Netherlands), as described elsewhere in more detail.[27-29] SBP and DBP were assessed in the left arm at 5-minute intervals with an oscillometric device (Colin Press-Mate, model BP 8800, Komaki-City, Japan) during the entire period of ultrasound imaging when the subjects were lying in a supine position. The mean value over this entire period was calculated. Resting heart rate was measured with the same device as used for the blood pressure measurement. The mean value over this measurement period was calculated and used in the analyses.

Serum LDL and HbA_{1c} (%) were measured from blood samples (10 ml) taken from the antecubital vein between 8.30 and 12.30 a.m. with subjects in a non-fasting state. Standard methods were used to analyse the LDL concentration and external quality control took place with target samples from a World Health Organisation reference laboratory (Lipid Standardization Laboratory, Atlanta, USA). HbA_{1c} was determined by non-exchange high performance liquid chromatography with a modular Diabetes Monitoring System (Bio-Rad, Veenendaal, the Netherlands).

Data-analyses

A t-test was used to analyse differences in birth weight between the *IGF-I promoter* polymorphism genotypes. Multiple linear regression analyses were applied to study the associations between birth weight and the risk factors for CVD and T2DM. The results of the regression analyses were presented as regression coefficients (β) and the corresponding 95% confidence intervals (CI) for two different models. The first model was a crude analysis, only adjusted for gender (and for SBP and DBP in case of IMT). The second was further adjusted for adult body weight.

To investigate whether the relationship between birth weight and the risk factors for CVD and T2DM were modified by the *IGF-I promoter* polymorphism, multiple linear regression was performed between birth weight and all risk factors (all as continuous variables) for the two genotypes separately.

All analyses were performed with the Statistical Package of Social Science (SPSS, Chicago, USA) version 10.1. The statistical significance was set at p-value ≤ 0.05.

Results

Table 1 gives allele and genotype frequencies in this cohort. As can be seen, of all 264 subjects, 189 (111 women) were WTs for the *IGF-I promoter* polymorphism. The remaining 75 subjects (41 women), 4 were homozygous for alleles with variant CA repeats and 71 were heterozygous for variant alleles. The distribution of genotypes was in Hardy-Weinberg equilibrium (p=0.17).

Table 1 Allelic and genotype frequencies for the bp repeat polymorphism at the promoter region of the IGF-I gene

Allele (bp length)	Frequency N (%)	Genotype		Frequency N (%)
176	3 (0.5)	WT	192/192	105 (39.8)
188	10 (1.9)		192/194	78 (29.5)
190	23 (4.4)		194/194	6 (2.3)
192	341 (64.6)		total	189 (71.6)
194	108 (20.5)	VC	192/x	53 (20.0)
196	38 (7.2)		194/x	18 (6.8)
198	5 (1.0)		x/x	4 (1.5)
			total	75 (28.4)

X, alleles with bp lengths other than 192 or 194; WT, Wild Type; VC, Variant Carrier

Table 2 Characteristics of the adult population of the Amsterdam Growth and Health Longitudinal Study

	Men		Women	
	WT	VC	WT	VC
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Birth weight (kg)	3.64 ± 0.47	3.40 ± 0.39	3.44 ± 0.53	3.41 ± 0.52
Height (m)	1.84 ± 0.08	1.83 ± 0.07	1.71 ± 0.06	1.72 ± 0.06
Weight (kg)	84.1 ± 10.8	85.2 ± 10.8	67.5 ± 9.3	69.2 ± 11.8
Body mass index (kg/m^2)	23.5 ± 2.6	24.5 ± 2.4	22.4 ± 2.9	22.8 ± 3.8
Fat-mass (kg)	13.7 ± 5.0	15.3 ± 5.6	17.7 ± 5.3	18.4 ± 6.4
Fat-free mass (kg)	66.1 ± 6.6	66.3 ± 5.7	47.3 ± 4.5	48.7 ± 5.9
Waist circumference (cm)	85.3 ± 7.5	85.9 ± 8.8	73.3 ± 8.9	73.4 ± 8.0
Waist-to-hip ratio	0.95 ± 0.04	0.96 ± 0.05	0.83 ± 0.08	0.82 ± 0.08
Systolic blood pressure (mmHg)	121.0 ± 8.9	122.7 ± 14.4	110.7 ± 10.6	111.8 ± 12.0
Diastolic blood pressure (mmHg)	66.4 ± 6.4	67.2 ± 8.6	63.1 ± 7.2	61.6 ± 6.9
LDL concentration (mmol/l)	3.22 ± 0.80	3.52 ± 0.87	2.80 ± 0.81	2.97 ± 0.70
Carotid intima-media thickness (mm)	0.628 ± 0.099	0.636 ± 0.095	0.616 ± 0.086	0.631 ± 0.101
Resting heart rate (b/min)	73 ± 12	69 ± 11	72 ± 12	70 ± 12
Glycated hemoglobin (%)	5.3 ± 0.5	5.3 ± 0.3	5.3 ± 0.4	5.3 ± 0.3

Data is presented as means ± standard deviations (SD),
WT, Wild type; VC, Variant carrier; LDL – low density lipoprotein

Table 3 Results of the linear regression analyses for the relationship between birth weight and adult risk factors for CVD and DM-2

Outcome	Crude† β	95%CI		Adjusted ‡ β	95%CI	
Body mass index (kg/m^2)	0.445	[-0.264;	1.154]			
Fat-mass (kg)	0.962	[-0.361;	2.285]	-0.663	[-1.605;	0.208]
Fat-free mass (kg)	2.553***	[1.224;	3.882]	0.714	[-0.085;	1.513]
Waist circumference (cm)	1.760	[-0.372;	3.892]	-1.013	[-2.253;	0.227]
Waist-to-hip ratio	0.012	[-0.005;	0.028]	0.006	[-0.011;	0.022]
Systolic blood pressure (mmHg)	-1.387	[-4.082;	1.309]	-3.050*	[-5.626;	-0.475]
Diastolic blood pressure (mmHg)	-0.692	[-2.445;	1.071]	-1.608	[-3.329;	0.113]
Resting heart rate (b/min)	1.941	[-0.932;	4.815]	1.861	[-1.081;	4.803]
Low density lipoprotein (mmol/l)	-0.079	[-0.277;	0.118]	-0.154	[-0.351;	0.044]
Carotid intima-media thickness (mm) |	0.011	[-0.012;	0.034]	0.011	[-0.012;	0.035]
Glycated hemoglobin (%)	-0.013	[-0.109;	0.082]	-0.025	[-0.123;	0.072]

Data is presented as regression coefficients and corresponding 95% confidence intervals (CI)
CVD – cardiovascular disease; DM-2 – type 2 diabetes mellitus
† Crude, only adjusted for gender; ‡, Adjusted, further adjusted for adult body weight
* $p < 0.05$ *** $p < 0.001$
|also adjusted for systolic and diastolic blood pressure

Population characteristics on all measured variables are presented in Table 2, stratified according to gender and genotype. In men, the mean birth weight was 0.2 kg lower in the VC group than in the WT group (p=0.009). In women, no significant differences in birth weights between the genotypes were observed (p=0.755).

Subjects in the VC group had significantly higher LDL concentrations (p=0.039). No other significant differences between the genotypes were observed.

Table 3 presents the results of the linear regression analyses for the relationship between birth weight and risk factors for CVD and T2DM. It was found that 1 kg higher birth weight was associated with 2.55 kg more FFM. However, this association decreased and lost significance after adjustment for adult body weight. In addition, birth weight was found to be associated with SBP in such a way that 1 kg lower birth weight was related to a 3.05 mmHg higher SBP. No other significant associations were observed between birth weight and risk factors for CVD and T2DM.

Table 4 shows the results of the linear regression analyses between birth weight and the risk factors for CVD and T2DM, stratified according to *IGF-I promoter* polymorphism genotypes. In most of the associations studied, the regression coefficient for birth weight was highest in the VC group, indicating a stronger effect of birth weight on the outcome variable. This difference was most marked in the association with SBP, in which a 1 kg lower birth weight was related to an 8.0 mmHg increase in adult SBP in the VC group, compared to a 1.4 mmHg increase in the WT group. Although, the difference between the VC group and WT group was not statistically significant (p=0.08) for these kind of 'interactions' normally a higher significance level is used. In addition, the relationships between birth weight and DBP and FM were significant in the VC group and not in the WT group (p = 0.06 for the difference between VCs and WTs for DBP). The differences between VCs and WTs regarding all other relationships showed p-values > 0.10.

For the associations between birth weight and adult FFM, waist circumference, LDL and resting heart rate, the regression coefficients were highest in the WT group, although none of these associations were statistically significant.

Table 4 Analyses stratified according to genotype for the relationship between birth weight and risk factors for CVD and DM-2

	Wild type			**Variant carrier**		
Risk factor	β	**95% CI**		β	**95% CI**	
BMI (kg/m^2)	0.475	[-0.312;	1.262]	0.777	[-0.835;	2.338]
Fat mass (kg)	-0.217	[-1.337;	0.904]	-1.929*	[-3.742;	-0.116]
Fat free mass (kg)	0.780	[-0.212;	1.722]	0.473	[-0.897;	1.844]
Waist circumference (cm)	-1.380	[-2.822;	0.061]	0.040	[-2.588;	2.668]
Waist-hip ratio	0.002	[-0.016;	0.021]	0.021	[-0.015;	0.057]
Systolic blood pressure (mmHg)	-1.497	[-4.265;	1.272]	-8.038*	[0.014;	-14.391]
Diastolic blood pressure (mmHg)	-0.599	[-2.553;	1.355]	-5.073**	[-8.845;	-1.301]
LDL cholesterol (mmol/l)	-0.138	[-0.369;	0.093]	-0.076	[-0.491;	0.339]
Resting heart rate (b/min)	2.540	[-0.865;	5.946]	-1.995	[-8.277;	4.286]
Carotid intima-media thickness (mm)‡	0.010	[-0.017;	0.036]	0.020	[-0.035;	0.075]
Glycated hemoglobin (%)	-0.019	[-0.138;	0.101]	-0.025	[-0.207;	0.157]

Data is presented as regression coefficients (β) and their corresponding 95% confidence intervals (CI)
CVD – cardiovascular disease; DM-2 – type 2 diabetes mellitus
Models were adjusted for gender and adult body weight
* $p < 0.05$ ** $p < 0.01$
‡ also adjusted for systolic and diastolic blood pressure

Discussion

In this study, the associations between a polymorphism in promoter region of the *IGF-I gene*, birth weight, (as a measure of intra-uterine growth), and risk factors for CVD and T2DM were investigated, in order to obtain more insight into the genetic aspects of the fetal origins hypothesis.[1;5;6;30;31] The results of the present study demonstrate that men who were carriers of one or two variant allele(s) of the *IGF-I gene* had significantly lower birth weights. However, this trend was not observed in women. It is not clear, why this association was absent in women, as no other study has reported gender differences in the association between *IGF-I* genotype and birth weight.[13;32;33] Therefore, the gender difference observed in this study might be a result of chance.

So far, results on the association between *IGF-I* genotype and birth weight have been conflicting. Vaessen et al. reported that absence of the wild type allele (192 bp) resulted in a lower birth weight, but subjects who were heterozygous for the wild type allele did not differ in birth weight from the homozygous subjects.[13] Nevertheless, Vos et al.,[33] Frayling et al.[32] and Day et al.[34] could not confirm these findings. These conflicting results could be due to differences in the population backgrounds, but also to the way in which subjects were classified per genotype. In the present study, an alternative method was used, in which the allele with 194 bp was also considered as a wild type allele, based on the observations made by Rietveld et al.[24] Subjects previously categorised as VC were now categorised as WT (e.g. subjects with genotype 192 bp/194 bp, or 194 bp/194 bp). Therefore, the VC group in other studies was actually heterogeneous, which may explain discrepant observations. To investigate this possible explanation, we investigated the characteristics of the subjects who would have been categorised as VC according to the traditional classification, and were now categorised as WT (84 subjects). The men in this group had a mean birth weight of 3.63 kg (± 0.51 kg), which is comparable with the men who were categorised as WT in both classification methods (3.64 ± 0.43 kg). The women in this group had a mean birth weight of 3.44 kg (± 0.53 kg), which is exactly the same as the women classified as WT in both methods. Besides this, the three groups were also different with regard to the interaction between birth weight and SBP and DBP in a way that the relationship between birth weight and adult SBP and DBP was strongest and significant in the 'constant' VCs (β = -8.0 for SBP and β = -5.1 for DBP), and weak and not significant in the two other groups. These results suggest that the alternative method used in the present paper discriminates better between the genotypes with regard to the observed health outcomes.

The results concerning risk factors for CVD and T2DM showed an increased risk in the VC group solely for LDL concentrations, all other risk factors did not differ between WT and VC groups. That only one risk factor was significantly different between the groups might be real or a result of chance, since we tested several associations.

Moreover, the relationship between *IGF-I genotype* and LDL concentrations disappeared after adjustment for BMI and there were no differences in HDL concentrations (p=0.255, data not shown). Several studies have shown that lower IGF-I bio-activity is related to higher incidence of atherosclerotic cardiovascular disease, higher carotid IMT values, lower levels of HDL cholesterol, and impaired glucose tolerance.[5;7;35] However, other studies have failed to show these associations.[36-38] Until now, observations have thus been inconsistent, which may be due to other factors that affect cardiovascular health, insulin metabolism and serum IGF-I levels, such as nutrition and endocrine factors. One should, however, realise that the subjects of the present study were still rather young (i.e. 36 years), and that a longer exposure to lower IGF-I bioactivity might be necessary to induce unfavourable levels of risk factors for CVD and/or T2DM.

Another aim of the present study was to investigate whether birth weight was associated with risk factors for CVD and T2DM. This was found to be the case for FFM, however this association decreased after adjustment for body weight. When studying this association within tertiles of BMI, it was found that only in the 2nd tertile the relationship between birth weight and FFM was significant (data not shown). Another significant association was found between birth weight and for SBP, which is in line with others and previously found in the AGAHLS.[39;40] No other significant associations were found, although the associations between birth weight and adult LDL, FM and waist circumference were in the expected (negative) directions.[41-46] No associations were found between birth weight and resting heart rate or carotid IMT. This latter finding is in contrast with what has been reported by Leeson et al.[47] However, their study focussed on an older population.

Birth weight is considered to be mainly dependent on the intra-uterine environment, such as the availability of nutrients and oxygen.[30] In the present population, the *IGF-I* genotype could only explain 6% of the variance in birth weight in men, and only 1% of the variance in birth weight in women. The magnitude of the relationship between birth weight and risk factors for CVD and T2DM, however, seems to be dependent on genes (i.e. *IGF-I* promoter polymorphism) (Table 4). This modification was strongest in the association between birth weight and blood pressure. In the VC group, a 1 kg lower birth weight was found to be related to an 8 mmHg increase in SBP, which is much more than has been reported in the literature (2 to 3 mm Hg).[39] The relationship between birth weight and DBP was also stronger than was expected (as 1 kg lower birth weight was related to a 5 mmHg increase in DBP). No other study has reported on interactions between genes and birth weight in the relationship with adult blood pressure, although IJzerman et al, in twins studies, have shown that the association between birth weight and blood pressure depends on genetic factors.[48;49] Interactions between birth weight and other genes have been reported before, which might suggest that some genotypes are more prone to

adverse circumstances during fetal growth, under the assumption that birth weight is mainly dependent on the intra-uterine environment.[15-17] On the other hand, the observed interactions between birth weight and *IGF-I promoter* polymorphism genotype could be a result of a gene-gene interaction, since birth weight is also determined by genes (other than the *IGF-I gene*).

This study was conducted in 264 subjects only, which is considered few in studies on genetic associations. This might be a reason that we did not find significant associations between genotype and the risk factors. Subjects born pre term were excluded, since gestational age may be another factor associated with risk factors for CVD and T2DM, but with another underlying mechanism.[21] We reanalysed the data including subjects born pre term but fulfilling the other inclusion criteria (N=22), which showed some different β's (birth weight was now significantly associated with FM, waist circumference (unadjusted model) and with DBP pressure (adjusted model)). However, the interaction between birth weight and *IGF-I genotype* was the same, with strong associations between birth weight and SBP and DBP in the VC (β=- 8.40 and β= -5.67, respectively). Furthermore, despite the fact that the AGAHLS is a longitudinal study, no data was available on infant growth, nor reliable data on birth length was available. If so, it was possible to study effects of *IGF-I genotype* on infant growth or interactions with infant growth.

Conclusions

From this study, it is concluded that *IGF-I promoter* polymorphism genotype is related to birth weight in men only, that this genotype is not associated with risk factors for CVD and T2DM, and, most interestingly, that the *IGF-I promoter* polymorphism genotype modifies the relationship between birth weight and risk factors for CVD and T2DM, especially for SBP and DBP.

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3

Glucocorticoid receptor gene polymorphism is less frequent in children born small for gestational age without catch-up growth

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Abstract

Background/aims: Glucocorticoids are important regulators of many processes involved in embryonal growth and development and fat and glucose metabolism. Glucocorticoids exert their effect through the glucocorticoid receptor (GR). The aim of this study was to investigate possible associations between 4 well known glucocorticoid receptor gene haplotypes and size at birth.

Methods: We investigated associations between GR haplotypes and size at birth in a Dutch reference cohort. This reference cohort consisted of 222 young healthy Caucasian subjects. Associations between size at birth and glucocorticoidreceptor gene haplotypes were tested. Furthermore we investigated a group of 119 children born SGA, without catch-up growth. Prevalence of the different GR haplotypes was compared between the SGA-group and the reference cohort.

Results: No associations were found between any of the GR haplotypes and birth weight or birth length in the reference group. The prevalence of GR haplotype2 (Bcl1) was significantly lower in the SGA group compared to controls.

Conclusion: Genetic variance in the glucocorticoid receptor seems not to be associated with intra uterine growth in the general population. However, GR haplotype might play a role in growth of children born SGA, reflected by the decreased prevalence of GR haplotype 2 (Bcl1) in this group.

Introduction

Children, born small for gestational age (SGA), are at increased risk of the metabolic syndrome.[1;2] Fetal programming of the hypothalamic-pituitary-adrenal (HPA)-axis is thought to play a role in this.[3] Prenatal exposure to exogenous glucocorticoids has been associated with low birth weight[4] and altered cardiovascular and metabolic programming.[5] Previous studies have shown that small size at birth is associated with increased fasting plasma cortisol and adrenal responsiveness to ACTH stimulation.[6;7] Glucocorticoids are important regulators of many processes involved in embryonal growth and development and fat and glucose metabolism. Exogenous glucocorticoids inhibit fetal growth and result in lower weight at birth. Human fetal blood cortisol levels are increased in intrauterine growth retardation, implicating a role of endogenous cortisol in retarded fetal growth.[8] High levels of glucocorticoids result in unfavourable cardiovascular risk factors, e.g. visceral obesity, steroid-induced diabetes mellitus and hypercholesterolemia.[9] Glucocorticoids exert their effect through the glucocorticoid receptor which is expressed in most cells of the human body and regulates the expression of multiple genes.[9] The sensitivity to glucocorticoids varies considerably between individuals.[10] Polymorphisms in the glucocorticoid receptor gene are thought to play a role in this and show associations with altered sensitivity.[11] The R23K[11] and GR9β[12;13] have been associated with a relative glucocorticoid resistance while the N363S and Bcl1 have been associated with a relative GC hypersentitivity[11] and with risk factors of the metabolic syndrome such as visceral obesity, hypertension, and insulin resistance.[14]

Recently a GR haplotype, characterized by a polymorphism in exon 9 alpha in combination with the Bcl1 polymorphism, was associated with lower birth weight and length and higher fasting plasma and mean 24-h salivary cortisol.[15] We hypothesized that glucocorticoid receptor haplotypes associated with increased GC sensitivity may lead to increased risk of intra-uterine growth retardation and the metabolic syndrome in later life.

Therefore an association between these polymorphisms and birth weight might be expected in the general population. Also a higher frequency of GR haplotypes associated with increased GC sensitivity might be expected in children born small for gestational age (SGA).

The first aim of this study was to investigate possible associations between the GR haplotypes and size at birth in a young healthy Dutch cohort. The second aim was to compare GR haplotype frequencies between a group of SGA children and controls.

Subjects & methods

Subjects

SGA group

This study included 148 children born small for gestational age (SGA), who were recruited from several medical centers in The Netherlands. Among the recruited children was a large proportion of children born SGA who participated in an ongoing study on the effects of growth hormone treatment in children born SGA. These children did not show any catch-up growth by the time of inclusion in the growth hormone trial. For this reason it was chosen for reasons of uniformity and comparability only to include children born SGA without any signs of catch-up growth (n = 124). Catch-up growth was defined as achieved height SDS for age above –2.0 SDS according to Dutch standards at last visit prior to start of GH therapy.[16] All data used in this study of children participating in the growth hormone study were obtained before the start of growth hormone therapy.

All children fulfilled the following inclusion criteria: 1) birth weight and/or birth height standard deviation score (SDS) below -2,0 for gestational age[17] an uncomplicated neonatal period, without signs of severe asphyxia, sepsis or long-term complications of respiratory ventilation. All children were apparently healthy at the time of recruitment. Children with endocrine or metabolic disorders, chromosomal defects or syndromes were excluded, with the exception of Silver-Russell syndrome. The ethnicity was Caucasian. The study was approved by the Committee of Ethics on Human Research.

Reference cohort

Reference data were drawn from a local cohort, the "Bone Study around Amsterdam in Kids" (Bonestaak) cohort, an observational mixed longitudinal study with repeated measurements. Participants were recruited from a number of primary and secondary schools in the villages around Amsterdam, The Netherlands (n=316: 161 boys and 155 girls). Subjects of non-Caucasian ethnicity were not included. Years of birth varied from 1981 to 1987 for girls and 1983 to 1989 for boys.

The study was approved by the Committee of Ethics on Human Research. Children above 12 years of age and one of the parents gave written informed consent.

Anthropometric measurements

Data on gestational age, birth weight and birth height were mostly obtained using birth data recorded at the national Community Health Service and partly by means of a questionnaire.

Genetic analysis

All subjects were genotyped for 4 glucocorticoid receptor gene polymorphisms as described before.[18-20] The glucocorticoid receptor gene polymorphisms studied were Bcl-1, identified as an intronic C-to-G nucleotide substitution 646 nucleotides downstream from exon 2.[21] The Asn363Ser polymorphism is an asparagine to serine polymorphism in codon 363 of exon 2 (rs 6195 in http://www.ncbi.nlm.nih.gov/SNP), located in the transactivation domain. The R23K polymorphism is a combination of two linked single-nucleotide variations in codons 22 and 23 (nucleotides 198 and 200; rs 6189 and rs 6190, respectively) leading to an arginine-to-lysine change in codon 23 in the transactivation domain. The 9β polymorphism is an A-to-G nucleotide substitution located in the 3' end of exon 9β, which encodes for the 3' UTR of the mRNA of the hGRβ isoform (nucleotide 3669 in X03348; rs 6198). The A to G nucleotide substitution is located in the 'ATTTA' motif (to GTTTA). This 'ATTTA' motif is known to destabilize mRNA and decrease receptor protein expression in vitro.[12] Haplotypes can be reconstructed from the genotypes of these 4 polymorphisms as described before.[19]

Statistical analysis

Data were analyzed using SPSS for Windows, release 11.0.1 (SPSS, Chicago, IL). Genotype was set as independent variable, whereas birth weight and birth weight sds were used as dependent variables. Associations of the variant alleles of the glucocorticoid receptor gene haplotypes with continuous variables were tested by analysis of covariance using the general linear model procedure and with linear regression analysis. Gene-dosage relationship was assumed on the basis of previous findings, especially for haplotype 2.[11] Comparison of the frequencies of the genotypes between SGA-group and controls was carried out using χ^2 test. Because of the low prevalence of homozygotes for most haplotypes, heterozygotes and homozygotes were summarized and defined as carriers before χ^2 test was performed. Statistical significance was set at p-value ≤ 0.05.

Results

For the analyses on the associations between the GR haplotypes and size at birth, 222 children (115 Boys, 107 girls) had complete datasets on birth anthropometric data and all glucocorticoid receptor gene haplotypes. No associations were found between any of the haplotypes and birth weight, birth weight sds, birth length or birth length sds. For example for the relationship between the different GR haplotypes and birth weight SD regression coefficient was for haplotype 2: -0.04 (95% CI, -0.25 to 0.17; P = 0.73), for haplotype 3: -0.25 (95% CI, -0.56 to 0.06; P = 0.11), for haplotype 4: 0.66 (95%

CI, -0.11 to 1.43; P = 0.09) and for haplotype 5: -0.08 (95% CI, -0.81 to 0.65; P = 0.83).

To compare the different haplotypes between the SGA group and the reference group, all children born SGA in the reference group (n = 18) were excluded from further analyses.

For 119 SGA children (66 boys, 53 girls) and 204 controls (109 boys, 95 girls) complete datasets on birth anthropometric data and glucocorticoid receptor gene polymorphisms were available. Baseline characteristics of these groups are shown in table 1. Of the 119 SGA children 9 were classified as having Silver-Russell syndrome. In controls, the distribution of genotypes for all individual glucocorticoid receptor gene polymorphisms was in Hardy-Weinberg equilibrium (p>0.05). Frequencies of the haplotype alleles are presented in table 2. The frequency of homozygotes of haplotype 3, 4 and 5 is very low or zero. Therefore the expected frequency would be too low for Chi Square test. For this reason homozygotes and heterozygotes were defined as carriers of their haplotype and Chi Square test was performed. The frequency of GR haplotype2 (Bcl1) carriers was significantly lower in the SGA group compared to controls (p = 0.0001). Exclusion from analyses of the SGA children having Silver-Russel syndrome did not affect the results significantly.

Table 1 Baseline characteristics of the study population

	Control group			SGA group		
	N	**Mean**	**SD**	**N**	**Mean**	**SD**
Gestational age (weeks)	204	39.5	1.0	119	36.1	3.8
Birth weight (grams)	204	3478	628	119	1643	630
Birth length (cm)	186	50.9	2.2	93	41.2	5.2
Birth weight sds	204	0.2	1.3	119	-3.0	0.8
Birth length sds	186	0.0	1.1	93	-3.8	1.7

Table 2 Results

Genotype, no. of haplotype copies	Reference group		SGA group		
	n	%	n	%	p-value*
Haplotype 2(Bcl1)					
0	61	30	61	51	0.0001
1	112	55	46	39	
2	31	15	12	10	
Haplotype 3 (9β)					
0	157	77	82	69	0.11
1	45	22	34	29	
2	2	1	3	2	
Haplotype 4 (Ans363Ser)					
0	197	97	110	92	0.10
1	7	3	9	8	
2	0	0	0	0	
Haplotype 5 (R23K + 9β)					
0	197	97	112	94	0.30
1	7	3	7	6	
2	0	0	0	0	

* Test for differences in prevalence of carriers of the different haplotypes between reference group and SGA group.

Discussion

In our study we found haplotype 2, which is characterized by the Bcl1 polymorphism, to be less frequent in the group of SGA children compared to controls. Bcl1 has been associated with glucocorticoid hypersensitivity and therefore this finding is in contrast with our a priori hypothesis. A possible explanation could be selection bias, because this group only consisted of SGA children without catch-up growth. Interestingly, the higher risk on metabolic syndrome seems to be associated with catch-up growth.[2] Thus the assumed risk on the metabolic syndrome would be smaller in a short SGA group than in a mixed SGA group or a SGA group with catch-up growth. Possibly non-carriers of the Bcl1 polymorphism in children born SGA are protected from the deleterious effects of being born SGA. It also might be possible that there is an association between this polymorphism and postnatal growth. To answer these

questions a larger group of children born SGA has to be studied, including both children with and without postnatal catch up growth.

In our study we did not find any association between one of the tested glucocorticoid receptor gene haplotypes and size at birth in our reference group. This finding suggests that there is no relationship between this polymorphism and intra uterine growth. Previously Rautanen et al.[15] found an association of a GR haplotype, characterized by a polymorphism in exon 9 alpha in combination with the Bcl1 polymorphism with lower birth weight and length. This haplotype is, however, different from our haplotypes, although it included the Bcl1 polymorphism. We only studied GR haplotypes that have been associated with cortisol sensitivity.

In a recently published study on a birth cohort of 249 premature born children, also no associations were found between birth anthropometry and GR polymorphisms R23K and N363S.[22] This is in line with our findings.

In conclusion, we were not able to find any association between several known polymorphisms in the glucocorticoid receptor gene and size at birth in a young Dutch population. However GR haplotype might play a role in growth of children born SGA, reflected by the decreased prevalence of GR haplotype 2 (Bcl1) in this group. Studies in larger groups of children born SGA, also comparing both children with and without catch-up growth, are needed to further clarify these findings.

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Part III

Genetic polymorphisms in growth and body composition

4

Association between an IGF-I gene polymorphism and body fatness: differences between generations

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Abstract

Objective: A polymorphism near the promoter region of the IGF-I gene has been associated with serum IGF-I levels, body height and birth weight. In this study we investigated whether this polymorphism is associated with body composition in young healthy subjects in two cohorts of different generations.
Design: Observational study with repeated measurements
Methods: The study group consisted of two comparable young Dutch cohorts with a generational difference of around 20 years. The old cohort consisted of 359 subjects born between 1961 – 1965. Measurements were performed from 13 until 36 years of age. The young cohort consisted of 258 subjects born between 1981 – 1989. Measurements were performed from 8 until 14 years of age. Height, BMI, fat mass, fat free mass, waist and hip circumference were compared between wild type carriers and variant type carriers of the IGF-1 polymorphism.
Results: In the young birth cohort body weight, BMI, fat mass and waist circumference were significantly higher in female variant carriers of the IGF-1 polymorphism. In male variant carriers a similar trend was observed. In contrast, in the old birth cohort these differences were not observed. Irrespective of genotype the young cohort showed a significantly higher total fat mass, body weight and BMI compared to the older birth cohort.
Conclusions: Because the found differences between both genotypes were small, it seems likely that the genetic variability due to this IGF-1 polymorphism impacts only slightly on body composition. Importantly, our study suggests that associations between this IGF-1 promoter polymorphism and body composition possibly reflect a gene-environmental interaction of this polymorphism and that an environment that promotes obesity leads to a slightly more pronounced fat accumulation in variant carriers of this IGF-1 polymorphism.

Introduction

Insulin-like growth factor-I (IGF-1) is a peptide that plays an important stimulatory role in skeletal growth, cell differentiation and metabolism. It is also known to influence body composition. [1]

Recently, a polymorphism in the promotor region of the IGF-1 gene has been identified, which is associated with IGF-1 serum levels, birth weight and body height in adults.[2;3] This polymorphism consists of a highly polymorphic microsatellite composed of variable cytosine-adenosine (CA) repeats situated in the promotor region 1 kb upstream from the transcription site of IGF-1. The number of CA repeats ranges between 10 and 24, with the most common allele containing 19 CA repeats in the Caucasian population. There is a functional relationship between this polymorphism and circulating IGF-1 levels. Carriers of the 192 bp allele (19 CA repeats) and/or 194 bp allele (20 CA repeats) of the IGF-1 promoter have higher circulating IGF-1 levels than non-carriers of these alleles.[4] More importantly, non-carriers of the 192 bp allele show an increased risk of type 2 diabetes and myocardial infarction.[2] Although these findings could not always be confirmed by others,[5] these observations may lead to the conclusion that genetically determined low IGF-1 levels might play a role in the pathogenesis of these diseases.

Unfavourable body composition, such as high body mass index (BMI) and raised waist hip ratio (WHR), is also associated with an increased risk to develop type 2 diabetes and cardiovascular disease. Earlier studies, though, did not show a relation between this IGF-1 gene polymorphism and body composition in the elderly.[2]
As we know from recent epidemiological studies the prevalence of overweight and obesity in adults and children is increasing worldwide, especially in the so called Western countries.[6;7] Also in the Netherlands the prevalence of overweight and obesity in children has increased.[8-10] This trend towards obesity seems primarily related to a changed lifestyle, as a result of changed environmental and socio-economic factors and will undoubtly lead to an increased morbidity and mortality at a younger age because of an expected sharp rise in the prevalence of type 2 diabetes mellitus and cardiovascular diseases.[11-16] Besides environmental factors, also genetic factors may play a role. But apart from a few rare mutations leading to extreme obesity, no major genes have been found to be responsible for the increase in prevalence of being overweight or obese.

In order to investigate gene environmental interaction, we examined the relationship between the polymorphism in the promoter region of the IGF-1 gne and body composition in two comparable young Dutch cohorts from differnt birth cohorts with an age differece of approximatey 15 to 25 years.

Subjects and methods

Subjects

The study group consists of two comparable young Dutch cohorts with a generational difference of about 20 years. The first and older cohort is the Amsterdam Growth and Health Longitudinal Study (AGAHLS) cohort, an observational study with repeated measurements.[17;18] Subjects were recruited among several schools in the city of Amsterdam. For the purpose of this study only Caucasian and apparently healthy subjects were included. Measurements were performed at mean ages of 12.9, 14.0, 15.0, 16.0 and 36.0 years of age (table 1). Years of birth varied from 1961 to 1965. On a total of 359 subjects (169 male, 190 female) we have complete data on IGF-1 genotype and anthropometry of at least 1 of 5 time points. The second and younger cohort is the "Bone Study around Amsterdam in Kids" (Bonestaak) cohort. Also in this study only Caucasian and apparently healthy subjects were included. The children were recruited from a number of primary and secondary schools in villages around Amsterdam. Measurements were performed at mean ages of 10.7, 11.2, 11.7 and 12.6 years for the girls and at 11.7, 12.3, 12.7 and 13.6 years for the boys (table 2). Years of birth varied from 1981 to 1987 for the girls and 1983 to 1989 for the boys. On a total of 258 subjects (132 male, 126 female) we have complete data on IGF-1 genotype and anthropometry of at least 1 of 4 time points.

Age distribution differed slightly between both cohorts, but as shown in figure 1, overlap is considerable. Both cohorts were recruited in socio economically comparable areas and schools.

At each visit height, weight and skin fold thickness were measured. Hip circumference and waist circumference were measured at each visit in the Bonestaak cohort, but only at 36 years in the AGAHLS cohort. In the AGAHLS cohort yearly X-rays of the left hand were performed as a measure for bone age development. In the Bonestaak cohort X-ray of the left hand was performed at first visit, and pubertal development (Tanner stages) was measured at each visit. From all participants blood samples were collected for DNA analyses.

The study protocols were approved by the Committee of Ethics on Human Research of the VU University Medical Centre. All subjects and at least one of the parents gave their written informed consent.

Anthropometric measurements

Standing height and body weight were measured using a stadiometer and a calibrated scale respectively, wearing underwear only. Body mass index (BMI) was calculated as body weight divided by body height squared. Waist (at umbilicus height) and hip circumference were measured using a flexible steel tape to the nearest 0.1 cm. Body fat distribution was assessed by the waist-to-hip ratio (WHR). Fat mass

Table 1 Baseline characteristics and anthropometric parameters in the old cohort (AGAHLS) during adolescence, and at the age of 36 years separately for boys and girls in IGF-1 wildtype (WT) and variant carriers (VC).

measurement		1 st				2nd				3rd				4th				36 years			
		Boys		Girls		Boys		Girls		Boys		Girls		Boys		Girls		Men		Women	
		mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
N	WT	117		123		92		113		91		110		88		115		117		133	
	VC	45		50		41		47		41		47		43		46		50		56	
Age (y)	WT	12.9	0.06	12.9	0.05	14.0	0.06	14.0	0.06	15.0	0.06	15.0	0.06	16.0	0.06	16.0	0.05	36.0	0.07	36.1	0.06
	VC	12.9	0.09	13.0	0.08	13.9	0.1	14.0	0.09	14.9	0.09	15.0	0.09	16.0	0.1	16.0	0.09	36.0	0.11	36.1	0.1
Height (cm)	WT	158*	0.7	160	0.7	166	0.9	164	0.6	174**	0.9	167	0.6	178**	0.8	169	0.5	184.3	0.6	170.4	0.5
	VC	157	1.3	161	1.2	164	1.4	165	1.1	172	1.3	169	1.0	176	1.1	170	1.0	182.8	1.0	170.1	0.9
Weight (kg)	WT	42.7	0.5	45.7	0.8	48.8*	0.7	50.3	0.7	55.5	0.8	54.0	0.7	61.1	0.8	56.8	0.7	84.3	1.0	67.6	0.9
	VC	42.5	1.1	46.2	1.1	48.6	1.3	51.2	1.2	55.6	1.4	55.1	1.1	60.7	1.2	57.6	1.0	83.6	1.5	68.6	1.5
BMI (kg/m2)	WT	17.0	0.1	17.8	0.2	17.6	0.1	18.5	0.2	18.3	0.1	19.3	0.2	19.1	0.2	19.9	0.2	24.8	0.3	23.3	0.3
	VC	17.2	0.2	17.8	0.3	17.9	0.3	18.6	0.3	18.7	0.3	19.3	0.3	19.4	0.3	19.9	0.3	25.0	0.4	23.6	0.5
FM (kg)	WT	6.8	0.2	11.2	0.3	7.5**	0.2	12.7	0.4	8.5	0.2	14.4	0.4	9.6	0.2	15.5	0.4	17.2	0.4	19.8	0.5
	VC	7.0	0.4	11.0	0.4	8.0	0.4	12.9	0.5	9.2	0.5	14.5	0.5	10.2	0.5	15.5	0.5	17.6	0.7	20.1	0.8
FFM (kg)	WT	35.9	0.4	34.5	0.5	41.3	0.6	37.5	0.4	46.9	0.7	39.6	0.4	51.5	0.7	41.2	0.4	66.9	0.7	47.7	0.5
	VC	35.5	0.8	35.1	0.7	40.6	1.0	38.3	0.8	46.5	1.0	40.6	0.7	50.5	0.8	42.1	0.7	65.9	0.9	48.9	0.8
Waist circ	WT	ND		ND		ND		ND		ND		ND		ND		ND		85.5	0.7	73.1	0.8
	VC	ND		ND		ND		ND		ND		ND		ND		ND		84.7	1.2	73.1	1.1
Hip circ	WT	ND		ND		ND		ND		ND		ND		ND		ND		89.5	0.7	88.6	0.7
	VC	ND		ND		ND		ND		ND		ND		ND		ND		88.6	1.0	89.2	1.3
WHR	WT	ND		ND		ND		ND		ND		ND		ND		ND		0.96	0.0	0.83	0.01
	VC	ND		ND		ND		ND		ND		ND		ND		ND		0.96	0.01	0.82	0.01

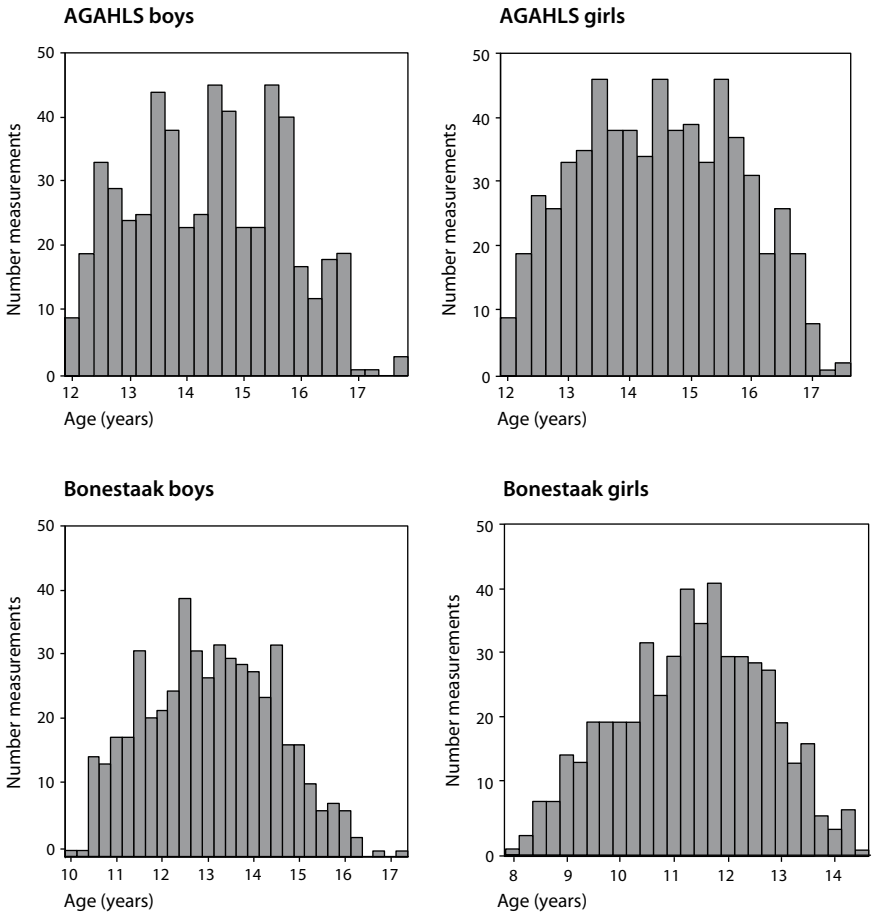
* = p< 0.1, ** = p< 0.05 between genotypes WT and VC. Data are expressed as means ± standard error (SE)
ND = not determined

Table 2 Baseline characteristics and anthropometric parameters in the young cohort (Bonestaak) for boys and girls in IGF-1 wildtype (WT) and variant carriers (VC).

Measurement		1 st				2nd				3rd				4th			
		Boys		Girls		Boys		Girls		Boys		Girls		Boys		Girls	
		mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
N	WT	98		90		98		89		97		89		77		88	
	VC	34		36		33		36		34		36		30		36	
Age (y)	WT	11.7	0.13	10.7	0.12	12.3	0.13	11.2	0.12	12.8	0.13	11.7	0.12	13.6	0.14	12.6	0.12
	VC	11.7	0.22	10.7	0.2	12.2	0.23	11.2	0.2	12.7	0.22	11.7	0.2	13.7	0.23	12.6	0.2
Height (cm)	WT	153	1.0	147	0.9	156	1.1	150	0.9	160*	1.1	153	0.9	165	1.3	159	0.8
	VC	155	1.8	148	1.9	158	1.9	150	1.9	162	2.1	154	1.9	167	2.1	158	1.8
Weight (kg)	WT	40.8	0.8	36.5*	0.8	43.5	0.9	38.7**	0.8	46.2	0.9	41.5*	0.9	51.8*	1.1	46.2**	0.9
	VC	42.9	1.7	38.4	1.4	46.2	1.9	40.7	1.4	49.4	2.0	43.7	1.6	55.4	2.3	48.0	1.7
BMI (kg/m2)	WT	17.4	0.2	16.7**	0.2	17.8	0.2	17.0**	0.2	18.0	0.2	17.5*	0.2	18.9	0.3	18.2*	0.3
	VC	17.7	0.5	17.4	0.4	18.3	0.5	17.8	0.4	18.5	0.5	18.4	0.5	19.6	0.6	18.9	0.4
FM (kg)	WT	8.0	0.4	8.8**	0.4	8.4	0.4	9.4**	0.5	8.8	0.5	10.1**	0.5	9.5	0.6	11.5*	0.5
	VC	8.4	0.8	10.5	0.7	9.0	0.9	10.9	0.7	9.4	0.9	11.6	0.8	10.6	1.3	13.3	0.9
FFM (kg)	WT	32.8	0.6	27.7	0.5	35.0	0.7	29.3	0.6	37.3	0.8	31.5	0.6	42.3*	1.0	34.7	0.6
	VC	34.5	1.3	27.9	0.9	37.2	1.5	29.5	1.0	40.6	1.7	32.1	1.1	44.3	1.9	34.8	1.1
Waist circ	WT	63.7	0.5	60.4**	0.6	65.1	0.5	62.0	0.6	65.8*	0.5	62.7**	0.6	67.7**	0.6	65.3*	0.7
	VC	65.2	1.1	63.0	1.2	66.9	1.2	63.7	0.9	68.2	1.2	64.8	1.0	70.4	1.3	66.8	1.5
Hip circ	WT	70.2*	0.6	72.7	0.7	70.0	0.6	74.1*	0.7	72.0	0.7	77.5	0.7	74.4	0.8	80.2	0.8
	VC	72.8	1.1	74.2	1.2	71.3	1.2	76.1	1.2	74.7	1.5	78.9	1.3	76.6	1.6	81.1	1.6
WHR	WT	0.91	0.005	0.83	0.007	0.93	0.003	0.84	0.004	0.92	0.004	0.81	0.004	0.91	0.005	0.81	0.004
	VC	0.90	0.007	0.85	0.016	0.94	0.006	0.84	0.006	0.91	0.007	0.82	0.007	0.92	0.006	0.82	0.009

* = $p < 0.1$, ** = $p < 0.05$ between genotypes WT and VC. Data are expressed as means ± standard error (SE)

Figure 1 Distribution of numbers of measurements at given ages for the two cohorts



(FM) was estimated from four skin folds (biceps, triceps, subscapular and supra iliacal) using the equation of Durnin and Womersley.[19;20] These four skin folds were measured according to standard procedures.[21] Fat free mass (FFM) was calculated by subtracting the FM from the body weight.

As interindividual variations in skin fold measurements in both cohorts were not measured, it is not possible to give exact data on the comparability of the skin fold measurements between both cohorts. Interaction analysis was performed to determine

differences in the relation between fat mass and height, weight and BMI in both cohorts. These analyses showed significant differences which might indicate measurement variations between the two cohorts, but could also be contributed to differences in body composition between the two cohorts. Within both cohorts skin fold measurements were performed by the same investigators. Because in this study differences between genotypes were analyzed within (and not between) both cohorts, possible measurement variations between both cohorts could not influence associations within both cohorts.

In the Bonestaak cohort pubertal stage was recorded by visual assessment by a trained observer, using breast stages according to Tanner (B1-5) in girls or genital stages (G1-5) in boys.

Plain radiographs of the left hand and wrist in the AGAHLS cohort were made using a double wrapped Osray T-4 (Agfa-Gevaert, Mortsel, Belgium) film, with an exposure time varying from 0.3 – 0.5 s. Skeletal ages according to the Tanner-Whitehouse method (TW II) were evaluated by one well trained examiner.

To test for possible differences in sexual maturation between both cohorts, we compared differences in calendar age and bone age for subjects in both cohorts with overlapping ages. No statistical difference was found. For the Bonestaak cohort mean (calendar age – bone age) ± 1 SD = 0.18 ± 1.1 and for AGAHLS cohort mean (calendar age – bone age) = 0.18 ± 0.90 (p=0.98). This finding is in accordance with our national survey on pubertal development between 1965 and 1997, which showed that the timing of occurrence of the various stages of pubertal development had stabilized between 1980 and 1997. This despite the general trend of increasing overweight and obesity in the same population.[22]

Genetic analysis

Genotypes of the IGF-1 polymorphism were determined as described earlier.[2] In brief, DNA was isolated using standard methods. PCR was performed in a final volume of 10 μL containing 10 ng DNA, 10* Gold buffer (Perkins and Elmer, Life and Analytical Sciences, Boston, MA, USA), 200 M dNTP, 30 pmol of each primer, 3 mM MgCl2, 0.5 U Ampli Tag Gold polymerase (Perkins and Elmer). The PCR program consisted of 30 cycles of 30 sec 95 °C, 30 sec 55 °C and 30 sec 72 °C and additionally 5 min. of denaturation at 95 °C before the first cycle and an extension of 10 min. at 72 °C after the last cycle. Forward primers were labelled with FAM to determine the size of the PCR products by fragment analysis (ABI-Prism genetic analyzer with Genescan 2.1 software, Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). The Genescan 350/500 Tamra was used as internal size standard within the fragment analysis.

In earlier studies carriers of the most frequent 192 bp allele were defined as carriers, whereas all other variants were defined as noncarriers. Recent data have shown that

subjects who were homozygous for the 194 bp allele had comparable IGF-1 blood levels as those who were homozygous for the 192 bp allele.[4] IGF-1 levels were significantly lower in homozygous carriers of alleles with more than 194 bp or less than 192 bp in the IGF1 promoter region. Therefore, different from earlier studies, in this study it is assumed that all subjects who were homozygous for 192bp or 194 bp or were carrier of both the 192bp allele and the 194 bp allele can be regarded as wild type (WT) group. Subsequently, all subjects who were carrier of at least one variant allele (either more than 194 bp or less than 192 bp) are grouped as variant carrier (VC). Recently, this more physiological classification was also used in two other studies.[23;24]

Statistical analysis

Data were analysed using SPSS for Windows, release 10.1 (SPSS, Chicago, IL). Results were reported as mean ± SE. Differences between genotypes were analysed using multiple regression analysis for boys and girls separately. Genotype was set as independent variable, whereas height, weight, BMI, fat mass, fat free mass, waist circumference and hip circumference were used as dependent variables. Adjustments for age, pubertal stage, bone age and weight were made, if appropriate, by putting these variables into different regression models. To investigate the influence of missing values on the statistics, analysis was performed on the complete dataset and on a truncated dataset of subjects without missing data.

In addition, longitudinal analyses were performed for the differences in IGF-1 genotype, using linear generalised estimating equations (GEE).[25] GEE takes into account that the observations within each subject are correlated. By comparing analyses on different datasets it was shown before that GEE analyses behaves very well in datasets with missing data when the outcome is a continuous variable.[26] GEE analyses were performed with the Statistical Package for Interactive Data Analyses.[27] As a result, regression coefficients were estimated, which reflect the linear relationships between the predictor variable (i.e. genotype) and the outcome variables throughout the longitudinal period.

A statistical trend was defined as a p-value ≤ 0.1. Statistical significance was set at p-value ≤ 0.05.

Results

The overall difference in body composition between both cohorts, irrespective of genotype, was studied using regression analyses for the difference in fat mass, body weight and BMI. Results showed significantly higher fat mass, body weight and BMI in the young cohort after adjustment for age and height where appropriate ($p << 0.001$).

Tables 1 and 2 show separately for the old cohort (table 1) and the young cohort (table 2), baseline characteristics and anthropometric parameters in wild type and variant carriers of the IGF-1 polymorphism at the different measurement time points in boys and girls.

Older cohort

In the old cohort (AGAHLS) of totally 359 genotypings we identified 106 (29.5%) variant carriers of the IGF-1 polymorphism (50 males and 56 females) (table 1). The distribution of genotypes was in Hardy-Weinberg equilibrium ($p=0.27$). Figure 2 shows graphically differences in body composition estimates between genotypes at different time points.

In males, we found greater body height in WT carriers at age 15 and 16 ($p<0.05$). At final height these differences in height were not statistically significant any more. In females no statistically significant differences in height were found between genotypes. No significant differences were found between the genotypes in both males and females for weight, BMI, fat mass and fat free mass for nearly all measurements. Only in males at the age of 14 years a significant higher fat mass was found in variant carriers of the IGF-1 polymorphism ($p=0.04$).

Waist circumference, hip circumference and calculated waist hip ratio were measured only at 36 years. No differences were found between genotypes at that age.

Using longitudinal analysis for all measurements, male wild type carriers were 2 cm taller on average than variant carriers ($p=0.047$). For the other parameters in males as well as for all parameters in females no differences were found between both genotypes (data not shown).

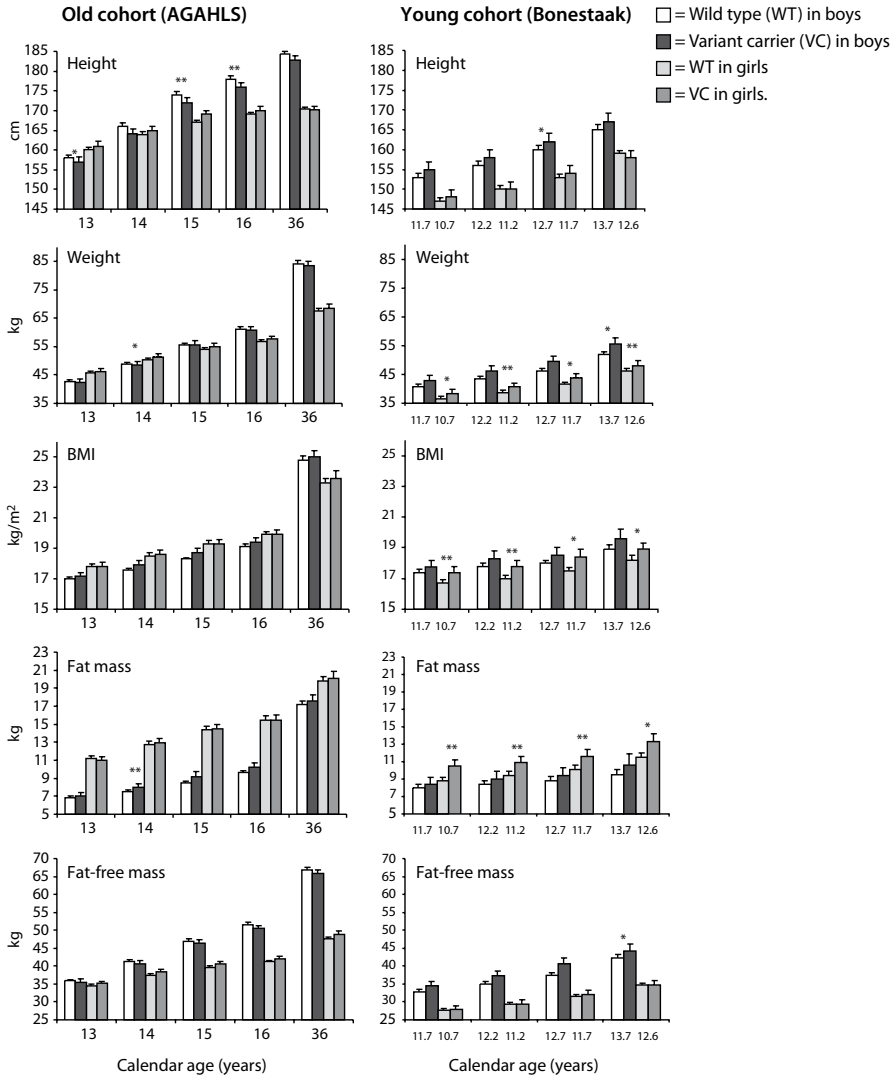
Younger cohort

In the young cohort (Bonestaak) out of a total of 258 genotypes (of participants) we identified 70 (27.1%) variant carriers of the IGF-1 polymorphism (34 males and 36 females) (table 2). Also in this group, distribution of genotypes was in Hardy-Weinberg equilibrium ($p=0.15$). Figure 2 shows graphically differences in body composition estimates between genotypes at different time points.

In males body height tended to be higher in variant carriers of the IGF-1 polymorphism at the age of 13 years ($p=0.096$). At other ages differences in height between genotypes were not significant. In females no differences in height were found. Using longitudinal analysis for all measurements, male variant carriers were on average 4.8 cm taller than wild type carriers ($p=0.013$) after adjustment for age and pubertal stage. For females also in longitudinal analysis no difference in height was found between genotypes.

In females body weight tended to be or was significantly higher in variant carriers at all ages. In males weight only tended to be higher in variant carriers at 14 years.

Figure 2 Left: Body composition parameters in the old (AGAHLS) cohort at young (age 13, 14, 15 and 16 years) and adult age (36 years). Right: Body composition parameters in the young (Bonestaak) cohort (mean age 11.7, 12.2, 12.7, 13.7 years in boys and 10.7, 11.2, 11.7, 12.6 years in girls).



Calculated differences are adjusted for bone age (AGAHLS cohort), pubertal stage (Bonestaak cohort) and age (and height in case of weight, FM and FFM) using regression analyses.

* = p< 0.1, ** = p< 0.05 between genotypes WT and VC. Data are expressed as means ± standard error (SE).

Longitudinal analysis for weight at all time points showed higher body weight in male variant carriers (β=5.06 kg, p=0.026) and female variant carriers (β=2.1 kg, p=0.07) after adjustment for age and pubertal stage.

In addition, BMI tended to be or was significantly higher in female variant carriers at all ages. In males the same pattern was seen, but differences were not significant at any time point. In longitudinal analyses, however, BMI tended to be higher in male variant carriers (β=1.05 kg/m^2, p=0.07) and was significantly higher in female variant carriers (β=0.77kg/m^2, p=0.04).

Also fat mass tended to be or was significantly higher in female variant carriers at all ages. In longitudinal analysis this difference was also statistically significant (β=1.31 kg, p=0.02). In males this was not seen at the different time points, but in longitudinal analyses a trend was seen towards higher fat mass in male variant carriers as well (β=1.84 kg, p=0.09). Fat free mass did not show any differences between genotypes in males and females.

Waist circumference was significantly higher in female variant carriers at first (p=0.024) and third (p=0.045) measurement (mean age 10.7 and 11.7 years). At fourth measurement (mean age 12.6 years) waist circumference tended to be higher in variant carriers (p=0.078). In males waist circumference tended to be higher in variant carriers at third measurement (p=0.08) and was significantly higher at fourth measurement (p=0.04). In longitudinal analysis waist circumference was significantly higher in female variant carriers (β=2.0 cm, p=0.029) and tended to be higher in male variant carriers (β=1.54 cm, p=0.077).

Hip circumference tended to be higher in male variant carriers at first measurement (p=0.065) and in female variant carriers at second measurement (p=0.067). In longitudinal analysis a tendency towards greater hip circumference was only found in female variant carriers (β=1.6 cm, p=0.051).

No differences in waist hip ratios were found between genotypes at any time point or in longitudinal analysis.

Analyses of the truncated dataset, only including subjects who had complete data (n = 230) on all measurements, showed weaker results but the same trends were found (data not shown). That the results were less strong was to be expected due to loss of data and thus loss of statistical power.

Discussion

In this population-based cohort study, the associations between a polymorphism in the promoter region of the IGF-1 gene and parameters of body composition and height were investigated in two young different birth cohorts of comparable origin and age. Important difference between both cohorts was a generational difference of about twenty years.

In the young "Bonestaak" cohort body weight, BMI, fat mass, waist circumference and hip circumference were higher in female variant carriers of the IGF-1 polymorphism. In boys, a similar trend towards higher weight, BMI, fat mass and waist circumference was also found in carriers of the variant IGF1 genotype. It seems unlikely that these differences between both genotypes are significant by chance because all tested parameters (BMI, weight, fat mass, waist and hip circumference) represent more or less the same properties. The found differences all point to the same direction in the whole cohort, at all time points for both sexes. If significance was only reached by the performed number of tests, this would not be the case. These findings were also confirmed by longitudinal analyses.

The differences in body composition between genotypes were not observed in the older "AGAHLS" cohort. Differences in cohort size did not appear to be the explanation since the group size of the AGAHLS cohort was larger than the young cohort. The only clear difference between genotypes in this cohort was found in male wild type carriers of the IGF-1 polymorphism who had higher standing height than variant carriers, which is in accordance with earlier findings in adults.[2] This, in contrast to male wild type carriers in the young "Bonestaak" cohort who were shorter than variant carriers. In addition, variant carriers in this cohort had a higher weight, which is not found in the old cohort. In childhood, it is known that an increase in fat mass or BMI is associated with an increased height gain.[28] Therefore, the finding of contrasting relationships between body height and genotype in the two separate cohorts can possibly be explained by the observation that in the young cohort fat mass was higher in variant carriers of the IGF-1 polymorphism, which may diminish the influence of the polymorphism specifically on growth.

As both groups are comparable concerning socio-economic, geographic, and genetic background, some other environmental factor might be responsible for the difference in body composition between both IGF-1 genotypes in both study cohorts. As is known from recent epidemiological studies the prevalence of overweight and obesity in adults and children is increasing worldwide, especially in the so called Western countries,[6;7] we studied the overall difference in body composition between both cohorts. Regression analyses of the difference in fat mass, body weight and BMI between both cohorts showed significantly higher fat mass, body weight and BMI in

the young cohort after adjustment for age and height where appropriate. Therefore it could be argued that, in contrast to the old cohort, the presence of an association between IGF-1 genotype and body composition in the younger, and fatter cohort, reflects a possible gene-environmental interaction of this polymorphism. Possibly an environment that promotes obesity leads to a more pronounced fat accumulation in variant carriers of this IGF-1 polymorphism.

As is known from earlier studies, carriers of the variant IGF-1 promoter have lower IGF-1 levels than wild type carriers.[4] Although the beneficial effects of growth hormone on lean body mass and body fat are known quite well, conflicting results on the direct influence of IGF-1 on body composition have been reported.[29-35] Although IGF-1 has no direct effects on adipocytes because of the lack of functional receptors in adipose tissue, it is known to enhance lipolysis by reducing insulin levels and thus releasing the brakes on lipolysis, which on theoretical grounds could lead to less fat accumulation in wild type carriers of this IGF-1 polymorphism.[36-40] Earlier studies on the same IGF-1 polymorphism in elderly did not show differences in BMI and waist hip ratio between both genotypes.[2;41;42] These studies differed from our study in the slightly different way that wild type carriage and non-carriage of the IGF1 polymorphism were defined, as well as the age of the study groups. Subjects in other studies were all between 55 and 75 years of age, a cohort from an even older generation than our AGAHLS-cohort, who certainly did not live in an environment that promotes obesity like today' s children. Since Vaessen et al.[2] found an increased risk for type 2 diabetes and myocardial infarction in non carriers of the 192 bp allele in these old cohorts, one could speculate that there is an increased risk on an earlier development of type 2 diabetes and cardiovascular disease for young individuals living in the present overweight promoting environment and being carrier of this polymorphism

In conclusion, we found that carriage of a genetic polymorphism in the promoter region of the IGF-1 gene is associated with higher body weight, BMI, fat mass and waist circumference in young subjects. Because the found differences between both genotypes were small, it seems likely that the genetic variability due to this IGF-1 polymorphism impacts only slightly on body composition in this cohort. These associations were not found in comparable young, but generally leaner, subjects of an older generation. We hypothesize that these differences possibly reflect a gene-environmental interaction of this polymorphism and that an environment that promotes obesity leads to a more pronounced fat accumulation in carriers of this IGF-1 polymorphism. To test this hypothesis, future studies are needed in even younger or more obese cohorts.

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5

The ER22/23EK polymorphism in the glucocorticoid receptor gene is associated with a beneficial body composition and muscle strength in young adults

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Abstract

Glucocorticoids play an important role in determining body composition. A polymorphism of the glucocorticoid receptor gene (in codons 22 and 23) has previously been found to be associated with relative glucocorticoid resistance, low cholesterol levels, and increased insulin sensitivity. In this study we investigated whether this ER22/23EK polymorphism is associated with differences in body composition and muscle strength. We studied a cohort of 350 subjects who were followed from age 13 until 36 years. We compared noncarriers and carriers of the ER22/23EK variant in anthropometric parameters, body composition, and muscle strength, as measured by arm pull tests and high jump from standing. We identified 27 (8.0 %) heterozygous ER22/23EK-carriers. In males at 36 yrs of age, we found that ER22/23EK-carriers were taller, had more lean body mass, greater thigh circumference, and more muscle strength in arms and legs. We observed no differences in body mass index (BMI) or fat mass. In females, waist and hip circumferences tended to be smaller in ER22/23EK-carriers at the age of 36, but no differences in BMI were found. Thus, the ER22/23EK polymorphism is associated with a sex-specific, beneficial body composition at young adult age, as well as greater muscle strength in males.

Introduction

Glucocorticoids are important regulators in numerous tissues throughout the human body and they also influence body composition. Their effects are mainly mediated by the glucocorticoid receptor (GR), a ligand-activated transcription factor.[1] Thus, changes in the gene coding for this receptor can play an important role in determining glucocorticoid sensitivity.[2] Within the normal population, several polymorphisms in the *GR* gene have been described.[3] One of these polymorphisms - N363S - was shown to be associated with an increased sensitivity to glucocorticoids and a higher body mass index,[4] as well as central obesity in males.[5] Lin et al.[6] confirmed this finding of higher BMI in N363S carriers and showed an allele-dosage effect of this polymorphism. In contrast, several other studies showed no effect on BMI.[7;8] A *Bcl*I polymorphism has previously been shown to be associated with a relative hypersensitivity to glucocorticoids in vivo,[9] an increased cortisol response to a standardized lunch, and abdominal obesity in middle-aged subjects.[10]

Previously, we identified another polymorphism that consists of two linked point mutations in codons 22 and 23 of the *GR* gene (GAG AGG→ GAA AAG). The first mutation in codon 22 is silent, both GAG and GAA coding for glutamic acid (E). The second mutation changes codon 23 from AGG to AAG, resulting in an amino acid change from arginine (R) to lysine (K).[3] This polymorphism was associated with a relative resistance to glucocorticoids.[11] We also showed in a population-based study in the elderly that carriers of this ER22/23EK polymorphism had a better insulin sensitivity and lower total and low-density lipoprotein cholesterol levels.[11] In addition, we found the frequency of the 22/23EK allele to be higher in the elder half of the studied population, which suggests a survival advantage. In order to investigate whether the ER22/23EK variant is indeed associated with survival we studied a separate population of 402 elderly Dutch men.[12] After a follow-up of 4 years we found that 19.2 % of the non-carriers had died, while none of the ER22/23EK-carriers (n=21) had died, which was a statistically significant difference. In this same population we also found ER22/23EK-carriers to have lower C-reactive protein (CRP) levels, which in turn were also associated with a better survival. These lower CRP levels in ER22/23EK-carriers possibly reflect a beneficial cardiovascular status.[12]

A well-known effect of glucocorticoids is to negatively influence body composition, including redistribution of body fat with deposition of adipose tissue on the abdomen and trunk, and muscle atrophy.[13] It is known that body composition plays an important role in lipid metabolism and insulin sensitivity, and as a consequence influences the risk on cardiovascular disease.[14] At present, it is not known what the effects of this ER22/23EK polymorphism are at a young age or whether there are any effects on body composition.

Therefore, in the present study we investigated a cohort of 350 subjects who were followed from 13 until 36 years, and studied whether there is an association between the ER22/23EK polymorphism of the *GR* gene and body composition during puberty and at young adult age.

Subjects and Methods

Subjects

350 healthy participants were drawn from the Amsterdam Growth and Health Longitudinal Study (AGAHLS), a population-based observational study with repeated measurements at the age of 13, 14, 15, 16, 27, 29, 32 and 36 years.[15] Subjects of non-Caucasian race were excluded from the analyses (5 males and 5 females, all noncarriers of the ER22/23EK polymorphism). On a total of 337 (158 males) we had complete data on GR genotype and adult anthropometry. Data were not complete at all measurements in puberty: a total of 332 subjects (26 ER22/23EK-carriers) participated at age 13, 290 (22 ER22/23EK-carriers) at age 14, 286 (21 ER22/23EK-carriers) at age 15, and 287 (19 ER22/23EK-carriers) at age 16 years. All subjects gave their written informed consent to participate in the study, which received the approval of the Medical Ethical Committee of the "Vrije Universiteit" of Amsterdam.

Anthropometric measurements

Body weight (kg) was measured to the nearest 0.1 kg using a spring balance scale (Van Vucht, Amsterdam, the Netherlands), with subjects dressed only in underwear. Standing height was measured with a stadiometer to the nearest 0.001 m. Body mass index (BMI) was calculated as body weight divided by body height squared. To assess fat distribution (abdominal versus gluteo-femoral), we measured waist (at the umbilicus) and hip circumference with a flexible steel tape to the nearest 1 mm and the waist-to-hip ratio (WHR) was calculated. Fat mass (FM) was estimated from four skinfold thickness measurements (biceps, triceps, subscapular and supra iliacal) by the equation of Durnin et al.[16-18] Lean body mass was measured by dual X-ray absorptiometry (DEXA), with the Hologic QDR-2000 (S/N 2513; Hologic, Inc., Waltham, MA, USA). Calf and thigh circumferences were measured with a steel tape to the nearest 0.1 cm.

Muscle strength

Was assessed by two physical fitness tests from the MOPER fitness test battery.[19;20] The first was the static arm pull test: the subjects were given two attempts to pull maximally with their arm of preference, the strength of which was measured (in kg) with a dynamometer (Bettendorf, Brussels, Belgium), fixed to the wall at a horizontal

level. The higher score of the two was recorded. The second test was the standing high jump. The subjects had two attempts to jump as high as possible (higher value recorded) from a platform, having been allowed only to bend their knees before jumping. The height they jumped (in cm) was measured by a tape, which was fixed to a belt around the subjects' waist to the platform on the ground.

Physical activity

A structured interview based on a physical activity questionnaire (PAQ) was used to investigate the amount of physical activity. The questionnaire comprises questions about duration, frequency and metabolic equivalent (MET) intensity of all physical activities during the last three months preceding the interview. From this information a total weighted activity score (METs/week) was calculated.[21;22]

Genetic analysis

Restriction fragment length polymorphism analysis was carried out to determine GR genotypes. DNA was extracted from peripheral blood leukocytes by standard techniques. PCR amplification of the *GR* gene was carried out employing primer sequences and amplification conditions as described previously.[3] The PCR-products were digested with 1 U *Mnl*I (PerkinElmer Life and Analytical Sciences, Boston, MA) at 37 ºC for 1 hour. *Mnl*I cleaves at 5'-CCTC(N)7-'3 and at 3'-GGAG(N)6-'5. Fragments were visualized with ethidium bromide on a 3% agarose -gel (Roche Diagnostics, Mannheim, Germany). We re-analysed all heterozygous and 10 wild type samples and found identical genotypes.

Statistical analysis

Data were analyzed by SPSS for Windows, release 10.1 (SPSS, Chicago, IL). Differences in means between the ER22/23EK-carriers and the noncarriers were adjusted for height if appropriate and tested by ANCOVA using the general linear model procedure. High jump scores were corrected for body weight. Results are reported as mean ± SE. *P* values are two-sided throughout, and $p \leq 0.05$ was considered to indicate a significant difference.

Results

Anthropometric parameters at young adult age

In the group of 337 participants we identified 27 (8 %) carriers of the ER22/23EK polymorphism (16 males and 11 females). Table 1 shows anthropometric parameters determined in noncarriers and carriers of the ER22/23EK polymorphism at the last measurement (at the age of 36 years).

Table 1 Mean and standard error (SE) of anthropometric parameters, muscle strength and MET activity score in non-carriers of both sexes, and in male (n= 16) and female (n=11) ER22/23EK-carriers at the age of 36 years

	Men (n= 158)					Women (n= 179)				
	non-carriers		ER22/23EK-carriers			non-carriers		ER22/23EK-carriers		
	Mean	SE	Mean	SE	P	Mean	SE	Mean	SE	P
Height (m)	1.83	0.01	1.87	0.02	**0.05**	1.71	0.01	1.70	0.02	0.48
Weight (kg)	83.5	0.9	89.8	3.1	0.14	68.5	0.8	63.2	1.8	0.13
BMI (kg/m2)	24.8	0.2	25.7	0.8	0.20	23.5	0.3	22.0	0.8	0.18
% total fat	21.5	0.5	21.1	2.0	0.87	32.3	0.6	30.6	2.1	0.36
Total fat mass (kg)	18.1	0.6	19.5	2.4	0.66	22.1	0.6	19.4	1.7	0.26
Total lean mass (kg)	61.4	0.5	66.2	1.5	**0.02**	42.7	0.4	41.0	1.2	0.38
Thigh circumf (cm)	57.4	0.4	60.4	1.1	**0.03**	57.2	0.4	55.7	1.0	0.38
Calf circumf (cm)	37.8	0.2	38.7	0.9	0.31	36.1	0.2	35.6	0.6	0.56
Upper arm circumf (cm)	30.5	0.2	31.2	7.3	0.45	27.6	0.2	26.3	0.7	0.15
Waist circumf (cm)	85.0	0.7	87.5	2.1	0.43	73.5	0.7	68.6	1.3	**0.07**
Hip circumf (cm)	89.0	0.6	91.9	1.8	0.31	89.3	0.7	84.7	2.2	**0.09**
Arm pull (kg)	70.9	1.1	77.8	3.9	**0.06**	38.8	0.6	36.2	2.2	0.32
High jump (cm)	51.9	0.6	55.7	1.8	**0.04**	38.7	0.4	40.1	2.5	0.64
MET-score (mets/week)	4243	267	4678	796	0.60	5316	297	6119	1733	0.49

Test for the difference between non-carriers and ER22/23EK-carriers. All parameters, except the arm pull strength, high jump from standing, and MET score, were corrected for height. High jump scores were adjusted for body weight. SE, Standard Error of the mean. BMI, Body Mass Index. Circumf, circumference, MET. Metabolic Equivalent.

In males, we found a greater body height in ER22/23EK-carriers (p=0.05), as well as a higher body weight (p=0.03). However, the latter was not significant after adjustment for height (p=0.14). BMI was not different between the genotypes. Total lean mass was significantly higher in ER22/23EK-carriers compared with noncarriers (respectively 66.2 ± 1.5 and 61.4 ± 0.5 kg, p=0.006, after additional correction for height p=0.02). The circumference of the thigh was also greater in ER22/23EK-carriers (ER22/23EK: 60.4 ± 1.1 and noncarriers: 57.4 ± 0.4 cm, p=0.03), while no differences were found in total fat mass or percentage fat.

Table 1 also shows anthropometric parameters in female noncarriers and ER22/23EK-carriers at the age of 36 years. In females, body weight tended to be lower in ER22/23EK-carriers (63.2 ± 1.8 and noncarriers: 68.5 ± 0.8 kg), although this was not statistically significant after adjustment for height (p=0.13). BMI was also not significantly different between the two genotypes (ER22/23EK: 22.0 ± 0.8 and noncarriers: 23.5 ± 0.3, p=0.18). Waist and hip circumferences tended both to be lower in female ER22/23EK-carriers compared to noncarriers (p=0.07 and p=0.09, respectively, table 1). No differences were found in height, fat mass and lean body mass, or circumferences of the thigh, calf and upper arm.

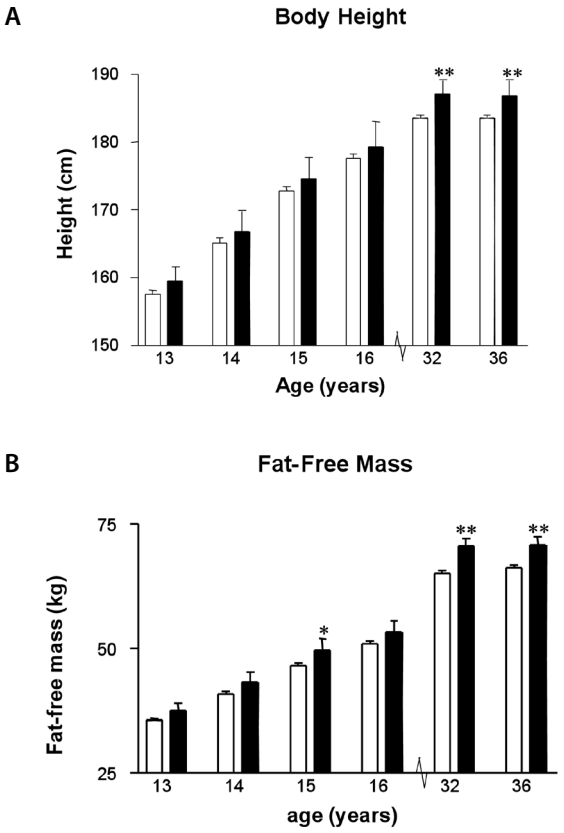
At the age of 32 years we found similar results (not shown in table). Male ER22/23EK-carriers had a greater body height (p=0.035, figure 1A), higher lean body mass (p=0.02, figure 1B), and higher weight (p=0.006, after adjustment for height (p=0.08)), while total fat mass was not different (p=0.12).

In females, we found a tendency towards a smaller waist circumference in female ER22/23EK-carriers (ER22/23EK: 67.5 ± 1.4 and noncarriers: 71.1 ± 0.5, p=0.08). No differences in hip circumference (ER22/23EK: 87.2 ± 2.1 and noncarriers: 90.1 ± 0.7, p=0.27) or in height, weight, BMI, body composition and muscle strength were observed at the age of 32.

Anthropometric parameters in puberty

During puberty we also measured anthropometric variables, body composition and muscle strength in the same subjects. Figure 1A shows the height of male noncarriers and carriers of the ER22/23EK polymorphism during puberty. Although the pattern of greater height in male ER22/23EK-carriers is similar to that at adult age, these differences were not statistically significant. The same applied to the amount of lean mass in males: no significant differences during puberty between genotypes, although a similar pattern as at adult age (higher lean mass in male ER22/23EK-carriers) could be observed (Figure 1B). At the age of 15, we found tendencies towards higher body weight (p=0.10), BMI (p=0.06), and lean mass (0.09) in ER22/23EK-carriers. On average, male noncarriers grew an additional 5.9 cm after the age of 16, while ER22/23EK-carriers grew 7.8 cm till they reached their final height, however this was not a significant difference. In males, no differences were found in other anthropometric

Figure 1 Height (A) and Fat free mass (B) in male noncarriers (white bars) and carriers of the ER22/23EK polymorphism (black bars) during puberty (age of 13, 14, 15, and 16 years) and adult age (32 and 36 years).



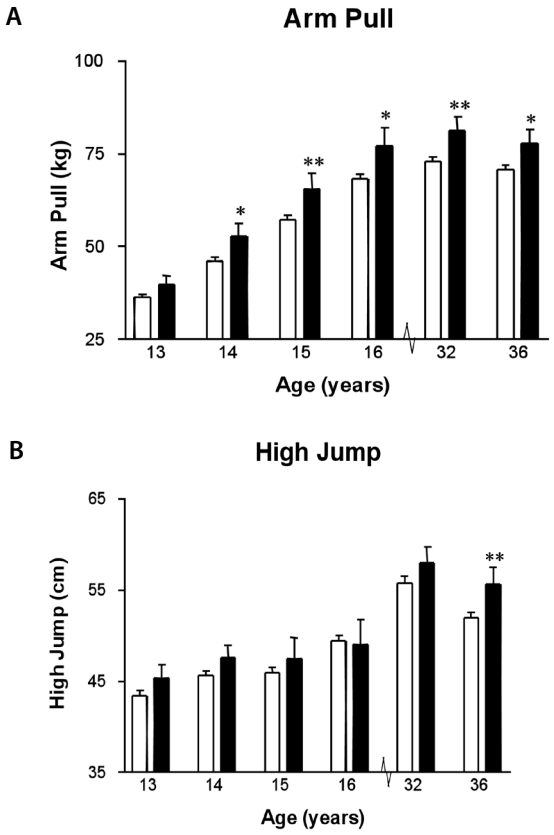
** p≤ 0.05, * p< 0.10

parameters or body composition variables during this period. In females, no differences were observed in the measured parameters at these four measurements during puberty.

Muscle strength at adult age and during puberty

Male ER22/23EK-carriers tended to perform better in the test of arm pull strength (p=0.06 figure 2A), as well as in high jump from standing (adjusted for body weight, p=0.04, figure 2B) at the age of 36 years (see also table 1). Arm pull strength was

Figure 2 Arm pull strength (A) and standing high jump (B) in male noncarriers (white bars) and carriers of the ER22/23EK polymorphism (black bars) during puberty (age of 13, 14, 15, and 16 years) and adult age (32 and 36 years). High jump scores were corrected for body weight.



** p≤ 0.05, * p< 0.10

significantly greater in males at the age of 32 (figure 2A, ER22/23EK: 81.2 ± 3.3 and noncarriers: 73.0 ± 1.1, p=0.02). During puberty, we found the same tendencies towards better arm strength in male ER22/23EK-carriers (figure 2A). Performance on high jump from standing was not significantly different between the genotypes in males at the age of 32 or during puberty (figure 2B). In females, we did not observe any differences in muscle strength of the arm or leg at the age of 36 or 32 years nor during puberty.

Physical activity

At both measurements at young adult age metabolic equivalent (MET)- scores were determined to evaluate physical activity in daily life of the participants. No differences in MET scores between the genotypes were observed in both sexes at age 36 (males, p=0.60; and females, p=0.49; table 1) and 32 years (males, ER22/23EK, 2755 ± 279, noncarriers, 3236 ± 255 METS/week, p=0.52, and females, ER22/23EK, 3687 ± 795 and noncarriers, 3547 ± 202 METS/week, p=0.85).

Discussion

In this population-based cohort study in young subjects we identified 8% heterozygous carriers of the ER22/23EK polymorphism. In males at young adult age, we found ER22/23EK-carriers to be on average 4 cm taller than noncarriers and to have significantly more lean body mass, while there were no differences in fat mass. In addition, male ER22/23EK-carriers had greater thigh circumferences, indicating more muscle mass. Functional muscle strength tests showed a better performance of ER22/23EK-carriers in arm strength, with the greatest difference at the age of 32, as well as a better performance in tests concerning strength of the legs. MET-scores did not differ between genotypes, so differences in physical activity did not underlie the greater amount of muscle mass in male ER22/23EK-carriers.

These differences in body composition in males were not yet clearly present during puberty. However, a tendency could be observed towards greater arm strength in male ER22/23EK-carriers during this period, which suggests that the differences already might have existed in puberty. The lack of statistical significance of the other body compositional parameters could possibly be due to the lower numbers of subjects who participated at pubertal age. These incomplete data during puberty might also explain a minor part of the difference in mean height between the age of 16 and age 32 yrs. However, most of this difference in height is explained by natural growth. In The Netherlands boys grow an additional 5.5 cm after the age of 16. Interestingly, male ER22/23EK-carriers grew on average almost 2 cm more than noncarriers after the age of 16. This increased growth suggests that puberty in ER22/23EK-carriers might be extended compared to non-carriers. It is known that glucocorticoids inhibit growth during puberty. Since we found the ER22/23EK polymorphism to be associated with relative glucocorticoid resistance, we would expect less inhibition of growth and thus a greater height. This is in accordance with our finding of a greater height in male carriers of the ER22/23EK polymorphism. Taken together, it remains unclear at what developmental stage exactly these differences between the genotypes that we observed in young adults in height, lean mass and thigh circumference arise. Although the mean heights in this population-based study

appear rather tall, these heights are in accordance with the mean height at these ages in The Netherlands. This suggests that our findings are very well applicable to the Dutch population as a whole.

In young adult females, we found in ER22/23EK-carriers tendencies towards smaller waist and hip circumferences and lower body weight, suggesting a lower amount of subcutaneous fat. These differences could not be detected during puberty. No statistically significant differences were found in measures of body composition or muscle strength between the genotypes.

Longterm exposure to high levels of glucocorticoids are known to negatively influence muscle mass and growth.[23;24] Thus, the findings of greater height and more muscle mass in male ER22/23EK-carriers could be explained by the observation that ER22/23EK-carriers are relatively resistant to the effects of glucocorticoids, as we recently demonstrated.[11] Another well-known chronic effect of glucocorticoids is redistribution of fat mass to the abdominal region. In line with a glucocorticoid insensitive effect of the ER22/23EK polymorphism we found at the age of 32, as well as at 36 years a tendency towards smaller waist circumference in female ER22/23EK-carriers.

The associations between the ER22/23EK polymorphism and body composition appear to be different between sexes. However, there could be subtle anabolic effects in female ER22/23EK-carriers as well, in line with a relative cortisol resistance and as a result possibly higher androgen levels. When we consider the mean weight difference (more than 5 kg) between female noncarriers and carriers of the ER22/23EK polymorphism, the difference in lean body mass is quite small (less than 2 kg), which indicates that female ER22/23EK-carriers also have relatively more lean body mass. However, the number of female carriers of the polymorphism is relatively small, which might explain that we found no statistically significant differences. Besides more muscle mass, we would also expect less fat mass in subjects with slightly higher androgen levels. In females, we observed tendencies towards smaller waist and hip circumferences, which might reflect a lesser amount of subcutaneous fat mass.

On the other hand, the ER22/23EK polymorphism could have sex-specific effects on body composition. We speculate that differential effects of sex steroid hormones and/ or growth hormone could play a role. It is known, that in rodents the hypothalamic-pituitary-adrenal (HPA) axis is differently regulated in males and in females, both in basal conditions and in response to psychological or physical stress conditions.[25] In this context, androgens inhibit and estrogens enhance the HPA responsiveness to stress.[26;27] In addition, in a relative glucocorticoid resistant condition, as is the case in carriership of the ER22/23EK variant allele, ACTH production is expected to be slightly higher than in noncarriers due to the lower negative feedback inhibition at the pituitary level. As a consequence, ER22/23EK-carriers might have slightly higher circulating androgen concentrations, which could also, besides a smaller direct

(negative) effect of glucocorticoids, contribute to the observed beneficial body composition. The differential effects of sex steroid hormones might explain the gender dimorphism in the associations we observed between genotype and body composition. However, in the present study we did not measure any serum hormone concentrations.

The exact mechanism of this polymorphism at the molecular level is unknown. The amino acid change in codon 23 (arginine to lysine) might affect the tertiary structure of the receptor. Since the ER22/23EK variant is located near the transactivation domain, this could influence the transactivational and/or transrepressional activity on target genes.[28;29] Recently, it has been shown that two different GR isoforms (A and B) exist, due to two different methionine (M) codons in the GR mRNA, which both can be used as initation codon (M1 and M27). The GR-B protein has a stronger transactivating effect in transient transfection experiments, but no difference in transrepression.[30] The secondary structure of the GR mRNA might be affected by the ER22/23EK polymorphism, which could result in a different usage of the initiation codons. A change in GR-A/GR-B ratio could then explain the decreased sensitivity to glucocorticoids.[29] Indeed, secondary structure prediction (M-*fold*) showed different structures for the wildtype and the polymorphic mRNA.[29] A third possibility is that the ER22/23EK polymorphism might lead to differences in binding of proteins, which could affect mRNA stability and thereby influence glucocorticoid sensitivity.

In summary, we found that the ER22/23EK polymorphism of the *GR* gene is associated with greater body height and more muscle mass and strength in young adult males. In females, we found a tendency towards smaller waist circumference, and to a lesser extent smaller hip circumference. Thus, we conclude that the ER22/23EK polymorphism is associated with a sex-specific, beneficial body composition at young adult age, as well as more muscle strength in males.

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6

Glucocorticoid receptor gene variant is associated with increased body fatness in youngsters

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Summary

Objective: Sensitivity to glucocorticoids is known to be highly variable between individuals and is partly determined by polymorphisms in the glucocorticoid receptor (GR) gene. We investigated the relationship between 4 GR gene polymorphisms and body composition during puberty and at young adult age.
Design: An observational study with repeated measurements.
Patients: Two comparable young Dutch cohorts with a generational difference of about 20 years. The first cohort consisted of 284 subjects born between 1961 and 1965. Measurements were performed from 13 until 36 years of age. The second cohort consisted of 235 subjects born between 1981 and 1989. Measurements were performed from 8 until 14 years of age.
Measurements: Associations between height, weight, BMI, fat mass and fat free mass and 4 well known functional polymorphisms were investigated.
Results: In boys in the younger cohort, the G-allele of the BclI polymorphism (haplotype 2) was associated with a higher body weight, weight-SDS, BMI, BMI-SDS and fat mass. These associations were not observed in the older cohort. Irrespective of genotype, the younger cohort showed a significantly higher total fat mass, body weight and BMI compared to the older cohort.
Conclusions: Because the associations between the G-allele of the BclI polymorphism in the glucocorticoid receptor gene and body fat mass in boys were only found in a healthy young population, but not in a comparable, but generally leaner cohort from an older generation, it is suggested that carriers of this polymorphism are likely to be more vulnerable to fat accumulation in today's obesity promoting environment, than non-carriers.

Introduction

Glucocorticoids (GCs) are important regulatory hormones in many tissues throughout the human body and are known to influence body composition. Response to endogenous or exogenous GCs is known to be highly variable between individuals, whereas the range of intra-individual sensitivity is rather narrow, suggesting that glucocorticoid sensitivity is largely determined by genetic factors.[1] The effects of GCs are predominantly mediated by the glucocorticoid receptor (GR), a ligand-activated transcription factor.[2] Changes in the gene coding for this receptor play an important role in determining glucocorticoid sensitivity.[3] Within the normal adult population, several functional polymorphisms in the GR gene have been described.[4;5] One of these polymorphisms, BclI, has been shown to be associated with a relative hypersensitivity to glucocorticoids in vivo,[6;7] an increased cortisol response to a standardized lunch, and abdominal visceral obesity, but not with general obesity.[8-10] In a group of elderly males a lower BMI was found for carriers of the G-allele, which was largely explained by a loss of lean body mass.[7]

Almost all previous studies were performed in adults. One study in adolescents showed in female heterozygous CG-allele carriers a greater increase of subcutaneous fat, as measured by skinfolds, when compared to both CC- and GG-carriers during a 12 years follow-up period.[11] No differences were found in baseline or post follow-up subcutaneous fat mass, or in total fat mass or trunk fat mass.

At present, it is not known what the effects of this BclI polymorphism are in childhood and adolescence. Neither data are available on the possible gene-environment interaction that might play a role in the expression of this polymorphism. Therefore, in the present study, we investigated the relationship between haplotypes of 4 known functional polymorphisms, one of them being the Bcl1 polymorphism, and body composition during puberty and at young adult age in two comparable Dutch cohorts from different birth cohorts with a birth date difference of approximately 15 – 25 years.

Methods and procedures

Subjects

The investigated population is composed by two comparable young Dutch cohorts with a generational difference of about 20 years. The first and older cohort is the Amsterdam Growth and Health Longitudinal Study (AGAHLS) cohort, an observational study with repeated measurements.[12;13] Subjects were recruited among several schools in the city of Amsterdam. For the purpose of this study only Caucasian and apparently healthy subjects were included (n = 350). Measurements were performed at mean

ages of 12.9, 14.0, 15.0, 16.0 and 36.0 years of age. Years of birth varied from 1961 to 1965.

The second and younger cohort is the "Bone Study around Amsterdam in Kids" (Bonestaak) cohort. Also in this study only Caucasian and apparently healthy subjects were included (n = 316). The children were recruited from a number of primary and secondary schools in villages around Amsterdam. Measurements were performed at mean ages of 10.8, 11.3, 11.8 and 12.7 years for the girls and at 11.8, 12.3, 12.8 and 13.8 years for the boys. Years of birth varied from 1981 to 1987 for the girls and 1983 to 1989 for the boys.

Age distribution differed slightly between both cohorts, but as described previously, overlap is considerable.[14] Both cohorts were recruited in socio economically comparable areas and schools.

At each visit, body height, body weight and skin fold thickness were measured. In the Bonestaak cohort also Dual-energy X-ray absorptiometry (DEXA) of the total body was performed at each visit. In the AGAHLS cohort yearly X-rays of the left hand were performed as a measure for bone age development. In the Bonestaak cohort X-ray of the left hand was performed at first visit, and pubertal development (Tanner stages) was measured at each visit. From all participants blood samples were collected for DNA analyses.

The study protocols were approved by the Committee of Ethics on Human Research of the VU University Medical Centre. All subjects and at least one of the parents gave their written informed consent.

Anthropometric measurements

Standing height and body weight were measured using a stadiometer and a calibrated scale respectively, wearing underwear only. Fat mass (FM) was estimated from the sum of four skin folds (biceps, triceps, subscapular and supra iliacal) using the equation of Durnin and Rahaman.[15;16] These four skin folds were measured according to standard procedures.[17] Fat free mass (FFM) was calculated by subtracting the FM from the body weight. Within both cohorts skin fold measurements were performed by the same investigators.

Interaction analyses were performed to determine differences in the relation between fat mass and body height, body weight and body mass index (BMI) in both cohorts.

In the Bonestaak cohort pubertal stage was recorded by visual assessment by one trained observer, using breast stages according to Tanner (B1-5) in girls or genital stages (G1-5) in boys. In the AGAHLS cohort skeletal ages were assessed according to the Tanner-Whitehouse method (TW II) by one well trained examiner.

DEXA scan

In the Bonestaak cohort fat mass and fat free mass were also measured with total body dual-energy X-ray absorptiometry using the Hologic QDR-2000 (Hologic Inc., Waltham, MA, USA). All scans were carried out in the array mode and analysed by the same investigator. The reproducibility of the different scans has been described previously.[18]

As the DEXA scan method is known to be more reliable than skin fold measurements, we compared both methods in the Bonestaak cohort. We performed reliability analysis calculating intraclass correlation coefficients (ICC).[19] For all 4 visits $ICC_{agreement}$ was excellent, varying between 0.89 and 0.92. All reported analyses on fat mass and fat free mass in this study were performed using both methods and showed comparable results.

Genetic analysis

To be able to correct for possible influences of other known functional GR gene polymorphisms, all participants were genotyped for four known functional GR gene polymorphisms. Only subjects with complete GR gene polymorphism genotyping were included for analyses. Genotype data were used to construct haplotypes as described previously.[20] For each haplotype, three genotype combinations were distinguished, with 0, 1, or 2 copies of the haplotype allele. Haplotype 1 represents the wild type variant of all four GR gene polymorphisms. Haplotype 2 is characterized by the G allele of the BclI polymorphism, identified as an intronic C→G nucleotide substitution (no rs number) 646 nt downstream from exon 2.[7] Haplotype 3 is characterized by the G allele of the nucleotide substitiution located in the 3' end of exon 9β, which encodes for the 3' untranslated region of the mRNA of the hGRβ isoform (nt 3669 in X03348; rs6198 in the dbSNP database [available at: http://www.ncbi.nlm.nih.gov/SNP].[21] Haplotype 4 is characterized by the S allele of the N363S polymorphism of exon 2 (nt 1220 in NM_000176; rs6195), located in the transactivation domain.[22] Haplotype 5 is characterized by the A allele of the ER22/23EK polymorphism, which is a combination of 2 linked single-nucleotide variations in codons 22 and 23 (nt 198 and 200; rs6189 and rs6190, respectively), leading to an arginine-to-lysine change in codon 23 in the transactivation domain,[5] in combination with a third polymorphism, the G allele of the 9β polymorphism that is also present in haplotype 3.

DNA was isolated from peripheral blood leucocytes by use of standard techniques. Genotyping of the N363S and ER22/23EK polymorphisms were performed using RFLP analysis, as previously described.[22;23] For the BclI and 9β polymorphisms polymerase chain reaction amplification and genotyping were performed using 5 ng of genomic DNA for the Taqman allelic discrimination assay. Primer and probe sequences were optimized using the single-nucleotide-polymorphism assay-by-design service of

Applied Biosystems. For details, see the Applied Biosystems Products and Services Web page (available at: http://store.appliedbiosystems.com). Reactions were performed on the TaqMan Prism7900HT (Applied Biosystems) in a 384-well format.

Statistical analysis

Data were analyzed using SPSS for Windows, release 11.0.1 (SPSS, Chicago, IL). Results were reported as mean ± SE. Boys and girls were analyzed separately. Genotype was set as independent variable, whereas body height, body weight, BMI, fat mass, fat-free mass were entered as dependent variables. Association of the G-allele of the BclI polymorphism (haplotype 2) with continuous variables was tested by analysis of covariance using the general linear model procedure and by linear regression analysis to test whether a trend is present. Adjustments for age (height, weight, weight-SD, BMI, fat mass, fat free mass), height (weight, fat mass, fat free mass) and pubertal stage or bone age (all variables except age) were made. Bonferroni post hoc tests were used to test for differences between each genotype and to correct for multiple comparisons. A statistical trend was defined as a p-value > 0.05 and ≤ 0.1. Statistical significance was set at p-value ≤ 0.05.

Results

Table 1 shows baseline characteristics of both cohorts after exclusion of subjects with incomplete GR gene polymorphism genotyping. For 284 participants in the AGAHLS cohort and 235 participants in the Bonestaak cohort, a complete data set and all GR gene polymorphisms were available. The distribution of genotypes for all GR gene polymorphisms was in Hardy-Weinberg equilibrium (P > 0.05).

We studied the overall difference in body composition between both cohorts, irrespective of genotype. Regression analyses of the difference in fat mass, body weight and BMI between both cohorts showed significantly higher fat mass, body weight and BMI in the Bonestaak cohort after adjustment for age and height where appropriate (p<<0.001).

Haplotype 2: In the AGAHLS cohort we found for haplotype 2 (BclI) 118 CC-carriers (41.6 %), 121 CG-carriers (42.6 %) and 45 GG-carriers (15.8 %). In the Bonestaak cohort we found 73 CC-carriers (31.1 %), 127 CG-carriers (54.0 %) and 35 GG-carriers (14.9 %). In the AGAHLS cohort no significant differences in age, body height and height-SDS, body weight, weight-SDS, BMI, BMI-SDS, fat mass and fat-free mass between the three BclI genotype groups were found (data not shown).

In girls of the Bonestaak cohort also no associations were found. In the boys however, regression analyses showed that the G-allele was associated with a higher weight, weight-SDS, BMI, BMI-SDS and fat mass at all visits (p_{trend}<0.05) (table 2). These

Table 1 Description of the two study populations at the start of the study.

Study group (n)	AGAHLS (284)		Bonestaak (235)	
	Mean	SE	Mean	SE
Age (years)	12.9	0.04	11.3	0.08
Males/females	132/152		120/115	
Body height (cm)	158.6	0.5	150.6	0.7
Body height (SD)	-0.22	0.06	-0.02	0.07
Body weight (kg)	44.1	0.4	39.4	0.6
Body weight (SD)	-0.34	0.05	-0.17	0.07
BMI (kg/m^2)	17.5	0.12	17.2	0.15
BMI (SD)	-0.48	0.05	-0.18	0.06
Fat mass (kg)	9.1	0.2	8.5	0.3
Fat-free mass (kg)	35.0	0.3	28.9	0.4
Allele frequencies for haplotype 2 (BclI)				
C-allele	63%		58%	
G-allele	37%		42%	

Subjects with incomplete GR gene polymorphism genotyping were excluded.

results strongly suggest an allele-dosage relationship as is also shown in figure 1 for fat mass. Homozygous G-allele carriers have a higher mean fat mass compared to CC-carriers ($p = 0.023$) and to CG-carriers, although the latter did not reach statistical significance ($p = 0.45$), probably because of lack of power. Mean difference in fat mass between GG-carriers and CC-carriers was 3.0 kg at baseline and 4.2 kg at the last visit. For GG carriers and CG carriers these differences were 1.5 kg and 2.5 respectively. No associations were found with body height, body height-SDS or fat-free mass.

Analyses for change in BMI and accumulation of fat mass in the follow up period did not show any association with haplotype 2 in both cohorts (data not shown).

To exclude possible influence of genetic clustering within families, repeated analyses without siblings did not change results significantly (Bonestaak cohort: 2 pairs of brothers and 6 pairs of sisters).

To correct for potential influences of two other GR polymorphisms which have a well known GC hypersensitive (haplotype 4) or GC resistant (haplotype 5) profile, we performed the same analyses omitting carriers of these polymorphisms. The results did not change significantly (data not shown).

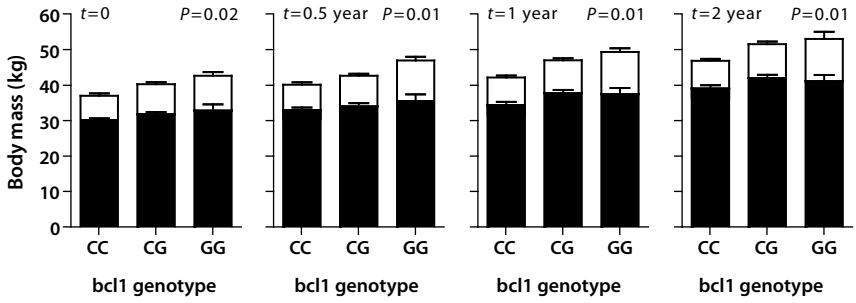
Table 2 Baseline characteristics and anthropometric parameters in the three genotypes for haplotype 2 (BclI) in boys in the Bonestaak study group.

Genotype	visit	CC		CG		GG		
		Mean	SE	Mean	SE	Mean	SE	P_{trend}
n	1	42		57		21		
	2	42		56		21		
	3	42		56		21		
	4	41		54		20		
Body weight (SD)	1	-0.46	0.17	0.10	0.14	0.31	0.21	**0.006**
	2	-0.45	0.19	0.20	0.13	0.44	0.21	**0.001**
	3	-0.46	0.19	0.17	0.13	0.39	0.22	**0.003**
	4	-0.12	0.17	0.44	0.12	0.51	0.23	**0.017**
BMI (kg/m^2)	1	16.6	0.27	17.8	0.33	18.4	0.58	**0.013**
	2	16.9	0.29	18.3	0.35	19.1	0.69	**0.003**
	3	17.2	0.32	18.6	0.35	19.3	0.75	**0.007**
	4	18.2	0.34	19.7	0.39	20.2	0.82	**0.025**
BMI (SD)	1	-0.39	0.15	0.06	0.13	0.27	0.23	**0.019**
	2	-0.38	0.16	0.16	0.13	0.38	0.24	**0.006**
	3	-0.41	0.17	0.13	0.13	0.32	0.25	**0.011**
	4	-0.16	0.17	0.35	0.13	0.39	0.26	**0.047**
Fat mass (kg)	1	6.8	0.42	8.3	0.60	9.8	1.1	**0.018**
	2	7.3	0.47	8.6	0.62	10.9	1.4	**0.008**
	3	7.7	0.49	9.1	0.66	11.5	1.6	**0.008**
	4	7.9	0.52	9.6	0.80	12.1	2.0	**0.008**
Fat-free mass (kg)	1	29.8	0.7	31.5	0.6	32.5	1.9	0.12
	2	31.8	0.9	33.0	1.0	34.6	2.0	0.35
	3	33.5	1.0	36.7	1.0	36.2	2.1	0.28
	4	37.8	1.3	40.9	1.2	40.0	2.1	0.68

Fat mass and fat free mass were calculated using DEXA-scan. Bold values signify $P < 0.05$.

Analyses of the other individual haplotypes for the same parameters of body composition showed for haplotype 1 in both cohorts at some timepoints a trend in both boys and girls towards lower weight, lower BMI and lower fat mass for carriers. These results were not as consistent as for haplotype 2 (data not shown). For haplotype 3 and haplotype 4 no associations were found. Results for haplotype 5 were published earlier.[24]

Figure 1 Fat free body mass and fat body mass in boys at first (t = 0), second (t = 0.5 year), third (t = 1 year) and last visit (t = 2 years) in the various BclI genotypes.



Black bars: fat free body mass, white bars: body fat mass.
Data are expressed as mean ± standard error (SE). P-values apply to body fat mass.

Discussion

In this population-based study, involving two different young cohorts, the associations between haplotypes of glucocorticoid receptor gene and parameters of body composition were studied. Both cohorts are of comparable origin and age, but differ for the fact that a generational difference of about twenty years exists between them.

We found an association between the G-allele of the BclI polymorphism (haplotype 2) and both body weight and BMI in boys in the Bonestaak cohort. Further analyses showed the same association for fat mass but not for lean body mass, suggesting that the increased body weight and BMI could be explained by the increase in fat mass only. In girls from the same cohort, and in all subjects from the AGAHLS cohort, these associations were not found. The findings in the boys are in line with several other reports. Rosmond *et al.* found an association of the G-allele with increased abdominal sagittal diameter, BMI, waist hip ratio (WHR) and leptin levels in a cohort of men.[9] In two other studies, also an association between the G-allele and increased abdominal fat mass was found, but no relation with BMI or total fat mass.[8;10] These studies were performed in middle-aged individuals. The study of Van Rossum *et al.* in elderly subjects showed apparently opposite results: a lower BMI in G-allele carriers. However, in additional analyses in an independent group of 370 Dutch males with a mean age

of 78 years, a tendency towards lower lean body mass in G-allele carriers in an allele-dosage way was found.[7] This lower lean body mass in elderly is attributed to a more pronounced muscle atrophy in G-allele carriers due to the hypersensitivity to glucocorticoids in G-allele carriers.

To our knowledge the only published study in adolescents comparable to our AGAHLS cohort was the study of Tremblay *et al.*[11] This study showed that female heterozygous CG-allele carriers experienced a greater increase in subcutaneous fat, as measured by skin folds when compared to both homozygous CC carriers and GG carriers during a 12-year follow-up period. No statistically significant differences were found in baseline or post-follow-up subcutaneous fat mass, total fat mass or trunk fat mass. Although we did not find a greater increase in BMI and fat mass in CG or GG carriers in our cohort, the findings at baseline are comparable in this cohort that was slightly older, but born in the same period (1960's). Probably we were not able to find differences in fat accumulation in both cohorts because of our shorter follow up period.

The findings in our study suggest that the hypersensitivity to GCs might first lead to fat accumulation and increased BMI, which starts in the first two decades of life. Due to the extra weight bearing in these individuals and a relatively high level of physical activity at young age, muscle mass is protected from atrophy. This effect might be sustained in middle aged individuals, but apparently changes in the elderly.

In contrast to the observation in boys, we found in girls from the same cohort no comparable associations. A possible explanation for this could be the fact that the girls were slightly younger than the boys and did not have time to accumulate enough fat mass. Another explanation could be the effect of increasing oestrogen levels in puberty, promoting the deposition of fat. Possibly this outweighs the effect of their GR haplotype. In future research it would be interesting to investigate the role of estrogen levels or estrogen sensitivity, which seems to be related to estrogen receptor polymorphisms.[25]

Also in the AGAHLS cohort no comparable associations were found in girls or boys. As both groups are comparable concerning socio-economic, geographic, and genetic background, some other environmental factor might be responsible for the difference in body composition among BclI genotypes in both study cohorts. As is known from recent epidemiological studies, the prevalence of overweight and obesity in adults and children is increasing worldwide, especially in the so-called Western countries.[26;27] Also in our present study higher fat mass, body weight and BMI were found in the second, more recently born cohort. Therefore it could be argued that, in contrast to the older (AGAHLS) cohort, the presence of an association between Bcl1 genotype and body composition in the younger, and fatter (Bonestaak) cohort, reflects a possible gene-environmental interaction of this polymorphism. Possibly an

environment that promotes obesity leads to more pronounced fat accumulation in carriers of this polymorphism, at least in boys.

With now a days available high-throughput genotyping technologies, association studies in large, prospective, population-based cohorts have become more popular to study genetic determinants of growth and body composition. The limitation of this study is that it describes associations. At least the found associations should be reproduced in other larger cohorts. Also further future research should lead to better insight into underlying mechanisms. In addition, our study was performed in Dutch Caucasian subjects. Therefore, results might not apply to groups of different genetic or environmental backgrounds.

In summary, we found an association between the presence of the G-allele of the Bcl1-polymorphism in the glucocorticoid receptor gene and higher body weight, BMI and, most importantly, body fat mass in boys in a healthy young population. These associations were not found in comparably young, but generally leaner individuals from an older generation. These findings suggest that young carriers of this polymorphism are more vulnerable to fat accumulation in today's obesity promoting environment, than non-carriers.

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7

Estrogen receptor α gene polymorphisms and body composition in children and adolescents

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Abstract

Background/aims: Gender differences in body composition are largely explained by differences in sex hormones, such as estrogens. Associations between 2 polymorphisms in the estrogen receptor α gene (ESR1) and body composition in children and adolescents were investigated.

Methods: Two comparable Dutch cohorts with a generational difference of about 20 years were investigated. The first consisted of 350 subjects (184 girls), the second of 316 subjects (155 girls). Associations between height, weight, BMI, fat mass (FM) and fat-free mass and two polymorphisms in the ESR1 gene were investigated.

Results: In girls in the recent cohort, the PvuII-XbaI haplotype 2 polymorphism in the ESR1 gene was associated with a lower body weight, BMI, and FM. These associations were not observed in the older cohort. The younger cohort had a significant higher total FM, body weight and BMI compared to the older cohort.

Conclusion: Because the associations between the PvuII-XbaI haplotype 2 polymorphism and body FM in girls were only found in the recent cohort, but not in a comparable, generally leaner cohort from an older generation, it is suggested that non-carriers of this polymorphism are likely to be more vulnerable to fat accumulation in today's obesity promoting environment, than carriers.

Introduction

As is known from recent epidemiological studies, the prevalence of overweight and obesity in adults and children is increasing worldwide, especially in several Western countries including the United States of America and Europe.[1-3] The prevalence of overweight and obesity in children has also increased over the past decades in The Netherlands.[4;5] This trend towards obesity seems to be primarily related to a changed lifestyle as a result of changed environmental and socio-economic factors. This trend will undoubtedly lead to an increased morbidity and mortality at a younger age because of an expected sharp rise in the prevalence of type 2 diabetes mellitus and cardiovascular diseases.[6-8] In interaction with environmental factors, genetic factors play a major role. However, apart from a few rare mutations leading to extreme obesity, no major genes have been found to be responsible for the increase in the prevalence of overweight and obesity, although novel findings in large cohorts using different strategies have shed new light on the knowledge of the genetics of childhood and adolescent obesity.[9-11]

Gender differences in body composition, but also in cardiovascular disease risk, are largely explained by differences in sex hormone levels, especially estrogens.[12-14] Estrogen exerts its effects by binding to the estrogen receptors α and β. The estrogen receptor α gene (ESR1), located on chromosome 6q25, has been shown to play a predictive role in cardiovascular disease and fat tissue deposition.[15-18]

Several studies have reported associations between polymorphisms in the ESR1 gene and cardiovascular risk factors and phenotypes, including body mass index (BMI),[19] fat mass,[20] waist circumference,[21] hypertension,[22] ischemic heart disease and myocardial infarction.[23] Studies in children or adolescents on such associations are scarce.[24-26]

Of the polymorphisms identified in the ESR1 gene, the c.454-397T>C (also known as IVS1-397 T/C, rs2234693, and the PvuII restriction site) and c.454-351A>G SNPs (also known as IVS1-351 A/G, rs9340799, and the XbaI restriction site) are the most widely studied. These polymorphisms are located in the first intron of the ESR1 gene, 397 and 351 base pairs upstream of exon 2.

The aim of this study was to determine whether these polymorphisms in the ESR1 gene are associated with body composition in children and adolescents.

Materials and methods

Study population

The investigated population is composed of two similar young Dutch cohorts with a generational difference of about 20 years. The first and older cohort is the Amsterdam

Growth and Health Longitudinal Study (AGAHLS) cohort, an observational study with repeated measurements.[27;28] Subjects were recruited among two schools in greater Amsterdam area. For the purpose of this analysis only Caucasian and apparently healthy subjects were included (n = 350; 184 girls). Measurements were performed at mean ages of 12.9, 14.0, 15.0, 16.0 and 36.0 years of age. Years of birth varied from 1961 to 1965.

The second and younger cohort is the "Bone Study around Amsterdam in Kids" (Bonestaak) cohort. Also in this analysis only Caucasian and apparently healthy subjects were included (n = 316, 155 girls). The children were recruited from a number of primary and secondary schools in villages around Amsterdam. Measurements were performed at mean ages of 10.8, 11.3, 11.8 and 12.7 years for the girls and at 11.8, 12.3, 12.8 and 13.8 years for the boys. Years of birth varied from 1981 to 1987 for the girls and 1983 to 1989 for the boys.

Age distribution differed slightly between both cohorts, but as described previously, overlap is considerable.[29] Both cohorts were recruited in socio-economically similar areas and schools.

At each visit, body height, body weight and skin fold thickness were measured. In the Bonestaak cohort also Dual-energy X-ray absorptiometry (DEXA) of the total body was performed at each visit. In the AGAHLS cohort during adolescence yearly X-rays of the left hand were performed as a measure for bone age development. In the Bonestaak cohort pubertal development (Tanner stages) was measured at each visit. From all participants blood samples were collected for DNA analyses.

The study protocols were approved by the Committee of Ethics on Human Research of the VU University Medical Center. All subjects and at least one of the parents gave their written informed consent.

Anthropometric measurements

Standing height and body weight were measured using a stadiometer and a calibrated scale respectively, wearing underwear only. Height-SDS and BMI-SDS, adjusting for sex and age, and weight-SDS, adjusting for sex and height, were calculated using Dutch standards.[30;31] Fat mass (FM) was estimated from the sum of four skinfolds (biceps, triceps, subscapular and supra iliacal) using the equation of Durnin and Rahaman.[32;33] These four skinfolds were measured according to standard procedures.[34] Fat free mass (FFM) was calculated by subtracting the FM from the body weight. Within both cohorts skinfold measurements were performed by the same investigators.

In the Bonestaak cohort pubertal stage was recorded by visual assessment by one trained observer, using breast stages according to Tanner (B1-5) in girls or genital stages (G1-5) in boys. In the AGAHLS cohort skeletal ages were assessed according to the Tanner-Whitehouse method (TW II) by one well trained examiner.

DEXA scan

In the Bonestaak cohort fat mass and fat free mass were measured also with total body dual-energy X-ray absorptiometry using the Hologic QDR-2000 (Hologic Inc., Waltham, MA, USA). All scans were carried out in the array mode and analysed by the same investigator. The reproducibility of the different scans has been described previously.[35]

As the DEXA scan method is known to be more reliable than skinfold measurements, we compared both methods in the Bonestaak cohort. We performed reliability analysis calculating intraclass correlation coefficients (ICC).[36] For all 4 visits $ICC_{agreement}$ for FM and FFM was excellent, varying between 0.80 and 0.95. All analyses on fat mass and fat free mass in this study were performed using both methods and showed comparable results. Reported results are the DEXA scan results.

Genetic analysis

Genomic DNA was isolated from peripheral blood leucocytes by use of standard techniques.

All participants were genotyped for the c.454-397T>C (*PvuII*) and c.454-351A>G (*XbaI*) polymorphisms. The PvuII and XbaI RFLPs are located in intron 1 of the ESR1 gene, 397 and 351 bp, respectively, upstream of exon 2. These polymorphisms have also been described at http://www.ncbi.nlm.nih.gov/projects/SNP under identification numbers rs2234693 (c.454-397T>C) and rs9340799 (c.454-351A>G).

Genotypes were determined in 5-ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, Calif). Primer and probe sequences were optimized by using the SNP assay-by-design service of Applied Biosystems (for details,see http://store.appliedbiosystems.com). Reactions were performed with the Taqman Prism 7900HT 384 wells format.

To increase genetic resolution, we constructed haplotypes in this area of the ESR1 gene as described earlier (see table 1).[37] The alleles were defined as haplotypes such as Px, with capitals denoting the absence and lowercase letters denoting the presence of the restriction sites for the PvuII (P/p) and XbaI (X/x) enzymes on each of the alleles. The haplotype alleles were coded as haplotype numbers 1-4 in order of decreasing frequency in the population (1 = px, 2 = PX, 3 = Px, and 4 = pX). Haplotype 4 has been shown to be nearly not existent in the general population.[38;39] Genotypes are analyzed as combinations of two alleles.

Statistical analysis

All statistical analyses were performed using SPSS version 16.0.2 (SPSS, Inc., Chigago, IL). Results are reported as mean ± SE. Boys and girls are analyzed separately.

To analyze the relationship between ESR1 genotypes, parameters of body composition, and potential confounders, we stratified subjects by allele copy number (0, 1, or 2) for the PvuII-XbaI haplotype in the ESR1 gene.

Allele dose was defined as the number of copies of a certain allele in the genotype. Associations with continuous variables were tested by analysis of covariance. If a consistent linear trend was found, indicating an allele dose effect, linear regression analysis was used to quantify a possible association.

Adjustments for the potential confounding effects of age, height, pubertal stage or bone age, were made where appropriate. For this purpose pubertal stage was expressed as SDS-score using stage line diagrams.[40]

A statistical trend was defined as a p-value > 0.05 and ≤ 0.1. Statistical significance was set at p-value ≤ 0.05.

Results

Table 1 shows both cohorts with numbers of subjects analyzed, and allele frequencies for all haplotypes. All genotypes and haplotypes were in Hardy-Weinberg equilibrium. Frequencies of haplotypes were similar to those in other population studies.[38;41-43] Table 2 shows baseline characteristics of both cohorts after exclusion of subjects with incomplete or failed ESR haplotyping (n = 311 for the AGAHLS cohort and n = 256 for the Bonestaak cohort).

First we analyzed the difference in body composition between both cohorts, irrespective of genotype. In a fused database with both cohorts, regression analysis, with fat mass, body weight and BMI as dependant variables, showed significantly higher fat mass (regression coefficient = 2.7 for boys and 1.3 for girls), body weight (regression coefficient = 4.7 for boys and 2.4 for girls) and BMI (regression coefficient

Table 1 Polymorphisms analyzed in this study.

Polymorphism	Alleles	No. of subjects analyzed	Allele frequency (%)
AGAHLS			
PvuII RFLP (intron 1-397 C/T)/	Haplotype 1 (px/T-A)	311	321/622 (52)
XbaI RFLP (intron 1-351 G/A)	Haplotype 2 (PX/C-G)	311	226/622 (36)
	Haplotype 3 (Px/C-A)	311	73/622 (12)
	Haplotype 4 (pX/T-G)	311	-/622 (0)
Bonestaak			
PvuII RFLP (intron 1-397 C/T)/	Haplotype 1 (px/T-A)	256	271/512 (53)
XbaI RFLP (intron 1-351 G/A)	Haplotype 2 (PX/C-G)	256	182/512 (35)
	Haplotype 3 (Px/C-A)	256	59/512 (11)
	Haplotype 4 (pX/T-G)	256	-/512 (0)

Table 2 Description of the two study populations at the start of the study.

	AGAHLS				Bonestaak			
	Boys (n=147)		Girls (n=164)		Boys (n=126)		Girls (n=130)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Age (years)	12.9	0.05	12.9	0.04	11.8	0.1	10.7	0.1
Body height (cm)	157.6	0.01	159.9	0.01	153.6	0.9	147.7	0.8
Body height (SD)	-0.3	0.07	-0.05	0.08	0.05	0.1	0.01	0.08
Body weight (kg)	42.5	0.5	45.8	0.6	41.8	0.8	37.3	0.7
Body weight (SD)	-0.4	0.06	-0.3	0.07	-0.04	0.1	-0.2	0.08
BMI (kg/m^2)	17.0	0.1	17.8	0.2	17.6	0.2	16.9	0.2
BMI (SD)	-0.5	0.06	-0.4	0.08	-0.03	0.1	-0.2	0.08
Fat mass (kg)	6.9	0.2	11.1	0.3	8.2	0.4	9.1	0.3
Fat-free mass (kg)	35.6	0.4	34.7	0.4	31.4	0.6	26.4	0.4

Subjects with incomplete ESR gene polymorphism genotyping were excluded.

= 1.9 for boys and 0.9 for girls) in the Bonestaak cohort after adjustment for age and height where appropriate ($p<0.001$).

In the Bonestaak cohort in girls a consistent, statistically significant association was found at all time points between haplotype 2 and body weight (regression coefficient at first measurement -1.61 (95% CI, -2.71 to -0.50; p = 0.005) , body weight-SDS (regression coefficient at first measurement -0.23 (95% CI, -0.46 to 0.0002; p = 0.05), BMI (regression coefficient at first measurement -0.72 (95% CI, -1.20 to -0.24; p = 0.004), BMI-SDS (regression coefficient at first measurement -0.28 (95% CI, -0.50 to -0.06; p = 0.01) and fat mass (regression coefficient at first measurement -1.69 (95% CI, -2.56 to -0.83; p = 0.0002). An increased number of haplotype 2 copies is associated with a reduced body weight (mean 1.61 kg / allele copy), body weight-SDS (mean 0.23 / allele copy), BMI (mean 0.72 / allele copy), BMI-SDS (mean 0.28 /allele copy) and fat mass (mean 1.69 kg / allele copy) (Table 3). These differences were consistently observed at the first, second (0.5 year), third (1 year) and last (2 year) visits (Figure 1). Stepwise regression analyses showed that the variance in fat mass in girls is explained for 6 – 9% by haplotype 2 (R Square = 0.08 at first visit). In boys no clear associations were found. For haplotype 1 in girls, associations were absent or marginally significant (data not shown): slight opposite trends were observed in girls between haplotype 1 and BMI, BMI-SDS and fat mass; however these trends were not consistent throughout the time of observation.

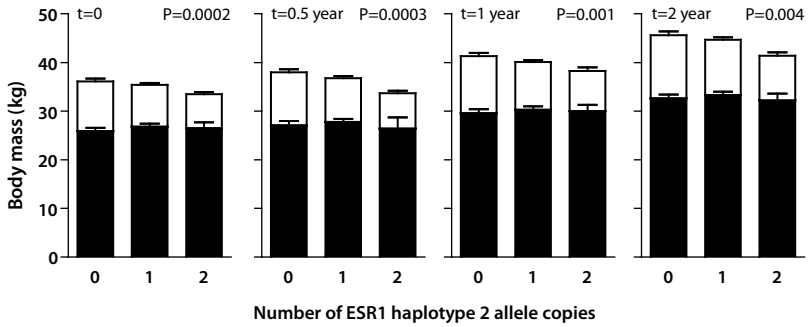
Also for haplotype 3 no associations were found, neither in boys, nor girls (data not shown).

Table 3 Characteristics of carriers of ESR1 haplotype 2 at first measurement in the Bonestaak Study.

	Girls				Boys			
	No. of allele copies ESR1 haplotype 2				No. of allele copies ESR1 haplotype 2			
	0	1	2	P value	0	1	2	P value
No. (%)	53 (40.8)	62 (47.7)	15 (11.5)		56 (44.4)	50 (39.7)	20 (15.9)	
Age ± SE (yr)	10.7 ± 0.1	10.7 ± 0.1	11.1 ± 0.3	0.5[a]	12.0 ± 0.2	11.5 ± 0.2	11.7 ± 0.3	0.2[a]
Weight ± SE (kg)	38.0 ± 1.2	37.2 ± 0.9	35.1 ± 1.5	0.005[b, d]	43.2 ± 1.2	40.3 ± 1.3	41.8 ± 1.7	0.8[b, d]
Weight ± SE (SD)	-0.08 ± 0.1	-0.3 ± 0.1	-0.5 ± 0.2	0.05[b, c]	-0.08 ± 0.2	-0.01 ± 0.1	-0.02 ± 0.3	0.6[b, c]
Height ± SE (cm)	147.3 ± 1.2	148.4 ± 1.2	146.5 ± 2.3	0.2[b, c]	155.8 ± 1.4	151.2 ± 1.5	153.3 ± 2.0	0.2[b, c]
Height ± SE (SD)	0.01 ± 0.1	0.1 ± 0.1	-0.5 ± 0.2	0.2[b, e]	0.2 ± 0.1	-0.08 ± 0.2	0.09 ± 0.2	0.3[b, e]
BMI ± SE (kg/m2)	17.3 ± 0.3	16.8 ± 0.2	16.2 ± 0.4	0.004[b, c]	17.7 ± 0.3	17.4 ± 0.3	17.8 ± 0.6	0.7[b, c]
BMI ± SE (SD)	-0.08 ± 0.1	-0.3 ± 0.1	-0.6 ± 0.2	0.01[b, e]	-0.04 ± 0.1	-0.04 ± 0.1	0.02 ± 0.2	0.8[b, e]
Fat mass ± SE (kg)	10.2 ± 0.6	8.6 ± 0.4	7.0 ± 0.4	0.0002[b, d]	8.1 ± 0.5	8.1 ± 0.6	8.4 ± 1.3	0.6[b, d]
Fat free mass ± SE (kg)	25.9 ± 0.7	26.8 ± 0.6	26.5 ± 1.2	0.6[b, d]	32.7 ± 0.9	30.1 ± 0.8	31.2 ± 1.2	0.7[b, d]

a ANOVA
b Linear regression
c Adjusted for age and pubertal stage
d Adjusted for age, height and pubertal stage
e Adjusted for pubertal stage

Figure 1 Fat-free body mass and fat body mass in girls in the Bonestaak Study at first (t=0), second (t=0.5 year), third (t=1 year) and last visit (t=2 years) according to the number of copies of ESR1 haplotype 2.



Black bars: fat-free body mass, white bars: body fat mass. Data are expressed as mean ± SE. P-values apply to body fat mass (linear regression).

In the AGAHLS cohort, no clear consistent associations or trends were found between any of the haplotypes and parameters of body composition (data not shown).

In an additional analysis we merged both cohorts and re-analysed for the same parameters. Because no uniform puberty staging was performed in both cohorts, no corrections for pubertal stage could be made. Comparable results, although less strong, as in the Bonestaak cohort were found for haplotype 2 at all visits in girls for body weight (regression coefficient at first visit -1.0 (95% CI, -1.90 to -0.16; p = 0.02) , body weight-SDS (regression coefficient at first visit -0.17 (95% CI, -0.32 to -0.01; p = 0.04), BMI (regression coefficient at first visit -0.46 (95% CI, -0.82 to -0.10; p = 0.01), BMI-SDS (regression coefficient at first visit -0.20 (95% CI, -0.37 to -0.04; p = 0.02) and fat mass (regression coefficient at first visit -0.98 (95% CI, -1.53 to -0.42; p = 0.001). Stepwise regression analysis in the merged cohorts showed that haplotype 2, although statistically significant, explains only approximately 2 to 3% of the variance of the fat mass.

Discussion

In this population-based study, involving two different young cohorts, the associations between haplotypes of the estrogen receptor α gene and parameters of body composition were studied. Both cohorts are of similar origin and age, but differ for the fact that a generational difference of about twenty years exists between them.

In the Bonestaak cohort, in girls, the ESR1 PvuII-XbaI haplotype 2 (-397 C and -251 G) was significantly associated with a reduction in body weight per allele copy (mean 1.65 kg / allele copy). No association was found with lean body mass. The reduction in body weight can almost be fully explained by a reduction in fat mass (1,69 kg / allele copy). Homozygous carriers of PvuII-XbaI haplotype 2 had 3.2 kg less fat mass than subjects who did not carry this haplotype. These associations were statistically powerful and consistent at all four observational time points during the two year follow up. An opposite trend, which was not consistent during the observation period, was seen between haplotype 1 and BMI and fat mass. All these associations were not found in boys.

In the AGAHLS cohort however, we did not find these associations in similarly calendar aged young girls or boys. Because this cohort is of an older generation, also data were available at age 36 years. Also at 36 years no clear significant associations were found.

When both cohorts were merged together, the associations between haplotype 2 and weight and fat mass stayed the same in girls, although less significant.

Studies on the associations between these polymorphisms and body composition are conflicting. The study of Fox *et al.* found lower waist circumference in haplotype 2, which is in accordance with our findings, although they only found this association in men.[21] Also in another study homozygous carriers of haplotype 2 had lower FM and waist circumference during post-menopause, but not before menopause.[20] A positive association between the PvuII polymorphism and BMI was reported in post menopausal Caucasian women.[19] Later studies, especially in younger subjects, did not find any associations between PvuII and XbaI polymorphisms and body composition.[24;26]

The findings in the Bonestaak cohort were powerful in the girls, and absent in boys. There are two possible explanations for this gender difference. Firstly, the circulating estradiol levels in puberty are much higher in girls than in boys. Possibly a slight difference in estradiol sensitivity may become only clinically relevant with increasing levels of estradiol. A second reason might be the fact that the higher testosterone levels in boys outweigh the estradiol effect on fat tissue.

Remarkably, the clear associations among girls in the Bonestaak cohort were not found in the AGAHLS cohort. Both cohorts were of similar size, origin and age. The main difference between both cohorts was the generational difference of approximately 20 years. A possible explanation for the difference could be the risk of

chance finding due to the lack of enough power in the AGAHLS cohort to find statistically significant differences, or to find associations by chance in case of the Bonestaak cohort. The latter does not seem to be very likely, as the associations were all quite strong and consistent over time. Furthermore, the same associations were found using different modes of measurements: anthropometry and DEXA scan. Also by merging both cohorts the same associations were found, although weaker. An important difference between both cohorts in general is the difference in overall weight and fat mass. Some associations of clinical parameters with genetic ones are only revealed in extreme conditions where there is an interaction of several genetic parameters with environmental ones. Possibly the associations are there in both cohorts, but they can only be revealed in the obese cohort, leading to a more pronounced fat accumulation in non-carriers of the ESRα haplotype 2, at least in girls. Of course this is speculative and cannot be proven with our study design.

In summary, we found a gender specific association between the presence of the PvuII-XbaI haplotype 2 polymorphism in the ESR1 gene and body weight and fat mass in girls in a healthy young population. An increase in number of haplotype 2 copies is associated with a reduction in body mass, which is largely attributable to a reduction in body fat mass (mean 1,69 kg fat / allele copy). These associations were not found in comparably young, but generally leaner individuals from an older generation. These findings might suggest that young non carriers of this polymorphism are more vulnerable to fat accumulation in today's obesity promoting environment, than carriers.

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8

Androgen receptor gene CAG repeat polymorphism in longitudinal height and body composition in children and adolescents

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Summary

Objective: The number of CAG repeats within the CAG repeat polymorphism of the androgen receptor gene correlates inversely with the transactivation of the receptor. We investigated the relationship between the AR CAG repeat polymorphism and longitudinal growth, puberty and body composition from pre-puberty until young adult age.
Design: Observational study with repeated measurements.
Subjects: Two comparable young Dutch cohorts. The first cohort consisted of 226 subjects. Measurements were performed from 13 until 36 years of age. The second cohort consisted of 244 subjects. Measurements in this cohort were performed from 8 until 14 years of age.
Measurements: Associations between height, height velocity, weight, BMI, fat mass, fat free mass and pubertal development and CAG repeat length were measured.
Results: Height-standard deviation scores (SDS) were inversely associated with AR CAG repeat length in boys at young, pre pubertal and early pubertal age. This association diminishes in the following years and completely disappears after the age of 16 years. No associations were found with pubertal stage or any of the other parameters for body composition.
Conclusions: AR CAG repeat length is inversely associated with longitudinal height in young boys, already before the onset of puberty. During puberty these differences disappear, possibly overruled by a strong developing hypothalamic-pituitary-gonadal axis.

Introduction

The length of CAG microsatellite repeat sequence in exon 1 of the androgen receptor (AR) gene correlates inversely with AR activity and the strength of androgen action. Associations with several androgen-dependant clinical endpoints have been studied extensively in adults.[1] Associations between CAG repeat length and serum testosterone levels also have been found, indicating that weaker AR activity is compensated for by higher androgen levels.[2-4]

Conflicting findings have been reported on the associations of the AR CAG repeat length and body composition. Previous studies have observed that CAG repeat length correlates negatively with BMI[5] or positively with fat free mass.[6] However, also positive correlations of CAG repeat length with BMI or body fat content were found.[7;8] Another study found unfavourable effects of low testosterone levels on body composition among elderly men with longer CAG repeats.[9] A recent study in young men (20 – 29 years) found that AR CAG repeat length correlated negatively with muscle mass of the thigh and lower trunk and total lean body mass, but positively with subcutaneous adipose tissue and total fat mass.[10]

Data on the associations of the AR CAG repeat length polymorphism and growth and body composition are lacking in children and adolescents. Therefore, in the present study, we investigated this relationship during puberty and at young adult age in two comparable Dutch cohorts.

Subjects and Methods

Subjects

The investigated population is composed by two comparable young, healthy and Caucasian Dutch cohorts. Both cohorts were described earlier.[11;12] The first and older cohort is the Amsterdam Growth and Health Longitudinal Study (AGAHLS) cohort, an observational study with repeated measurements *(n* = 350*)*.[13;14] Measurements were performed at t=0, t= 1 years, t= 2 years, t= 3 years, t= 19 years and t= 23 years (mean ages of 12.9, 14.0, 15.0, 16.0, 32.0 and 36.0 years of age. Years of birth varied from 1961 to 1965.

The second and younger cohort is the "Bone Study around Amsterdam in Kids" (Bonestaak) cohort *(n* = 316*)*. Measurements were performed at t= 0, t= 0.5 years, t= 1 years and t= 2 years (mean ages of 10.8, 11.3, 11.8 and 12.7 years for the girls and at 11.8, 12.3, 12.8 and 13.8 years for the boys respectively). Years of birth varied from 1981 to 1987 for the girls and 1983 to 1989 for the boys.

The study protocols were approved by the Committee of Ethics on Human Research of the VU University Medical Centre. All subjects and at least one of the parents gave their written informed consent.

Anthropometric measurements

Body weight, height, waist to hip ratio and skin fold thickness (biceps, triceps, subscapular and supra iliacal) were measured, and fat mass (FM) and fat free mass (FFM) were calculated as described previously.[12]

In the Bonestaak cohort fat mass and fat free mass were measured also with total body dual-energy X-ray absorptiometry using the Hologic QDR-2000 (Hologic Inc., Waltham, MA, USA) as described previously.[12]

In the Bonestaak cohort pubertal stage was recorded using breast stages according to Tanner (B1-5) in girls or genital stages (G1-5) and volume of the testes in boys. Also staging of pubic hair development (P1-5) was recorded. In the AGAHLS cohort skeletal ages, using X-ray of the left hand, instead of pubertal staging, were assessed according to the Tanner-Whitehouse method (TW II).

Genetic analysis

Genomic DNA was isolated from peripheral blood leucocytes by use of standard techniques.

For the Bonestaak cohort, the CAG repeat in the AR was amplified in a 2 µl polymerase chain reaction (PCR) containing approximately 2,5 ng of DNA, 0,4 pmol of each of the primers (Isogen Life Science , De Meern, the Netherlands), 1x AmpliTaq Gold® PCR Mastermix (Life Technologies, Carlsbad, CA, USA) and 1,5 nmol additional $MgCl_2$ (Life Technologies). The primers used for amplification were 5'-TCCAGAATCTGT-TCCAGAGCGTGC-3' (sense) and 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3' (antisense). The antisense primer was labeled with FAM fluorescent dye. Amplifications were performed using a Auto-Lid Dual 384-Well GeneAmp® PCR System 9700 (Life Technologies) applying the following conditions: initial denaturation step at 95 °C for 5 minutes; 30 cycles of 15 seconds denaturation at 95 °C, primer annealing at 54 °C for 15 seconds and primer extension at 72 °C for 30 seconds; and a final extension step of 72 °C for 7 minutes.

For the AGAHLS cohort samples a similar protocol was used, with slight modifications.

Each sample was analyzed by capillary electrophoresis on a 3100 genetic analyzer according to details provided by the manufacturer (Life Technologies). The results were analyzed with GENEMAPPER software version 4.0 (Life Technologies). The number of repeats was calculated from the size of the PCR products.

Statistical analysis

Subjects were grouped as harbouring short repeat lengths (< median repeat length) or long repeat lengths (≥ median repeat length). Only females homozygous for a short allele or homozygous for a long allele were included in the analysis; females carrying both a short allele and a long allele were excluded. Boys and girls were

analyzed separately. Dependant variables were analyzed by using analysis of covariance. Adjustments for age (height, weight, weight-SD, BMI, fat mass, fat free mass), height (weight, fat mass, fat free mass) and pubertal stage or bone age (all variables except age) were made. In addition, analyses were performed by using several "extremes" of the spectrum of repeat numbers: subjects with very short repeat numbers were compared to subjects with very long repeat numbers. We defined short as repeat numbers below the 5th, 10th, 15th percentile or below the median value minus 1. Long repeats were defined as respectively numbers above the 95th, 90th, 85th percentile or above the median value plus 1. Furthermore analyses were performed using approximate tertiles of the repeat numbers.

To explore possible associations between the AR CAG repeat number and the appearance or progression of puberty in the Bonestaak cohort, Mann-Whitney test was used to compare pubertal stages among genotypes. Comparison of the onset of puberty between genotypes was carried out using the χ^2 test. In the AGAHLS cohort possible associations between AR CAG repeat number and bone age were tested using analysis of covariance.

Data are reported as mean ± SE. A statistical trend was defined as a p-value > 0.05 and ≤ 0.1. Statistical significance was set at p-value ≤ 0.05. Analyses were performed by using SPSS for Windows, release 16.0.2. (SPSS, Chicago, IL, USA)

Results

Table 1 shows baseline characteristics of both cohorts with successful genotyping of the AR CAG repeat polymorphism. The mean (± SD) repeat length for boys was 20.9 ± 2.9 in the AGAHLS cohort and 20.8 ± 3.3 in the Bonestaak cohort. For girls the mean repeat length of allele 1 and 2 was 21.0 ± 2.1 in the AGAHLS cohort and 20.9 ± 2.2 in the Bonestaak cohort.

Bonestaak cohort.

The median of the distributed repeat number was 21 repeats both for boys and girls. A trend for height-SD was found in the same direction at all time points (t=0, t=0.5 years, t=1 years and t=2 years) in boys, indicating a higher height-SD for subjects with shorter AR CAG repeat number (figure 1). Additional analyses in boys of the "extreme" AR CAG repeat numbers showed similar results for height-SD, although analyses in the very extremes of the repeat spectrum became unreliable due to low numbers (data not shown). Especially grouping as carriers of short or long alleles based on the median plus or minus 1, showed statistically significant differences in height-SD (t=0: p=0.04, t=0.5 years: p=0.03, t=1 year: p=0.1, t=2 years: p=0.03). Also analyses using approximate tertiles of the repeat numbers showed significant lower height-SD in the

Table 1 Description of the two study populations at the start of the study.

	AGAHLS (n=226)		Bonestaak (n=244)	
	Mean	**SE**	**Mean**	**SE**
Age (years)	12.9	0.04	11.3	0.08
Males/females	115/111		127/117	
Body height (cm)	159.1	0.01	151.0	0.6
Body height (SD)	-0.2	0.06	0.02	0.07
Body weight (kg)	44.3	0.5	39.7	0.5
Body weight (SD)	-0.4	0.05	-0.2	0.06
BMI (kg/m2)	17.4	0.1	17.2	0.1
BMI (SD)	-0.5	0.06	-0.2	0.06
Fat mass (kg)	9.0	0.2	9.1	0.3
Fat-free mass (kg)	35.3	0.3	29.1	0.4

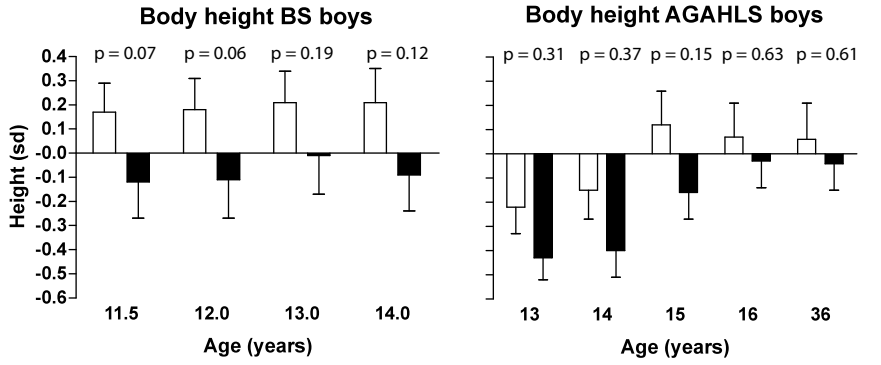
Only subjects with successful genotyping of the AR CAG repeat polymorphism were included.

third tertile (AR CAG repeat ≥ 23): (t=0: -0.33 SD vs 0.15 SD; p=0.066, t=0.5 years: -0.35 SD vs 0.15 SD; p=0.048, t=1 year: -0.20 SD vs 0.19 SD; p=0.17, t=2 years: -0.33 SD vs 0.22 SD; p=0.044).

No significant differences were found for weight, weight-SD, BMI, BMI-SD, fat mass, fat free mass or waist to hip ratio (data not shown). Also no significant associations between AR CAG repeat number and pubertal staging were found in boys for genital stage, pubic hair or testicular volume (data not shown). Additional analyses on growth velocity, measured as difference in height between the measurements, did not show statistically significant differences between AR CAG repeat numbers. Because AR CAG repeat number did not appear to be associated with pubertal development or height velocity during the period of observation, it was assumed that the difference in height-SD already existed before the onset of puberty. To further explore this, we analysed height-SD in boys who were in Tanner stage 1 or 2 at first measurement, before the onset of a growth spurt. Boys with short AR CAG repeat number had significantly higher height-SD than boys with long AR CAG repeat number (height-SD = 0.06 versus -0.4 respectively (p = 0.04)).

In girls no trends or significant associations were found for any of the investigated parameters. Due to the exclusion of carriers of both a short and a long allele, numbers for analyses in girls were often too low for reliable analyses.

Figure 1 Body height-SD in boys at different ages according to AR CAG repeat length in the AGAHLS cohort and the Bonestaak cohort.



Black bars: AR CAG repeat long allele (≥ median repeat length). White bars: AR CAG repeat short allele (< median repeat length).
Data are expressed as mean ± standard error (SE).

AGAHLS cohort.

The median of the distributed repeat number was 20 repeats for boys and 21 repeats for girls. As in the Bonestaak cohort, height-SD in boys was higher at all time points in subjects with short repeat numbers, although this was never statistically significant (see figure 1). This difference in height-SD seems to disappear at higher age. Analyses in boys of the "extreme" AR CAG repeat numbers showed similar results for height-SD, although, as in the Bonestaak cohort, analyses in the very extremes of the repeat spectrum became unreliable due to low numbers (data not shown). Analyses using approximate tertiles of the repeat numbers also only showed a visible trend for height-SD, which was never statistically significant.

No significant differences were found for weight, weight-SD, BMI, BMI-SD, fat mass, fat free mass or waist to hip ratio; waist to hip ratio only available at age 32 and 36 years (data not shown). Also no associations were found between bone age and any of the AR CAG repeat number groups (data not shown).

Analyses of growth velocity did not show significant associations between AR CAG repeat length and growth velocity between age 13 and 14 years or between 14 and 15 years. Between age 15 and 16 years though, a lower growth velocity was found in subjects with short AR CAG repeat number, compared to subjects with long AR CAG repeat numbers (difference 1.0 cm/year (p=0.01)).

In girls no trends or significant associations were found. Because of the exclusion of carriers of both a short and a long allele, numbers for analyses in girls were very low for reliable analyses.

Discussion

In this population-based cohort study involving two young cohorts we studied the associations of the CAG-repeat polymorphism of the androgen receptor with height, body mass, total fat mass, total fat free mass and pubertal staging. To our knowledge, this is the first study on this subject in this young age group, in which also pubertal development is included.

Longitudinal height, measured as height-SD, was inversely associated with AR CAG repeat length in boys at young age. This association diminished in the following years and had completely disappeared after the age of 16 years. Adult height was not associated with AR CAG repeat length. Also other studies in adults never found associations with adult height.[10]

Apparently AR CAG repeat length only influences longitudinal height in the growth phase. To address this further, we additionally analysed the association between AR CAG repeat length and pubertal development or bone age, prepubertal height and height velocity at different ages. Firstly, we did not find any association between AR CAG repeat length and the timing, or stage of, pubertal development, which excludes increased growth due to earlier or progressive pubertal development. Also analyses of height velocity did not reveal an association with AR CAG repeat length before the age of 15 years. However at the end of the growth phase, between 15 and 16 years, a lower growth velocity was found in subjects with short AR CAG repeat length, indicating earlier cessation of growth in this group. Moreover, height-SD in boys who were in Tanner stage 1 or 2, before the onset of a growth spurt, was already inversely associated with AR CAG repeat length, indicating that the association of AR CAG repeat length already exists before the onset of puberty. Apparently this difference in height-SD eventually disappears at the very end of the growth phase, by earlier cessation of growth in the group with short AR CAG repeat length.

It is undisputed that the length of the AR CAG repeat polymorphism correlates inversely with AR activity. In a normal functioning adult hypothalamic-pituitary-gonadal axis a diminished testosterone feedback, in case of a long AR CAG repeat, is compensated for by increased androgen production, due to increased LH stimulation.[2-4;6] Due to this phenomenon it was suggested that several phenotypic effects are probably more attributable to estrogen action than to androgen action, due to the increased effective bioactive estrogen/androgen ratio in increasing AR CAG length.[3] In pre-pubertal boys, the hypothalamic-pituitary-gonadal axis is not responding as in

adulthood, because the axis is relatively silent and the gonads only produce minor amounts of androgens. In pre-puberty also a very small amount of androgens is produced by the adrenals, which are not under control of the hypothalamic-pituitary-gonadal axis. One could argue that in these boys the compensatory mechanism of increasing androgen production in increasing AR CAG repeat length is absent. Therefore pre-pubertal boys with long AR CAG repeats are relatively androgen deficient in pre-puberty compared to boys with short AR CAG repeat, leading to subtle differences in pre-pubertal growth as shown in our study. In the pubertal phase the hypothalamic-pituitary-gonadal axis becomes increasingly responsive, which in case of a relatively small AR resistance leads to higher compensatory androgen production resulting in equal height velocity and possibly to longer sustained growth due to earlier relative androgen deficiency. Because an increased estrogen/androgen ratio is only expected in a (fully) functional hypothalamic-pituitary-gonadal feedback system, it is understandable that no different estrogen effect is seen on pubertal growth and that final height is equal irrespective of AR CAG repeat length. Unfortunately, in our study we had no substantial data on hormone levels to verify this hypothesis.

In both cohorts no significant associations between AR CAG repeat number and the other measured parameters of body composition were found: weight, BMI, fat mass, fat free mass or waist to hip ratio. Conflicting findings have been reported on the associations of the AR CAG repeat length and body composition.[1;3;5-10] Several explanations can be generated about the lack of these associations in our study. Firstly, our cohorts are relatively small and from a different genetic background than other studies. Possibly our cohorts lack enough power to detect any weak existing associations. Another explanation could be that the parameters used are not specific enough for androgen or estrogen actions. The study of Nielsen et al.[10] for example, also could not detect any associations with weight, BMI, fat mass or lean body mass, but found robust associations with thigh and lower trunk muscle areas using magnetic resonance imaging (MRI). Furthermore, our cohorts are the youngest studied for the effects of the AR CAG repeat polymorphism so far. Possibly the effects of this polymorphism on phenotype, apart from longitudinal growth, have not come to full development yet, due to the very low androgen or estrogen levels at this age. Possibly further and full activation of the hypothalamic-pituitary-gonadal axis allows for further development of phenotypic differences over years due to the subtle differences in androgen sensitivity. Whether these differences are caused by direct androgen action or by indirect estrogen action still remains to be elucidated.

The significance of the AR CAG repeat polymorphism in women is still unclear. Due to the presence of two alleles, most studies only use homozygous short or long alleles in their analyses. We used this same strategy, which resulted in too low numbers for reliable analyses in girls in our cohorts.

Taken together, in our study AR CAG repeat length was inversely associated with longitudinal height in young boys, already before the onset of puberty. No associations were found with pubertal development, height velocity during growth spurt or final height. It is hypothesized that the slight androgen insensitivity in boys with long AR CAG repeat length results in difference in growth in pre-puberty under an immature hypothalamic-pituitary-gonadal axis and that further and compensatory development of the axis overrules these effects at later age. These results need to be confirmed in other young and larger cohorts.

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General discussion and summary

Part IV

9

General discussion and future perspectives

General discussion and future perspectives

9.1 Rationale

Genetic variations promote diversity within a population and can be common in nature. From an evolutionary point of view such polymorphisms are important for biodiversity, genetic variation, adaptation and natural selection in different or changing environments. Polymorphisms offer different advantages or disadvantages in different environments.

Numerous environmental and genetic factors can contribute to common characteristics, but also to risk profiles for future diseases in children and adults.
The research described in this thesis aimed at studying associations between several genetic polymorphisms in well known endocrine genes and certain risk factors for future health and disease in children and adolescents.

In this general discussion some of the major points raised by the different parts of this thesis will be explored:

9.2 Birth weight

IGF-1 polymorphism

IGF-1 is considered a major growth factor in pre- and postnatal growth.[1;2] The IGF-1-gene is therefore thought to be an important candidate gene for children born SGA. Point-mutations and deletions in the IGF-1 gene have been described in several case reports.[3-5]

In recent years many IGF-1 and IGF1R polymorphisms have been studied for associations with birth weight and SGA-related outcomes as extensively reviewed by Ester et al.[6] Especially, the 192bp CA-repeat in the IGF-1 promoter, as studied in this thesis, was found most likely to associate with SGA-related outcomes. Conflicting results arise mainly in "normal" cohorts, especially with associations between birth size measures and polymorphisms in this gene region. Also our study was performed in a healthy cohort of children and adolescents. Our study found a lower birth weight for males who were carriers of one or two variant allele(s) of the IGF-I gene (i.e. non 192bp and/or 194bp carriers). Our results are largely in line with the results of Vaessen et al., but others found no associations.[7-10] These conflicting results might be explained by pitfalls of association studies as discussed later, by the absence of a clear association, or by the alternative method of allele classification we used, which was not used by other authors.

Apart from low birth weight as a risk factor for later CVD and T2DM, we found slightly higher LDL cholesterol levels in variant carriers, although this disappeared after correction for BMI. Because also no other risk factors were associated with

genotype, this observation seems rather questionable in this very young population. Birth weight is generally considered to be mainly dependant on the intra-uterine environment, such as the availability of nutrients and oxygen.[11] Genes most likely only play an additive or modifying role. In our study, the IGF-1 genotype could only explain 6% of the variance in birth weight in men, and only 1% of the birth weight in women. However, in our study, the relationship between birth weight and certain risk factors for CVD and T2DM seems to be more dependant on genes. This modification was strongest in the association between birth weight and blood pressure: a lower birth weight in the variant carrier group is associated with a significant higher blood pressure than in the non-carrier group. This finding is in line with results in twin studies, which showed that the association between birth weight and blood pressure depends to a large extent on genetic factors.[12;13]

Glucocorticoid receptor polymorphisms

Glucocorticoids are important regulators of many processes involved in embryonic growth and development and fat and glucose metabolism. Prenatal exposure to exogenous glucocorticoids is associated with low birth weight and altered cardiovascular and metabolic programming.[14;15] Small size at birth is associated with increased fasting plasma cortisol and adrenal responsiveness to ACTH stimulation.[16;17] High levels of glucocorticoids result in unfavourable cardiovascular risk factors, e.g. visceral obesity, steroid induced diabetes mellitus and hypercholesterolemia.[18] We hypothesized that genetic variants leading to increased glucocorticoid sensitivity are associated with fetal growth retardation.

However, we were not able to find any association between several well known polymorphisms in the GR gene and size at birth in a healthy Dutch population (Bonestaak cohort). This finding suggests that there is no relationship between these polymorphisms and intra uterine growth, although possibly our cohort was too small to pick up small influences. This was recently confirmed by a much larger study in the Netherlands, which could not find evidence for an effect of the same GR polymorphisms on fetal growth, birth weight and early postnatal growth.[19] Also in a study in prematurely born children no associations were found between birth anthropometry and the GR polymorphisms ER22/23EK and N363S.[20] Rautanen et al. found an association of a GR haplotype, characterized by a polymorphism in exon 9α in combination with the BclI polymorphism with lower birth weight and length. This haplotype is, however, different from our haplotypes which makes it difficult to compare the results.[21]

In our study in a selected group of children born SGA, without catch-up growth, we found the GR haplotype 2 (BclI) to be less frequent compared to controls. BclI has been associated with glucocorticoid hypersensitivity, and therefore this finding is in contrast to our a priori hypothesis. A possible explanation could be selection bias,

because this group only consisted of SGA children without catch-up growth. Interestingly, the higher risk of metabolic syndrome seems to be associated with catch-up growth.[22] Possibly, non-carriers of the BclI polymorphism in children born SGA will be protected from the deleterious effects of being born SGA. It also might be possible that there is a relation between this polymorphism and postnatal growth. No other studies are available on the effects of glucocorticoid receptor polymorphisms in selected children born SGA. Only Geelhoed et al. performed a subgroup analyses in their large cohort study in children born SGA (n=55).[19] In this small subgroup no associations of the GR haplotypes with fetal and early postnatal growth characteristics were found. To answer these questions, larger groups of children born SGA have to be studied, including both children with and without postnatal catch-up growth.

9.3 Growth and body composition

IGF-1 polymorphism

Our study showed an association between the microsatellite 192bp/194bp CA-repeat polymorphism and body weight, BMI, fat mass and waist circumference at young age in the Bonestaak cohort. This association was significant in girls, but in boys the same trends were observed: subjects carrying the 192bp/192bp, 194bp/194bp or 192bp/194bp CA repeat polymorphism (wild type carriers) had lower weight, lower BMI, lower fat mass and lower waist circumference than variant carriers. These associations were not found in the AGAHLS cohort. Other studies in adults did not find associations between the 192bp CA repeat polymorphism and BMI.[23-25] Also a large study in children did not find associations between the 192bp CA repeat polymorphism and weight and BMI at age 7 years and fat mass at age 9 years.[26] These studies differed from ours for the fact that we used a categorization of genotype based on the finding that circulating serum IGF-1 levels were highest for subjects with 192bp and 194 bp alleles,[27] defining both 192bp and 194bp alleles as wild type instead of 192bp alleles only. Studies in larger young cohorts using the same classification will be needed to confirm our findings.

In our second cohort, the AGAHLS cohort, we were not able to reproduce our findings. A possible explanation could be the relatively small sample size of the cohort resulting in a lack of enough power to reproduce the findings. Also the findings in the Bonestaak cohort could be explained by chance although this seems less likely because all the parameters tested represent more or less the same properties and point in the same direction at all time points, which was also confirmed by longitudinal analyses. Because both groups are similar with regard to socio-economic, geographic and genetic background, we speculated that another environmental factor, interacting with this genotype, might be responsible for the difference in body composition

between the IGF-1 genotypes in both cohorts. Because both cohorts differ in their general weight and fat mass, in line with the worldwide increase in body weight and fat mass in children, it is possibly this obesogenic environment leading to a more pronounced fat accumulation in variant carriers of the IGF-1 polymorphism.

In the AGAHLS cohort male carriers of the 192bp/194bp CA-repeat carriers were taller than variant carriers before reaching adult height, which is in accordance with earlier findings in adults,[25;27] although others did not find this association.[26] This was in contrast to the male wild-type carriers in the Bonestaak cohort who were slightly shorter than variant carriers. Possibly variant carriers in this cohort have a better longitudinal growth due to their higher fat mass, leading to an increased height gain.

The sex-specific differences in genetic effects in our studies are not always well explainable, but it is known from earlier studies that sex-specific genetic effects underlie sexual dimorphism in several body composition traits, especially for fat percentage, lean mass, android fat, gynoid fat and waist-to-hip ratio.[28;29]

Glucocorticoid receptor polymorphisms

In adults four polymorphisms in the glucocorticoid receptor gene have been described to be associated with cortisol sensitivity,[30-32] and several studies analyzed the associations of these polymorphisms with body composition and obesity. Carriers of the ER22/23EK or R23K variant are known to be relatively more cortisol resistant than non-carriers, which results in a beneficial body composition and a better metabolic health profile in adults.[33] By contrast, the *Bcl*I and N363S polymorphisms showed opposite effects. In adults these polymorphisms showed hypersensitivity to glucocorticoids resulting in an increased BMI, increased body fat mass and or decreased lean body mass.[34-36] However, a large meta-analysis performed in almost 6,000 individuals concluded that there is no compelling evidence that the N363S polymorphism is associated with either average BMI or obesity risk,[37] although results are not consistent.[38;39] Recently, also the GR-9β polymorphism was found to be related to a decreased sensitivity to glucocorticoids, leading to an increased risk of cardiovascular disease.[40]

In this thesis these four different variants in the glucocorticoid receptor gene were investigated in relation to body composition in children, adolescents and young adults. During puberty we observed in the AGAHLS cohort that male carriers of the ER22/23EK polymorphism showed tendencies towards greater body height, lean mass and muscle strength. These differences between carriers and noncarriers were more pronounced and statistically significant at young adult age. In females in the AGAHLS cohort, we observed associations with smaller waist and hip circumferences, suggesting less central fat mass. Thus even at young age the influence of the ER22/23EK polymorphism is measurable, although the strength of the associations with body composition parameters seems to increase with age.

In the Bonestaak cohort measurements on circumferences of arms and legs, muscle strength and physical activity were not available at all visits. Also no data at adult age are yet available. Because we did not have good comparable parameters in both cohorts regarding muscle strength and related parameters of body composition, only the results in the AGAHLS cohort were published in a scientific paper (chapter5). Analyses in the Bonestaak cohort on the associations between the ER22/23EK polymorphism and parameters on growth and body composition did not show statistically significant associations or tendencies, although a higher mean body height and higher mean total lean body mass was seen at all visits in ER22/23EK male carriers. In male non-carriers a higher mean weight-SD, BMI-SD and total body fat mass was seen at all visits compared to ER22/23EK carriers. Although these differences are in line with the findings in the AGAHLS cohort, no conclusions may be drawn because these differences were never statistically significant or classifiable as a statistical trend ($p<0.10$). In girls in the Bonestaak cohort the number of ER22/23EK carriers was too low for reliable statistical analyses.

In line with the known hypersensitivity to glucocorticoids in BclI-carriers, we also found at young age in male carriers of BclI, associations with higher body weight, BMI and body fat mass. These associations were not found for carriers of the N363S or the GR-9β polymorphism. Remarkably we only found the associations between the BclI polymorphism and body composition in the heavier and fatter Bonestaak cohort and not in the leaner AGAHLS cohort. This led to the speculation that, at least for carriers of the BclI polymorphism, an obesogenous environment is necessary for a significant, measurable greater fat accumulation than in noncarriers. The concept that sustained environmental factors are important for the expression of these polymorphisms is supported by the absence of any associations between these polymorphisms and fetal and early postnatal anthropometry and growth characteristics in large recent studies in very young children.[19;20]

Estrogen receptor polymorphisms

Our study in children and adolescents showed that in the Bonestaak cohort, in girls, the ESR1 PvuII-XbaI haplotype 2 (-397 C and -251 G) was significantly associated with a reduction in body weight per allele copy (mean 1.65 kg / allele copy). The reduction in body weight could almost be fully explained by a reduction in fat mass (1,62 kg / allele copy). Homozygous carriers of PvuII-XbaI haplotype 2 had 3.2 kg less fat mass than subjects who did not carry this haplotype. These associations were statistically powerful and consistent at all four observational time points during the two year follow up.

In the AGAHLS however, we did not find these associations in similarly calendar aged young girls or boys. Because this cohort is of an older generation, also data were available at age 36 years. Also at 36 years no clear significant associations were found.

Previous studies on the associations between these polymorphisms and body composition are conflicting. The study of Fox et al. found lower waist circumference in haplotype 2 , which is in accordance with our findings, although they only found this association in men.[41] Also in another study homozygous carriers of haplotype 2 had lower FM and waist circumference during post-menopause, but not before menopause.[42] A positive association between the PvuII polymorphism and BMI was reported in post menopausal Caucasian women.[43] Later studies, especially in younger subjects, did not find any associations between PvuII and XbaI polymorphisms and body composition.[44;45]

Differences among the findings in these and our studies might be explained by methodological issues related to association studies (see also table 1 "pitfalls in polymorphism association studies" in chapter "Methodological considerations and limitations").

A more functional explanation could be that during puberty sex steroids increase from low pre-pubertal levels to high pubertal levels. Possibly ESRα gene polymorphisms have specific effects on estradiol sensitive targets like fat mass in this transition period, explaining the differences found in these studies, depending upon pubertal stage and therefore estradiol levels.

The findings in the Bonestaak cohort were powerful in the girls, and absent in boys. There are two possible explanations for this gender difference. Firstly, the circulating estradiol levels in puberty are much higher in girls than in boys. Possibly a slight difference in estradiol sensitivity may become only clinically relevant with increasing levels of estradiol. A second reason might be the fact that the higher testosterone levels in boys outweigh the estradiol effect on fat tissue.

Also in this study the clear associations among girls in the Bonestaak cohort were not found in the AGAHLS cohort. A possible explanation for the difference could be the risk of chance finding due to the lack of enough power in the AGAHLS cohort to find statistically significant differences, or to find associations by chance in case of the Bonestaak cohort. The latter seems not very likely though, because the associations were all quite strong and consistent over time. Furthermore, the same associations were found using different modes of measurements for fat mass: anthropometry and DEXA scan.

Another possible explanation could again be the difference in overall weight and fat mass between both cohorts; the Bonestaak cohort being heaver and having a higher fat mass than the AGAHLS cohort. Possibly the same associations are there in both cohorts, but can only be revealed in the more obese cohort, leading to a more pronounced fat accumulation in non-carriers of the ESRα haplotype 2, at least in girls.

Androgen receptor polymorphism

Also androgens are important regulators of linear growth and body composition in children, especially boys. In puberty androgens are responsible for the development of secondary sexual characteristics, linear growth spurt and several anabolic effects like promotion of muscle growth[46] and inhibition of subcutaneous fat deposition.[47] The androgen receptor (AR) is essential for signal transduction of androgens. The highly polymorphic CAG microsatellite repeat sequence in the androgen receptor has well known modulatory effects on androgen-dependant gene transcription: the basal and ligand-induced transactivational activity of the AR is inversely associated with the length of this CAG repeat chain.[48]

In our studies longitudinal height, measured as height-SD, was inversely associated with AR CAG repeat length in boys at young age. This association diminished in the following years and had completely disappeared after the age of 16 years. No associations were found with pubertal development, height velocity during growth spurt or final height. Also other studies in adults never found associations with adult height.[49] This inverse association of AR CAG repeat length with longitudinal height already existed before the onset of puberty, implying that this polymorphism only influences pre-pubertal growth. It is hypothesized that the slight androgen insensitivity in boys with long AR CAG repeat length results in difference in growth in pre-puberty under an immature hypothalamic-pituitary-gonadal axis and that further and compensatory development of the axis overrules these effects at later age.

In both cohorts no significant associations between AR CAG repeat number and the other measured parameters of body composition were found: weight, BMI, fat mass, fat free mass or waist to hip ratio. Conflicting findings have been reported on the associations of the AR CAG repeat length and body composition.[49-56] Several possible explanations can be generated about the lack of these associations in this study. Firstly, our cohorts are relatively small cohorts from a different genetic background than other studies. Possibly our cohorts lack enough power to detect any weak existing associations. Another explanation could be the fact that the used parameters are not specific enough for androgen or estrogen actions. The study of Nielsen et al.[49] for example, also could not detect any associations with weight, BMI, fat mass or lean body mass, but found robust associations with thigh and lower trunk muscle areas using magnetic resonance imaging (MRI). Furthermore, our cohorts are the youngest studied for the effects of the AR CAG repeat polymorphism so far. Possibly the effects of this polymorphism on phenotype, apart from longitudinal growth, have not become to full development yet, due to the very low androgen or estrogen levels at this age. Possibly further and full activation of the hypothalamic-pituitary-gonadal axis allows for further development of phenotypic differences over years due to the subtle differences in androgen sensitivity. Whether these differences are caused by direct androgen action or by indirect estrogen action still remains to be elucidated.

General summary of findings

The findings related to growth and body composition in the different investigated genetic polymorphisms are summarized in table 1.

Table 1 Summary of findings related to growth and body composition in the different investigated genetic polymorphisms in this thesis.

	cohort	
polymorphism	**AGAHLS**	**Bonestaak**
IGF-1 gene		
promoter 192/194bp CA repeat	♂↑ pubertal height adult height NA body comp NA	♂↓ pubertal height (trend) ♀ (♂ trend) ↓ weight, BMI, FM, WC
Glucocorticoid receptor gene		
ER22/23EK	♂↑ adult height, FFM, thigh circ, muscle strength ♀↓ WC (trend)	NA
BclI	NA	♂↑ weight, BMI, FM
N363S	NA	NA
9β	NA	NA
Estrogen receptor α gene		
PvuII-XbaI (haplotype 2)	NA	♀↓ weight, BMI, FM
Androgen receptor gene		
AR CAG repeat	♂↑ height with repeat length ↓ adult height NA body comp NA	♂↑ height with repeat length ↓ body comp NA

NA = no association; WC = waist circumference; body comp = body composition; thigh circ = thigh circumference.

9.4 Methodological considerations and limitations

Pitfalls in association studies in relation to our studies

(parts of this chapter are adapted from Van Rossum *et al.*[57])

The techniques to detect genetic variations, polymorphisms, in the human genome have developed rapidly in the recent years. Especially with the increasing use of high-

throughput techniques data are generated faster and faster.[58] These techniques improved the possibilities to search for polymorphisms in candidate genes in large numbers of individuals in a simple, cheap and fast manner. Association studies test whether a genetic polymorphism occurs more frequently in cases than healthy controls. Also, certain traits can be studied and compared between carriers and non carriers of a polymorphism. However, association studies have many limitations, which have to be accounted for when conducting these studies (table 2).[57]

Table 2 Pitfalls in polymorphism association studies.

• Lack of standardized genotyping
• Lack of standardized phenotyping
• Lack of statistical power
• Improper statistics
• Racial heterogeneity
• Population stratification (founder effect)
• Gender differences
• Age differences
• (no) functionality of the studied gene variation
• Publication bias

Because all studies in this thesis are based upon association studies in two Dutch cohorts, all pitfalls will be discussed in relation to our studies.

High quality association studies are very much dependant on reliable phenotyping. Participants have to be recruited very carefully to rule out a bias of incorrect inclusion. Phenotypic differences between studies may exist due to variable definitions for cases and controls in different studies, and the heterogeneous phenotypic expression of certain diseases.[59] Sensitivity and specificity of the methods used to characterize phenotype should also be taken into account. This applies to the observer who performs measurements, but also for the variance in measurement of routine parameters such as laboratory measurements. The occurrence of, frequently undetected, errors is a problem in large population studies, in which many steps of data-collection and many researchers are involved. These negative influences can be reduced by improving the requirements needed to assure high quality association studies (table 3).[57]

Table 3 Requirements to assure high quality of association studies.

• good phenotyping (careful recruitment of subjects and data collection, high sensitivity and specificity of tests, minimizing inter researcher variation, double check measured parameters)
• high number of individuals studied (depending on the frequency of the studied gene variant)
• homogeneity of the study population, with respect to ethnicity, gender, age and environmental factors or using statistical corrections for these confounders.
• replication in different independent study populations
• a good rationale for the association under investigation to increase the a priori justification
• statistical analysis using multiple testing corrections
• confirming "in vivo" results in "in vitro" experiments
• unravelling the molecular basis of the mechanism of the studied gene variation
• Publication bias

In our cohort studies we investigated two well defined cohorts of healthy children in the area of Amsterdam and around Amsterdam. Subjects were recruited from several schools in and around Amsterdam. All children willing to participate were included. For our studies only Caucasian and healthy subjects were included. Both groups are similar with regard to socio-economic, geographic and genetic background. A major difference between both cohorts is the generational difference of about 20 years: the AGAHLS cohort started in 1976 at an age of 13 years, whereas the Bonestaak cohort started in 1995 at age 9 for girls and in 1998 at age 10 for boys. Parameters of growth, body composition and puberty were assessed by a minimum well trained staff of experts with high quality tools and well defined methods for measurements. Assessments of bone age were performed by one well-trained examiner and DEXA scanning was performed by the same machine of which the reproducibility was known. Skin fold measurements were available for both cohorts; DEXA scans only for the Bonestaak cohort, but fat mass calculations were comparable in the Bonestaak cohort for both skin fold measurements as DEXA measurements. Another difference between both cohorts was the availability of exact pubertal staging in the Bonestaak cohort, which was not available in the AGAHLS cohort. In the AGAHLS cohorts on the other hand, bone age assessments were available. To correct for pubertal stage, in the AGAHLS cohort, bone age was used as a marker for pubertal stage.

A drawback of both our cohorts is the relatively low number of individuals studied. Results would be more robust with higher numbers of participants. We often found statistical trends, but larger cohorts and replication are needed to exclude false

positive findings. The same accounts for borderline significant effects, which in a relatively small cohort are more likely to be the result of chance than in a large cohort. Large, well defined, young cohorts are difficult to collect and are rather rare compared to older cohorts.

A widely discussed topic is the problem of multiple testing. It is recognized that when performing a large number of statistical tests, the rate of false positive results (type I error) is relatively high.[60] One way to diminish this problem is to statistically correct for multiple testing, which we did in our studies. However, other factors can also help to reduce the amount of false positive results:

a) A good rationale for the association study, creating a high a priori justification, will increase the chance of finding true positive results. The polymorphisms we investigated are all quite well known polymorphisms which have been described in (adult) literature. All polymorphisms are located in endocrine genes, which are important for the production and/or function of several hormones in growth and body composition in children (IGF-1, glucocorticoids, androgens and estrogens). The functionality of these polymorphisms has been confirmed or speculated on in several reports.[27;33;34;36;52;61-66] Especially in vitro testing may help to distinguish between functional and non-functional polymorphisms.[67-69] Also the location of the polymorphism within the gene is important with respect to functionality. Polymorphisms leading to an amino acid change are more likely to have functional effects, but also microsatellite repeat variations are known to have functional effects.

Functionality of intronic polymorphisms remains a difficult issue. Intronic polymorphisms are often considered as non-functional, because they do not change the coding sequence. However, they can still be involved in the splicing process for example by changing the sequence of so-called intronic splicing silencers or enhancers or other mechanisms important for the expression of the gene.[70]

b) Confirmation of an observed association in a different study population diminishes the risk of findings by chance. However, it is important to realize that polymorphisms can exert different effects in different ethnicities. First, the frequency of polymorphisms is known to differ between ethnic groups. Second, the presence of a combination with other polymorphic genes, which is highly variable between ethnicities, can also lead to a different phenotype. Third, different environmental factors can contribute to the effects of a certain polymorphism on the phenotype. Furthermore, a "founder effect" can be a problem of association studies. This is the tendency that within populations high frequencies of both certain genes as well as certain diseases are present, possibly leading to false positive associations. Larger sample sizes, as well as studies with family-based controls can help to avoid this problem.[71]

Besides ethnicity, also factors as gender and age have to be taken into account when interpreting association studies: sex-specific or age-dependant associations of polymorphisms are well known. We also found several age- and gender specific

results in our studies. The reason why different associations with respect to growth and body composition are found in boys and girls remains unclear, although sex-specific hormonal factors may play a role in these differences.

Publication bias: journals tend to publish rather positive than negative results.[72] However publication bias seems not very likely to account for the inconsistency in the reproducibility of association study results.[71] More likely underpowered non-significant studies of real associations with modest genetic effects can reasonably account for much of the variability in replication.

In the recent years a shift towards whole genome association studies has become apparent. But also for these studies careful study design remains very important.

Genome wide association studies (GWAS) and birth weight

Large scale genome wide association studies have very recently brought new insights on the associations of genetic markers with birth weight. Meta-analysis of six genome-wide association studies in European pregnancy and birth cohorts found two loci near CCNL1 (encoding cyclin L1) and at ADCY5 (encoding adenylyl cyclise 5) in the fetal genotype, which were associated with birth weight in the general population.[73] The causal mechanisms are not yet known, but the ADCY5 locus been linked to glucose regulation and type 2 diabetes in adulthood, suggesting that the widely described association between lower birth weight and increased risk for type 2 diabetes has a genetic component.

Although these findings are very promising, it needs to be said that these two loci explained only 0.3% and 0.1% of the variance in birth weight, respectively. Given the fact that the fetal genetic contribution to birth weight is generally estimated between 10% and 40% according to twin and family studies,[74;75] much of the heritability of weight at birth is not fully understood.

GWAS and obesity

Many hypotheses have been proposed to explain the origin of the obesity epidemic in adults and children. Although the impact of changes in lifestyle and environment are likely to be significant, it is clear that obesity has a large underlying genetic component. Heritability estimates up to 0.81 for weight and BMI were found in several large studies.[76-79] The identification of a significant number of genes in rare forms of obesity has not translated to an explanation of the genetics underlying common obesity. This is a consequence of the fact that common obesity is polygenic with no simple Mendelian inheritance pattern.

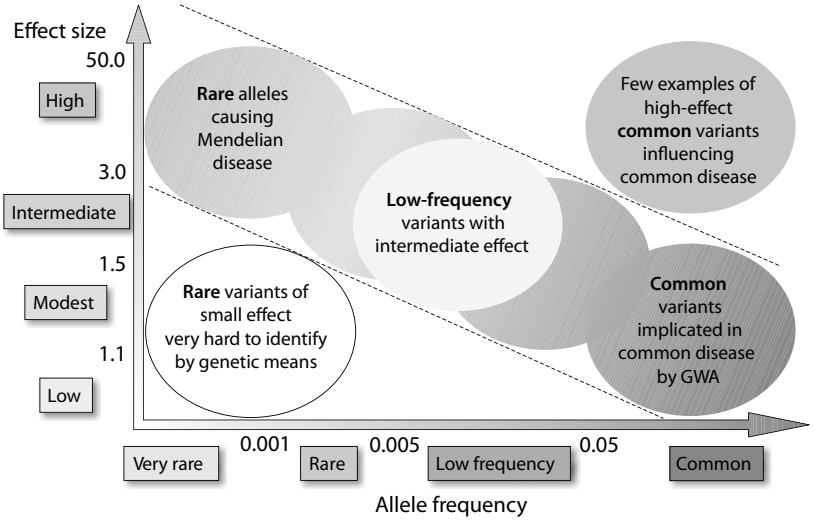
Several methods of identifying genes linked to obesity have been used, of which family-based (genome wide) linkage studies and candidate gene association studies have revealed many loci associated with common obesity.[80-82] However, replication of these results has proved very difficult. Recent meta-analyses of many studies, including

thousands of subjects, did not detect strong evidence for linkage for BMI or BMI-defined obesity at any locus.[83;84] The failure to replicate associations at many loci in studies of common disease suggests that the common variants only exert small genetic effects and although it is clear that candidate gene association studies have not provided unequivocal results, the evidence is strong enough and replicated enough times to suggest that many of these genes contain variants that have a modest effect on obesity. For example, even the most strongly associated gene variant so far, the rs9939609 SNP in the FTO-gene, has only a 1% effect on the variance observed in BMI.[85]

Unlike candidate gene approach studies, genome wide association study (GWAS) is a hypothesis-free approach, using a case-control design to increase the chances of recruiting large numbers of subjects and thereby enhancing statistical power. The GWAS approach has increased the known number of obesity-associated markers.[29;82;85-91] However, it is becoming clear that there is only a small overlap between found genes with genetic linkage and GWAS.[82] A potential explanation is that the linkage signals may be due to DNA variations of strong effect that are relatively infrequent compared with the observed associations from GWAS, which are statistically robust and common but are not strong effects (figure 1).[92] This was recently highlighted by the finding of the association between rare copy number variants (CNV's) with severe early-onset obesity in relatively small, but well defined cohorts.[93] The latest publication on a large meta-analysis on GWAS of BMI confirmed 32 BMI loci, explaining only 1,45 % of the interindividual variation in BMI.[91] In this study it is estimated that, if accounted for yet undiscovered variants with similar properties, these common variant signals account for only 6% - 11% of the genetic variation in BMI. The additional variance and biology of BMI will only be explained using other or complementary approaches that capture variants, such as lower frequency variants and short insertion-deletion polymorphisms. In addition, recent, large meta-analysis on GWAS of waist-hip ratio confirmed 14 loci associated with waist-hip-ratio.[29] Remarkably these loci are largely distinct from and show no overlap with those that influence BMI and risk of obesity, showing that BMI and fat distribution have different genetic backgrounds. Another interesting finding is the fact that fat distribution showed a largely genetic sexual dimorphism, with a greater genetic contribution to the overall variance in WHR in women than in man.[29]

This means that various study designs add value to the knowledge of the genetics of obesity, depending not only on sample size, but also on study design and especially on the phenotypic markers being studied.

Figure 1 Feasibility of identifying genetic variants by risk allele frequency and strength of genetic effect (odds ratio). Most emphasis and interest lies in identifying associations with characteristics shown within diagonal dotted lines. Adapted from McCarthy and Manolio.[92;94]



The large reported GWAS on BMI and WHR did not report associated loci which were in close vicinity to our studied polymorphisms.[29;91] Our studies were not very large, highly powered, studies using GWAS. Instead we used two very well defined cohorts of children and adolescents in which we performed a specific hypothesis approach, based on known or very likely functional polymorphisms in diverse endocrine genes. In addition, not only weight and BMI were used as markers of overweight or obesity (as used in most GWAS), but also fat mass and lean body mass were used. Furthermore two of our studied polymorphisms were microsatellite repeat sequences (IGF-1 promoter CA repeat sequence and the AR CAG repeat sequence), which will probably with the current GWAS techniques not be picked up (see also "limitations of current GWAS").

GWAS and height

Heritability of human height has been reported to be 80-90%, which is one of the highest among human physical quantative traits. Very recent GWAS have detected an increasingly amount of genetic loci associated with human height.[95-102] A very recent

meta-analyses of GWAS data from 46 studies, showed that hundreds of genetic variants, in at least 180 loci, influence adult height.[98] These data explain approximately 10% of the phenotypic variation in height. Even larger studies will be able to improve this potential explanation, although it is not expected that GWAS will be able to explain the full heritability of height by far. Additional approaches, including those aimed at less common variants, will likely be needed to dissect more completely the genetic component of complex human traits like height.

The strength of these huge studies is not only just the finding of a possible genetic background of body growth, but these studies are also able to find biologically relevant pathways and mechanisms. For example the 180 loci associated with height appear to be non-randomly clustered within biologically relevant pathways and are enriched for genes that are involved in growth-related processes, that underlie syndromes of abnormal skeletal growth and that are directly relevant to growth-modulating therapies (GH1, IGF1R, CYP19A1, ESR1). The large number of loci with clearly identified relevant genes suggests that the remaining loci could provide potential clues to important and novel biology.[98]

Our small studies in 2 well defined cohorts found modest associations with growth and body height for the IGF-1 microsatellite 192bp/194bp CA-repeat polymorphism, for the ER22/23EK variant in the glucocorticoid receptor gene and the CAG microsatellite repeat in the androgen receptor gene.

In the large GWAS, the IGF-1 locus was only identified as associated with height in a large Japanese study and not in the Caucasian population.[100] In our studies no clear association with final height was found, but only in the growth phase, which was not studied in the larger GWAS.

The AR-gene also was not found in the large GWAS as an important (final) height locus. Also in our study the CAG microsatellite repeat in the AR gene was not found to be associated with final height. An association was found in boys with growth before the onset of puberty, which was not taken into account in the large GWAS.

Another explanation for not finding associations in the GWAS for these genes is the fact that these polymorphisms were microsatellite repeat sequences, which will, with the current GWAS techniques, probably not be picked up (see also "limitations of current GWAS").

Also the glucocorticoid receptor locus was not found in the large GWAS as a height locus, although we found a clear association with a higher final height in male ER22/23EK carriers. Possibly the frequency of ER22/23EK carriers in the population (8%) is too low for the detection in GWAS.

GWAS and puberty

GWAS on the timing of puberty have primarily focussed on age at menarche, probably because of the well defined and easy to remember moment for almost every girl or

woman. Twin and family studies estimate that at least 50% of the variance in age at menarche is heritable.[103;104] Very recently four separate GWAS identified common variants in the height-related gene, LIN28B, on chromosome 6 as having significant associations with menarche timing.[105-108] This allele was also associated with earlier breast development in girls, earlier voice breaking and more advanced pubic hair development in boys, a faster tempo of height growth in girls and boys and shorter adult height.[107] Also some genomic regions related to adult body mass seem to affect menarcheal age.[105;106] Less definitively, some other loci also associated with age at menarche. Large-scale collaboration between these and other groups is in progress, and it is expected that many more novel loci for timing of puberty will be identified. Our studies did not reveal associations between any of the investigated polymorphisms and several markers of pubertal development. Our investigated polymorphisms were not closely related to the recently reported loci in the GWAS concerning pubertal timing.

Limitations of current GWAS

In GWAS several hundred thousand to more than a million SNPs are assayed in thousands of individuals. They represent a powerful tool for investigating the genetic architecture of complex diseases. These studies have been facilitated by the development of commercial "SNP chips" or arrays that capture most, although not all, common variations in the genome. As already mentioned before, although GWAS have identified hundreds of genetic variants associated with complex human diseases and traits, these variants only explain small increments in risks, and explain only a small proportion of familial clustering. Suggested explanations for this missing heritability are:[92]

1) Much larger numbers of variants of smaller effect are yet to be found to explain more of the missing heritability.
2) Rarer variants, with possibly larger effects, are poorly detected by available genotyping arrays that focus mainly on variants present in 5% or more of the general population (see also figure 1).
3) Structural variants are poorly captured by existing GWAS arrays. These variants include copy number variants (insertions, deletions, inversions, translocations and microsatellite repeat expansions) and copy neutral variations (inversions, translocations, complex rearrangements).
4) Low power to detect gene-gene interactions.
5) Inadequate accounting for shared environment among relatives.

Of our investigated polymorphisms two variants (ER2223EK and N363S in the GR gene) have a low frequency in the population (4 – 8%), which is probably not picked up with current GWAS techniques. Furthermore two of our studied polymorphisms

were microsatellite repeat sequences (IGF-1 promoter CA repeat sequence and the AR CAG repeat sequence), which also will probably not be picked up with current GWAS techniques. The latter problem may be solved by looking for a strongly linked SNP allele in the close vicinity of the repeat sequence. Using this strongly linked SNP, microsatellites may be picked up using GWAS.

9.5 Future perspectives

Birth weight

The relation between intrauterine growth retardation, birth weight, growth, body composition, genetic factors and the development of cardiovascular risk factors is complex. The aetiology of SGA is diverse, including underlying genetic and environmental causes. The results of our studies only hint at possible relations between several genetic polymorphisms and birth weight and SGA related outcomes. Also other studies have not been able to find important genetic variations in the genes which may serve as predictors for postnatal growth and metabolism. Not only larger studies are needed to confirm our findings; also studies in children born SGA with more detailed phenotypic descriptions, including groups with and without catch up growth, are needed to differentiate between SGA subgroups. Reliable sub typing in SGA subjects may possibly improve focused genetic research in children born SGA.[109]

Growth and body composition

Longitudinal growth and the development of body composition during childhood and adolescence are influenced by important genetic but also environmental factors. Our studies showed that common variations in several endocrine genes have small, but significant influences on growth and body composition. We also showed that environmental factors are likely to interact with these genetic factors and that the magnitude of the genetic influence might depend on the magnitude of environmental factors. In order to further elucidate these findings, these studies need to be repeated in (much) larger cohorts. Also large specific cohorts, for example comparing (extreme) obese groups with non-obese groups, might possibly magnify the influences of these polymorphisms. Furthermore better description of environmental and lifestyle factors in investigated cohorts is needed to differentiate between non-genetic influences. Because weight, BMI, total fat mass and waist/hip ratio are rather non-specific markers of body composition, more specific techniques are needed to improve the physical phenotype to be studied. For example by using advanced techniques like photonic scanners,[110] air-displacement plethysmography,[111] CT and MRI[112] and ultrasonography.[113]

Much attention in the field of obesity and body composition research has shifted

towards genome wide association studies. But also these GWAS only identified minor contributions to the genetic effects in obesity and body composition. Larger studies are needed, using novel phenotypes, rare SNP's, SNP's linked to microsatellite repeat sequences and studies on heritable changes in the genome (epigenetics) to further elucidate the mechanisms involved in our changing body composition in an ever changing environment from birth to adulthood.

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10

Summary
Samenvatting

Summary

Chapter 1 provides background on the different factors important for fetal growth and longitudinal growth and body composition in childhood and adolescence. Associations between size at birth and later risks for several diseases, like insulin resistance and cardiovascular diseases, are described. Body composition in childhood, especially overweight and obesity, as a marker for increased risk for early mortality and severe illnesses is discussed. The concept that both environmental factors as genetic factors are important for growth and body composition and for future diseases is discussed.

A general introduction in physiology and molecular mechanisms is provided for IGF-1, glucocorticoids, estrogens and androgens. Background is provided on the studied genetic polymorphisms: an IGF-1 promoter polymorphism, glucocorticoid receptor gene polymorphisms, estrogen receptor α gene polymorphisms and the androgen receptor CAG repeat polymorphism. The aim of this thesis was to study the role of these polymorphisms in relation to growth and body composition in healthy children and adolescents and in a subgroup of children born small for gestational age (SGA).

In **chapter 2** the influence of the CA-repeat polymorphism in the IGF-1 gene promoter region on birth weight was studied as well as the influence of this polymorphism on the relation between birth weight and risk factors for cardiovascular disease and diabetes. Results showed that male variant carriers of the IGF-1 polymorphism had lower birth weight than men with two wild type alleles. Of the risk factors for cardiovascular disease, male variant carriers at age 36 years had slightly higher LDL cholesterol concentrations than wild type carriers. Furthermore it was found that variant carriers of the polymorphism showed a stronger association between birth weight and risk factors for cardiovascular disease and T2DM than subjects with two wild type alleles. This was most evident for systolic blood pressure and diastolic blood pressure: in variant carriers 1 kg lower birth weight was related to an 8.0 mm Hg higher systolic blood pressure at age 36 years, compared to 1.5 mm Hg in the wild type carriers.

In **chapter 3** associations between 4 GR haplotypes and size at birth in a Dutch healthy reference cohort, the Bonestaak cohort, were investigated. No associations were found between any of the haplotypes and birth weight or birth length. In addition a group of children born small for gestational age (n=119), without catch-up growth was investigated before the start of growth hormone therapy. Prevalence of GR haplotype 2 (BclI) was significantly lower in the SGA group compared to controls. It is concluded that genetic variance in the GR seems not to be associated with

intrauterine growth in the investigated healthy cohort. However, GR haplotype might play a role in postnatal growth of children born SGA, reflected by the decreased prevalence of GR haplotype 2 in this group without catch-up growth.

In **chapter 4** possible associations between growth and body composition and the CA-repeat polymorphism in the IGF-1 gene promoter region were investigated in two comparable young healthy cohorts. The two study groups differed for the fact that they were born with a generational difference of around 20 years. In the youngest cohort (Bonestaak), body weight, BMI, fat mass and waist circumference were significantly higher in female variant carriers of the IGF-1 CA-repeat polymorphism, although the differences were quite small. A similar trend was observed in male variant carriers. These differences were not observed in the older cohort (AGAHLS). Irrespective of genotype, the younger cohort showed overall a significantly higher fat mass, body weight and BMI compared with the older cohort; a phenomenon which is known quite well in most young Western populations. Because the differences between both genotypes were quite small, it was concluded that the genetic variability due to this IGF-1 polymorphism impacts only slightly on body composition. Because the associations were only found in the most recent, heavier and fatter cohort, it is suggested that the associations between this IGF-1 polymorphism and body composition might reflect a gene-environmental interaction of this polymorphism and that an environment that promotes obesity leads to a slightly more pronounced fat accumulation in variant carriers of this IGF-1 polymorphism.

In **chapter 5** the role of the ER22/23EK, or R23K, polymorphism in the glucocorticoid receptor gene in body composition is described. On the basis of previous findings in elderly of relative glucocorticoid resistance and a better metabolic condition in carriers of the ER22/23EK polymorphism, it was hypothesized that this polymorphism might also be related to the regulation of body composition. Therefore we analysed data from the AGAHLS cohort from the age of 13 years until 36 years. In young adult males we found ER22/23EK-carriers to be taller, have more lean mass, and greater thigh circumferences (indicator of muscle mass), as well as increased muscle strength when compared to noncarriers. This phenotype was also present during puberty, however marked differences could only be detected at adult age. In females, waist and hip circumferences tended to be smaller, suggestive of less fat mass, but no differences in BMI or total fat mass were found. Thus, the ER22/23EK polymorphism seems to be related to a sex-specific, beneficial body composition at young adult age.

Chapter 6 further describes the relationships of four well known GR polymorphisms (ER22/23EK, N363S, BclI, 9β) with growth and body composition in two comparable young Dutch cohorts; the Bonestaak cohort (n=235) and the AGAHLS cohort (n=284).

In boys in the Bonestaak cohort, the G-allele of the Bcl1 polymorphism (haplotype 2) was associated with a higher body weight, weight-SD, BMI, BMI-SD and fat mass. For haplotype 3 (GR-9β) and haplotype 4 (N363S) no associations with body composition were found in the two cohorts. The results for ER22/23EK were discussed in chapter 5. Because the associations between the G-allele of the Bcl1 polymorphism and body fat mass were only found in the most recent Bonestaak cohort, but not in the comparable, generally leaner cohort from an older generation, it is speculated that carriers of this polymorphism are likely to be more vulnerable to fat accumulation in today's obesity promoting environment, than non-carriers.

Gender differences in body composition are largely explained by differences in sex hormones, such as estrogens. In **chapter 7** the role of 2 polymorphisms in the estrogen receptor α gene (ESR1) and body composition in puberty and at young adult age is described in the two comparable young Dutch cohorts; the Bonestaak cohort (n=311) and the AGAHLS cohort (n=256). In girls in the Bonestaak cohort, the PvuII-XbaI haplotype 2 polymorphism in the ESR1 gene was associated with a lower body weight, weight-SD, BMI, BMI-SD and FM. These associations were not observed in the AGAHLS cohort. Because the associations between the PvuII-XbaI haplotype 2 polymorphism and body FM in girls were only found in the most recent Bonestaak cohort, but not in the comparable, generally leaner AGAHLS cohort from an older generation, it is suggested that female non-carriers of this polymorphism are likely to be more vulnerable to fat accumulation in today's obesity promoting environment, than female carriers.

It is known that the number of CAG repeats within the CAG repeat polymorphism of the androgen receptor gene correlates inversely with androgen receptor activity and the strength of androgen action. In **chapter 8** the role of the CAG repeat polymorphism is investigated in relation to longitudinal growth, puberty and body composition from pre-puberty until young adult age in both the Bonestaak cohort (n=244) and the AGAHLS cohort (n=226). Height-SD scores were inversely associated with AR CAG repeat length in boys at young, pre pubertal and early pubertal age. This association diminished in the following years and completely disappeared after the age of 16 years. No associations were found with pubertal stage or any of the other parameters for body composition. It was concluded that the AR CAG repeat length is inversely associated with longitudinal height in young boys, already before the onset of puberty. During puberty these differences disappear.

In **chapter 9** the results of the studies described in this thesis are discussed in relation to the current literature and put into broader perspective. Methodological considerations and limitations are discussed, especially with regard to the pitfalls in

association studies. Furthermore, recent findings in large genome wide association studies (GWAS) with regard to birth weight, obesity, longitudinal height and puberty are discussed and compared to our findings. Future perspectives are discussed.

Samenvatting

Hoofdstuk 1 beschrijft de achtergrondinformatie van verschillende factoren, die van belang zijn voor foetale groei en lengtegroei en lichaamssamenstelling bij kinderen en adolescenten. De verbanden tussen geboortegewicht en geboortelengte en de risico's op latere ziekten, zoals insulineresistentie en cardiovasculaire aandoeningen, worden uiteengezet. Er wordt dieper ingegaan op het belang van lichaamssamenstelling op de kinderleeftijd, en dan in het bijzonder overgewicht en obesitas, als belangrijke factoren voor een verhoogd risico op ernstige ziektes en vroegtijdige dood. Tevens wordt de invloed van zowel omgevingsfactoren als genetische factoren op groei, lichaamssamenstelling en risico op toekomstige aandoeningen besproken.
Verder wordt een algemene inleiding gegeven over fysiologie en moleculaire mechanismen voor IGF-1, glucocorticoiden, oestrogenen en androgenen. Meer in detail wordt ingegaan op de onderzochte genetische polymorfismen: één polymorfisme in de promoter regio van het IGF-1 gen, vier polymorfismen in het glucocorticoid receptor gen, twee polymorfismen in het oestrogeen receptor α gen en het CAG repeat polymorfisme in het androgeen receptor gen. Doel van het onderzoek dat aan dit proefschrift ten grondslag ligt, was het beschrijven van de betekenis van de genoemde polymorfismen in relatie tot groei en lichaamssamenstelling bij gezonde kinderen en adolescenten en in een subgroep van kinderen met een te laag geboortegewicht voor de zwangerschapsduur.

Hoofdstuk 2 beschrijft het onderzoek naar de relatie tussen het CA-repeat polymorfisme in de promoter regio van het IGF-1 gen en geboortegewicht in een cohort gezonde jongens en meisjes (AGAHLS cohort). Tevens werd de invloed onderzocht van dit polymorfisme op de relatie tussen geboortegewicht en risicofactoren voor diabetes mellitus type 2 en hart- en vaatziekten. Alleen manlijke dragers van het zogenaamde variant polymorfisme ("variant carriers") van het IGF-1 gen bleken een lager geboortegewicht te hebben dan mannen met twee zogenaamde "wild type" allelen. Op de leeftijd van 36 jaar hadden manlijke "variant carriers" iets hogere LDL cholesterol spiegels in het bloed dan dragers van het "wilde type". Verder bleken "variant carriers" van het IGF-1 polymorfisme een sterker verband te hebben tussen geboortegewicht en risicofactoren voor hart- en vaatziekten en type 2 diabetes mellitus dan mensen met twee "wild type" allelen. Dit was het duidelijkst bij de systolische en diastolische bloeddruk: op de leeftijd van 36 jaar was bij "variant carriers" 1 kg lager geboortegewicht gerelateerd aan 8.0 mm Hg hogere systolische bloeddruk, vergeleken met 1.5 mm Hg bij de wild type dragers.

Hoofdstuk 3 beschrijft het onderzoek naar associaties tussen 4 GR haplotypes en geboortegewicht en geboortelengte in een gezond Nederlands referentie cohort

(Bonestaak). Er werden geen associaties gevonden tussen de verschillende haplotypes en geboortegewicht of geboortelengte. Ook werd onderzoek gedaan in een groep kinderen, die met een te kleine lengte en/of laag gewicht ten opzicht van de zwangerschapsduur werden geboren (SGA) en die daarna te klein waren gebleven (n=119). Het aandeel (prevalentie) van GR haplotype 2 (BclI) was significant lager in de SGA groep zonder inhaalgroei, vergeleken met de referentiegroep. De conclusie is dat de genetische variatie in het glucocorticoid receptor gen niet geassocieerd is met intra-uteriene groei in het onderzochte, gezonde, cohort. Echter, het GR haplotype zou wel van belang kunnen zijn voor de postnatale groei bij kinderen die SGA geboren zijn.

In **hoofdstuk 4** wordt het onderzoek naar de mogelijke associaties tussen groei en lichaamssamenstelling en het CA-repeat polymorfisme in de promoter regio van het IGF-1 gen beschreven in twee vergelijkbare cohorten met jonge en gezonde proefpersonen. Het grote verschil tussen beide cohorten is het generatieverschil van ongeveer 20 jaar. In het jongste cohort (Bonestaak) waren gewicht, BMI, vetmassa en tailleomtrek significant hoger bij meisjes die drager waren van het "variant gen" van het IGF-1 CA repeat polymorfisme; de verschillen waren echter vrij klein. Een zelfde trend bestond bij jongens die drager waren van het "variant gen". Deze verschillen tussen dragers en niet-dragers van het "variant-gen" werden niet gevonden in het oudere cohort (AGAHLS). Onafhankelijk van het genotype bleek het jonge cohort in zijn geheel zwaarder te zijn, een hogere BMI te hebben en een hogere vetmassa te hebben dan het oudere cohort. Dit fenomeen zien we in de meeste jonge Westerse populaties. Omdat de verschillen tussen beide genotypes vrij klein zijn, is de conclusie dat de genetische variatie door het IGF-1 polymorfisme waarschijnlijk slechts een kleine invloed heeft op de lichaamssamenstelling. Omdat de associatie alleen werd gevonden in het meest recente, zwaardere en vettere cohort, ligt de suggestie voor de hand, dat de associatie tussen dit IGF-1 polymorfisme en lichaamssamenstelling door een gen-omgevingsinteractie wordt gevormd. Mogelijk leidt een omgeving die aanzet tot obesitas, tot meer vetopbouw bij dragers van het "variant-gen" van dit IGF-1 polymorfisme dan bij niet-dragers.

Hoofdstuk 5 beschrijft de rol die het ER22/23EK, of R23K, polymorfisme heeft in relatie tot lichaamssamenstelling. Op basis van eerdere bevindingen bij ouderen, dat dragers van het ER22/23EK polymorfisme een relatieve resistentie tegen glucocorticoïden hebben en een betere metabole conditie hebben, werd de hypothese geformuleerd dat dit polymorfisme ook gerelateerd zou kunnen zijn aan de regulatie van de lichaamssamenstelling. Om dit te bestuderen werd het AGAHLS cohort onderzocht, dat gevolgd en getest werd van de leeftijd van 13 jaar tot 36 jaar. In jong volwassen mannen vonden we dat ER22/23EK-dragers gemiddeld langer zijn, meer

spiermassa en een grotere dijbeenomtrek (ook een indicator voor hoeveelheid spiermassa) hebben in vergelijking tot niet-dragers. Daarnaast bleken deze mannelijke dragers ook sterker te zijn dan niet-dragers. Deze verschillen waren al gedurende de puberteit in enige mate waarneembaar, echter duidelijke verschillen werden pas duidelijk op volwassen leeftijd. Bij vrouwelijke ER22/23EK-dragers zagen we een neiging tot kleinere omtrek van heup en taille, wat suggestief is voor een lagere vetmassa, al vonden we geen verschillen in BMI. Concluderend is het ER22/23EK polymorfisme geassocieerd met een sexe-specifieke, gunstige lichaamssamenstelling op jong volwassen leeftijd.

In aanvulling op de vorige studie worden in **hoofdstuk 6** de relaties onderzocht van vier bekende GR polymorfismen (ER22/23EK, N363S, BclI, 9β) met groei en lichaamssamenstelling in twee vergelijkbare, jonge Nederlandse cohorten; het Bonestaak cohort (n=235) en het AGAHLS cohort (n=284). Bij de jongens in het Bonestaak cohort bleek het G-allel van het BclI polymorfisme (haplotype 2) geassocieerd met een hoger lichaamsgewicht, gewicht-SD, BMI, BMI-SD en vetmassa. Bij dragers van haplotype 3 (GR-9β) en haplotype 4 (N363S) werden in geen van beide cohorten verbanden aangetoond met lichaamssamenstelling. De resultaten van ER22/23EK werden al grotendeels bediscussieerd in hoofdstuk 5. Aangezien de associatie tussen het G-allel van het BclI polymorfisme en vetmassa alleen werd gevonden in het meest recente Bonestaak cohort, en niet in het vergelijkbare, maar over het algemeen minder vette cohort van een oudere generatie, is het mogelijk dat dragers van dit polymorfisme gevoeliger zijn voor vetstapeling in de hedendaagse obesitas stimulerende omgeving, dan niet dragers.

Verschillen in de secretie van geslachtshormonen, zoals oestrogenen, verklaren voor een groot deel verschillen in lichaamssamenstelling tussen mannen en vrouwen. In **hoofdstuk 7** wordt de rol beschreven van 2 polymorfismen in het oestrogeen receptor α gen (ESR1) in relatie tot lichaamssamenstelling tijdens de puberteitsperiode en jong volwassen leeftijd in de twee vergelijkbare, jonge, Nederlandse cohorten: het Bonestaak cohort (n=311) en het AGAHLS cohort (n=256). Bij meisjes in het Bonestaak cohort werd een associatie gevonden tussen het PvuII-XbaI haplotype 2 polymorfisme in het ESR1-gen en een lager lichaamsgewicht, gewicht-SD, BMI, BMI-SD en vetmassa. Deze associaties werden niet gevonden in het AGAHLS-cohort. Aangezien de associaties tussen het PvuII-XbaI haplotype 2 polymorfisme en vetmassa bij meisjes alleen werden gevonden in het recentere Bonestaak cohort, maar niet in het vergelijkbare, maar over het algemeen minder vette AGAHLS-cohort van een oudere generatie, is het mogelijk dat vrouwelijke niet-dragers van dit polymorfisme gevoeliger zijn voor vetstapeling in de hedendaagse obesitas stimulerende omgeving, dan dragers.

Het is bekend dat een toename van het aantal CAG repeats, binnen het CAG repeat polymorfisme van het androgeen receptor gen, correleert met een afname van androgeen receptor activiteit en de sterkte van androgeen effect. In **hoofdstuk 8** wordt de rol van het CAG repeat polymorfisme onderzocht in relatie tot lengtegroei, puberteit en lichaamssamenstelling vanaf de prepuberteit tot jong volwassen leeftijd in zowel het Bonestaak cohort (n=244) als het AGAHLS cohort (n=226). Een toename van AR CAG repeat lengte bleek geassocieerd met een afname van de lengte-SD bij jongens op jonge, prepuberale en vroegpuberale leeftijd. Deze associatie bleek af te nemen in de navolgende jaren en was compleet verdwenen na de leeftijd van 16 jaar. Er werden geen verbanden gevonden met puberteits stadium en de diverse parameters voor lichaamssamenstelling. De conclusie is dat een kortere AR CAG repeat lengte geassocieerd is met grotere lengtegroei bij jongens vóór de start van de puberteit. Tijdens de puberteitsontwikkeling verdwijnen deze verschillen.

In **hoofdstuk 9** worden de resultaten van de verschillende studies in dit proefschrift bediscussieerd in relatie tot de huidige literatuur. De bevindingen worden tevens in een breder perspectief geplaatst. Methodologische overwegingen en beperkingen worden besproken, vooral met betrekking tot de valkuilen van het uitvoeren van associatie studies. Recente bevindingen in grote "genoom brede" associatie studies worden bediscussieerd, in het bijzonder in relatie tot geboortegewicht, overgewicht en obesitas, lengtegroei en puberteit. Deze resultaten worden vergeleken met onze bevindingen. Suggesties voor de toekomst worden besproken.

Dankwoord
Curriculum vitae
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Dankwoord

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Teun, jij bent papa's kleinste reus: nu gaan we vaker voetballen.

Curriculum vitae

Paul Voorhoeve werd geboren op 1 april 1967 te Leiden. In 1985 behaalde hij het VWO diploma aan de Rijks Scholengemeenschap te Epe. In 1985 begon hij met de studie geneeskunde aan de Rijks Universiteit Groningen. Tijdens zijn opleiding deed hij wetenschappelijk onderzoek aan de afdeling Obstetrie en Gynaecologie van het Academisch Ziekenhuis te Groningen (hoofd prof.dr. J.G. Aarnoudse). Aan het eind van zijn opleiding deed hij een klinische stage in verschillende ziekenhuizen in Ghana, West-Afrika. Het artsexamen werd behaald in 1993. Na een kort vakantie-assistentschap op de afdeling kindergeneeskunde van ziekenhuis de Weezenlanden te Zwolle, was hij werkzaam als arts-assistent kindergeneeskunde op de Neonatale Intensive Care Unit van het Universitair Medisch Centrum St Radboud te Nijmegen (hoofd dr. L.A.A. Kollée) tot eind 1993. Begin 1994 startte hij zijn werkzaamheden als arts-assistent kindergeneeskunde in het Wilhelmina Kinderziekenhuis te Utrecht, alwaar hij in 1995 startte met zijn opleiding tot kinderarts (opleiders prof.dr. J.W. Stoop, prof.dr. A. Okken, prof.dr. J.L.L. Kimpen). De opleiding in de tweede lijn werd gevolgd in het Catharina Ziekenhuis te Eindhoven (opleider dr. J.J.J. Waelkens). In juli 1999 vond registratie plaats als kinderarts en aansluitend volgde hij een fellowship kinderendocrinologie aan het VU Medisch Centrum te Amsterdam (opleider prof.dr. H.A. Delemarre-van de Waal). Tijdens zijn fellowship deed hij in 2001 een laboratoriumstage op het laboratorium Interne Geneeskunde, sectie Endocrinologie, van het Erasmus Medisch Centrum te Rotterdam, onder leiding van prof.dr. S.W.J. Lamberts en dr. J.W. Koper. De eerste ervaringen en bevindingen tijdens deze stage waren aanleiding om het daar verrichte onderzoek in de loop der jaren uit te breiden tot promotie onderzoek, uiteindelijk resulterend in dit proefschrift. Van januari 2002 tot mei 2005 was hij werkzaam als kinderarts-endocrinoloog aan de afdeling kinderendocrinologie van het VU Medisch Centrum te Amsterdam. Sinds mei 2005 is hij werkzaam als kinderarts-endocrinoloog in het Canisius-Wilhelmina Ziekenhuis en het Kinder Diabetes Centrum (KDCN) te Nijmegen.
Paul is getrouwd met Astrid Brouwer. Samen hebben zij drie kinderen: Anne, Hidde en Teun.

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