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Emile Hendriks

Fertility in Tall Men and Women Treated with High-Dose Sex Steroids during Adolescence and Genetic Determinants of Tall Stature

Vruchtbaarheid na hoge doses geslachtshormoonbehandeling tijdens de puberteit en genetische determinanten van lange gestalte

Emile Hendriks

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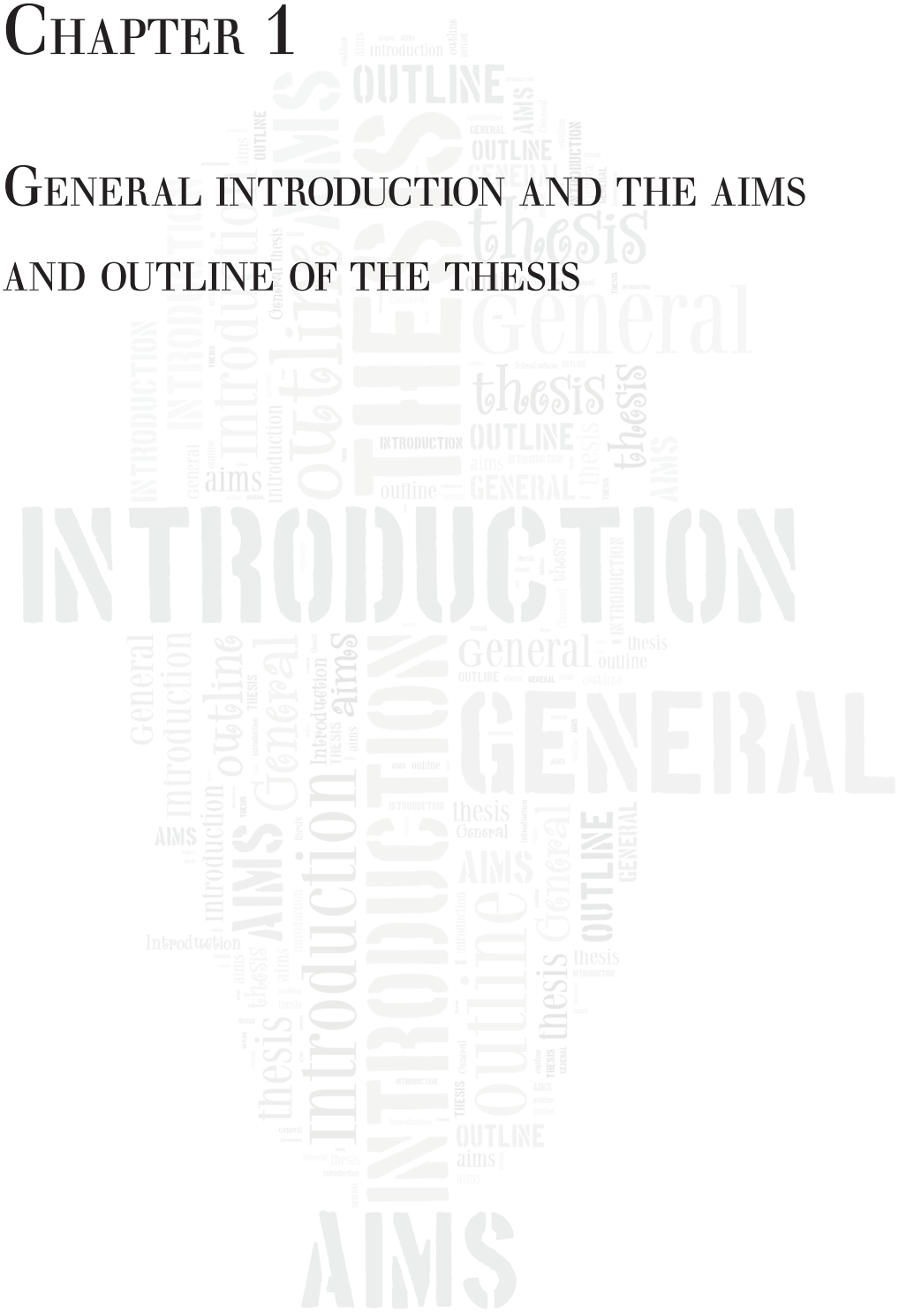
Chapter 1

General introduction and the aims and outline of the thesis



Part 1 - High-dose sex steroid treatment of constitutional tall stature

Populations of various ethnic origin differ substantially in growth and development.[1] In order to address normal growth of a particular population reference growth curves have been developed in many countries, for instance in the Netherlands and the United States.[2, 3] The extremes of growth, such as tall stature, can be defined following the normal distribution of these reference curves. Tall stature is usually defined as height of more than two standard deviations above the mean. While as many children grow above the 97th percentile (+1.8 SDS) as below the 3rd percentile, tall stature is a far less common reason for seeking medical attention than short stature. Tall stature is more easily accepted in society and may even be an advantage. This holds specifically true for boys, and girls are more often referred.[4] However from the late 1950s onwards many constitutional tall girls but also boys have been treated with sex steroids to reduce their adult height.[5] This treatment has been widely used in Europe, Australia and the USA.[6-9]

Constitutional Tall Stature (CTS)

CTS is a condition in which the subject's height reaches the upper limit of the normal pattern of childhood growth and constitutes three percent of the normal population. In CTS mean birth length is at or above the 75th percentile (+0.7 SDS) and tall stature becomes evident around the age of four years.[4] Growth velocity is accelerated in early childhood but slows down after about five years of age when the growth curve starts to parallel the normal curve.[10] No apparent abnormalities are present at physical examination which makes it possible to distinguish CTS from primary or secondary excessive growth syndromes (Table 1). Genetic and familial factors underlie CTS as almost always one or both of the subject's parents are tall and have followed a similar growth pattern.

Height prediction

Height prediction plays a crucial role in the management of CTS. Mean parental height and skeletal maturity are used as predictors of adult height. Methods developed by Tanner and Whitehouse and Bayley and Pinneau both use bone age as an indicator of skeletal maturity.[11, 12] Tanner and Whitehouse base bone age on hand and wrist radiography scored using a self developed method, while Bayley and Pinneau scored bone age according to the Greulich and Pyle atlas.[13] Both methods have their limitations and problems.[14] For instance, few investigators have studied the accuracy of these height prediction methods in tall stature.[15, 16] It is generally agreed that prediction models based on the

Table 1. The differential diagnosis of tall stature.

A.	**Variants of normal growth:** constitutional tall stature
B.	**Primary growth disorders**
	1. Sex-chromosome related disorders
	Klinefelter syndrome and variants
	XYY syndrome
	2. Overgrowth syndromes with advanced bone maturation
	Sotos syndrome
	Weaver syndrome
	Marshall-Smith syndrome
	Beckwith-Wiedemann syndrome
	Hyperinsulinism
	3. Syndromes with tall stature as outstanding feature
	Marfan syndrome
	Marfanoid phenotype
	Multiple endocrine neoplasia IIB
	Homocysteinuria
	Estrogen inactivity/resistance
C.	**Secondary growth disorders**
	1. GH excess
	2. Precocious (pseudo) puberty

[Adapted from Drop, S.L. *et al. Endocr Rev* 1998, 19, 540-558.]

general population should be cautiously applied at the extremes of the distribution.[12, 17] One Dutch study has developed a height prediction model specifically for boys and girls with tall stature.[18] Based on auxological data of untreated healthy tall children their model showed to be reliable and have better accuracy than the general methods. However, although prediction techniques may have small mean errors of prediction, individual variation and the chance of considerable errors in height prediction remains large.[19]

High-dose sex steroid treatment

Treatment of constitutional tall stature is generally based on psychological grounds. Although psychological investigations before or during height reductive therapy have never been performed, many pediatricians and pediatric endocrinologists share the experience that some children with excessive growth may suffer considerably from being much

taller than others.[5, 20-22] Many of them feel different from their peers and often are subject to hurtful remarks. Coping mechanisms such as kyphotic posture, social withdrawal and depression are not uncommon. In addition, practical problems such as clothing and shoes, but also career-planning are frequently reported.[23] On the other hand, many tall adolescents have no concern at all about their height. In fact in our culture tallness is generally valued positively.[24] Sex steroids have been widely used in the treatment of tall boys and girls.[25] Other treatment modalities, such as using Somatostatin or performing Epiphysiodesis, have been studied but only on a very limited scale.[26-28] Greater social acceptance of tall stature possibly explains the decline in recent years in the number of treated adolescents.

The use of sex steroids to limit adult height is based on the knowledge that in healthy pubertal development, gonadal steroids lead to epiphyseal fusion of the long bones. The pubertal growth spurt has long been considered to be an androgen-dependent process. However, experiments of nature clearly illustrate a dominant role for estrogens in bone maturation and thus the attainment of adult height: cases have been reported of men lacking estrogen activity as a result of aromatase deficiency or as a result of an inactivating mutation of the estrogen receptor. These conditions result in severely retarded maturation and growth continuing far into adulthood as the epiphyses do not close.[29, 30] Conversely, premature estrogen exposure, as seen in precocious puberty, leads to premature epiphyseal fusion and a limited adult height.[31, 32]

Since the first study in 1956, many reports have appeared describing the height reducing effect of high-dose sex steroid treatment in tall girls[6, 9, 33-48] and tall boys[8, 49-51]. Most experience has been gained with the administration of high doses of estrogens in girls (0.1-0.3 mg/day ethinylestradiol) and androgens in boys (testosterone depot preparations 250 mg/week).[4]

In recent years studies have reported on the cellular mechanisms by which estrogen causes epiphyseal fusion. One study suggests that epiphyseal fusion is triggered when the proliferative potential of growth plate chondrocytes is exhausted and that estrogen does not induce growth plate ossification directly. Instead, the auhors propose that estrogen accelerates the programmed senescence of the growth plate, thus causing earlier proliferative exhaustion and consequently earlier fusion.[52] In another report it was concluded that estrogen accelerates growth plate senescence without accelerating resting zone chondrocyte proliferation or accelerating the numerical depletion of these cells, suggesting that it might accelerate senescence by a proliferation-independent mechanism or by increasing the loss of proliferative capacity per cell cycle.[53]

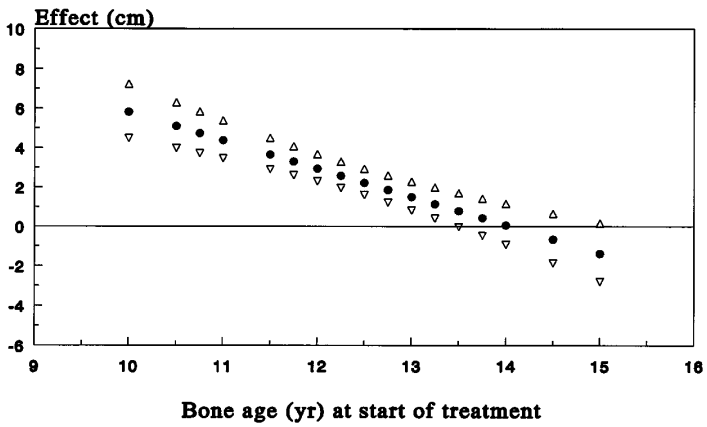


Figure 1a. Adjusted effect of estrogen treatment of tall girls.

The *solid dots* represent all patients with a given bone age, and the *open triangles* represent the 95% confidence interval of the calculated effect.[19]

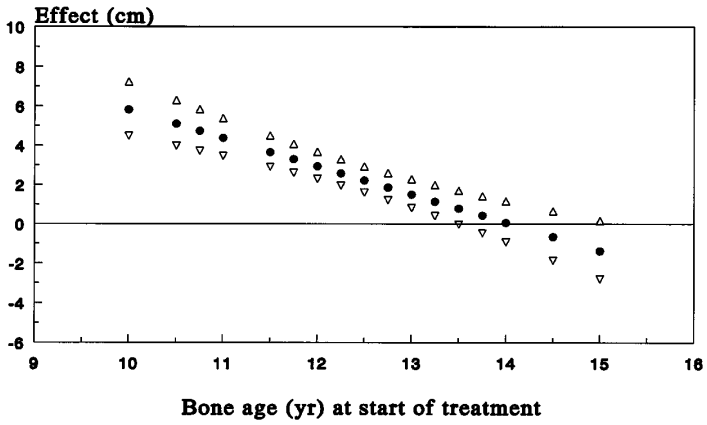


Figure 1b. Adjusted effect of androgen treatment of tall boys.

The *solid dots* represent all patients with a given bone age, and the *open triangles* represent the 95% confidence interval of the calculated effect.[19]

[Adapted from Drop, S.L. *et al. Endocr Rev* 1998*,* 19, 540-558.]

Effectiveness

Several studies have shown that high-dose sex steroid treatment reduces adult height. In general it is more effective in girls than in boys.[4] However, in the absence of randomized controlled trials, the true effectiveness of this treatment remains uncertain. Studies that accounted for the confidence intervals of the used height prediction methods report a mean effect of 1.1 - 2.4 cm in girls and 0.6 - 2.0 cm in boys.[4, 19] In addition, the height reduction is greater when the treatment is started at lower chronological age and lower bone age (Figure 1a & 1b).[54]

Side effects

It is known that sex steroids may have many effects on hemostasis, lipid metabolism and on the functioning of the hypothalamic-pituitary-gonadal axis.[55, 56] Short-term side-

Table 2. Reported side effects of high-dose sex steroid treatment of tall stature.

Short term	Women	Men
	Headache and/or migraine	Aggravation of acne
	Nausea and/or vomiting	Painfulness of injection
	Fluor vaginalis	Weight gain
	Pigmentation of areola & nipples	Gynecomastia
	Weight gain	Muscle ache
	Calf cramp	Edema
	Change in psychological behavior	Change in psychological behavior
	Hypertrichosis	Hypertrichosis
	Cysts in mammae/uterus/ovaries	Decreased stamina
	Thrombosis	
	Bleeding disturbances	
	Galactorrhea	
	Striae	
	Dizziness and/or orthostatic problems	
Long term	**Women**	**Men**
	Reduced per cycle rate of conception and increased risk of infertility	Marginally higher serum FSH levels and lower serum LH levels

[Adapted from Drop, S.L. *et al. Endocr Rev* 1998, 19, 540-558.]

effects of high-dose sex steroid treatment for tall stature are well documented (Table 2). Most studies report side effects of treatment only during or shortly after therapy and most of the time these effects were found to be mild and reversible.[6, 8, 9, 33-50, 57-65]

So far, little research has been done on the long-term effects of high-dose sex steroid treatment in CTS. However, several studies have been performed on the association of sex steroid use and possible health risks. In women long-term oral contraceptive use is associated with a higher risk of breast cancer, while hormone replacement therapy has been associated with epithelial ovarian malignancies.[66-68] In men an association of anabolic steroid use with azoospermia, testicular atrophy and decreased testosterone production was observed, both in athletes and chronically ill patients.[69-71] The reduced sperm production, although reversible in adult men, may sometimes take years until full recovery.[72, 73]

Regarding the long-term effects of high-dose sex steroid treatment, only two studies have attempted to assess the impact of such treatment in adult men and women previously treated for tall stature. In women, de Waal and colleagues reported in 1995 that,

10 years after treatment, menstrual cycle characteristics and reproductive outcome were equal between treated and untreated women.[74] However, a tendency towards an increased incidence of infertility was observed in treated women when compared to the known baseline prevalence of 85% for normal fertility in the general female population.[74, 75] In 2004, Venn and colleagues reported that high-dose estrogen treatment in adolescence reduces fertility in later life.[7] The latter study revealed that treated tall women have an increased risk of ever having tried for more than 12 months to become pregnant, to have seen a fertility doctor and to have taken fertility drugs compared to untreated tall women. They reported that treated women were 40% less likely to conceive in any given menstrual cycle of unprotected intercourse. Since the assessment of fertility was performed using a questionnaire and interview method, a causal relationship between treatment for tall stature and reduced fertility could not definitely be established. In men, de Waal and colleagues found marginally higher serum follicle stimulating hormone (FSH) levels and lower serum luteinizing hormone (LH) levels in treated compared to untreated tall men at an average follow-up of ten years. No significant effects on sperm quality or fertility were found.[76]

Part 2 - Genetic determinants of constitutional tall stature

Growth is one of the most important physiologic processes during childhood and adolescence and an important indicator of physical and emotional well being. Deviations from the normal range both for height and for height velocity may indicate an underlying congenital or acquired problem. A thorough understanding of the factors influencing the physiology of extremes of growth in healthy people may assist in better understanding the process of normal growth and pathologic forms of growth. This could lead to new treatment modalities for tall stature or for short stature.

Dutch height

At the end of the 20th century, the Dutch became the tallest in the world. This was the result of a spectacular increase in Dutch heights that began decades before in the second half of the 19th century and accelerated in the second half of the 20th century. The success in the Dutch biological standard of living in the late 20th century is greatly accountable to a high income growth that enabled the attainment of ample nutrition, and to a welfare state that provided health and social insurance to every single person in the society with a redistributive policy that kept income inequality within a modest range.[77] However, growth is a result of complex processes.

Remarkably, studies of fossil remains of our hominid ancestors demonstrate that the stature of individuals living during the last hundreds-thousands years reaches the range of heights seen today: The mean stature of early anatomically modern H. Sapiens in Europe was 184 cm in males and 167 cm in females.[78, 79] However by the mid-eighteen-hundreds the mean adult height of Dutch army recruits had decreased to 165 cm (Figure 2). Various hypotheses of probable phenomena responsible for this negative secular trend have been discussed, of which dietary considerations are favored. Early humans were hunter-gatherers but, due to the climatic changes, were forced to either follow the large animals they were accustomed to consume, or adapt culturally to the new and warmer environment. Increased migration brought more violence and endemic diseases. Those who stayed developed agriculture, changed their diets, and started to use cereals as the major source of starchy food. After the transition to agriculture populations rapidly expanded decreasing food supply and increasing risk of diseases.[80]

Equally, a positive secular trend that initiated in the middle of the 19th century has brought our mean height back up at 184 cm in males at the beginning of the 21st century. Several hypotheses have been put forward as to the causes of this positive secular trend.

Socioeconomic factors, such as social class, family size, birth rank, housing and crowding, genetic constitution, nutrition, endocrine function and psychosocial well-being are among the phenomena involved in the process of growth.[81, 82] Several popular beliefs have also developed, such as a role for landscape, level of child care or the Dutch love of milk. Although some of these hypotheses may be valid, genetic factors play a key role in the determination of height.[83, 84] The genetic component of height has been estimated to be 0.5-0.9, i.e., 50-90% of the height variation is accounted for by genetic factors.[85, 86] Assessment of the parental height as an indicator of the genetic component of growth and development of the child is therefore of clinical relevance.[87, 88] The latest nationwide growth study in the Netherlands showed no further increase of Dutch heights and thus it seems an end has come to the secular trend of the last 200 years. One may speculate that we have reached our genetic height potential.

Genetic determinants

Human growth and adult height are considered highly heritable polygenic traits that reflect the input of multiple genes interacting with environmental factors such as nutrition.[89-92] Even though estimates of heritability for stature are high, few genes have been

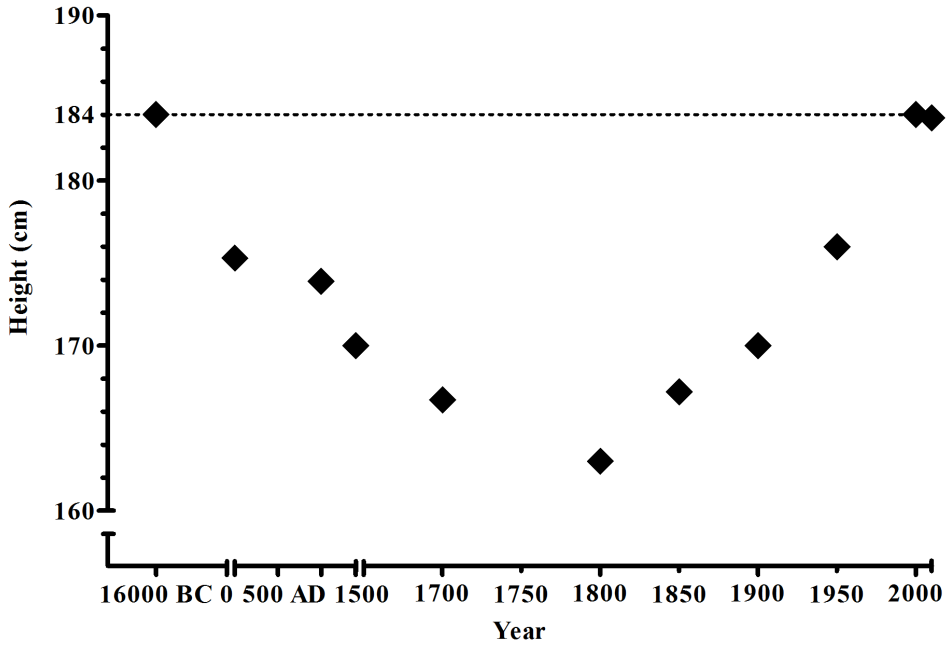


Figure 2. Mean height of Dutch males from 16000 BC till 2010 AD.

[Based on data from Maat, G.J.R. *Int J Osteoarchaeol* 2005, 15, 276-290; de Beer, H. *Econ Hum Biol* 2004, 2, 45-55; Styne, D.M *et al*. *Horm Res* 1993, 39 Suppl 3, 3-6.]

robustly associated with height variation in the general population.[93-95] The study of the genetics of height is important for several reasons. First, as one of the fundamental characteristics of childhood, deviation from a normal pattern of growth is a common cause of medical evaluation. In addition, epidemiological studies have shown that height is correlated with the risk of certain common cancers and diseases; thus genes regulating height may explain some of the familial clustering of these diseases.[96, 97] Finally, height represents a good phenotype with which to improve our understanding of the general genetic architecture of complex traits, for it is easily and accurately measurable.[91, 98] Among the unanswered questions in complex trait genetics is the degree to which common sequence variation in biologically plausible candidate genes contributes to trait variation.

Candidate gene analysis

Normal variations occur frequently throughout human DNA. These variations can occur as single base changes, single base deletions, insertions or variable numbers of a repeated sequence such as $(CA)_n$ or $(AAAG)_n$. Usually these variations or polymorphisms do not cause pathological changes and are considered silent markers. In comparison, "mutations" are DNA variations that are associated with a loss or gain of function. Although polymorphisms do not appear to be a direct cause of disease, these common variations are thought to influence susceptibility or resistance to disease. In the past, polymorphisms in several genes have been associated with observable changes in phenotype. These polymorphisms are often referred to as functional polymorphisms because the variation alters the function or expression of a gene product. Functional polymorphisms have been associated with increased risk of depression[99], osteoporosis[100], cognitive decline[101], myocardial infarction[102], diabetes[103] and other major illnesses.

Genetic association studies are used to identify genes associated with a disease or inherited trait. These studies assess correlations between genetic variants, such as polymorphisms, and trait differences on a population scale. The polymorphisms serve as markers for candidate regions or genes that can then be statistically linked to the trait. Genetic association studies require an appropriate sample size based on power calculations, a carefully selected control population and a valid statistical framework in order to detect real effects. Despite these recognized limitations, association studies represent an essential step in advancing the field to the definition of trait-mediating genetic variants.[104] Statural growth is controlled and influenced by complex cascades of hormones, regulatory factors, and intracellular signaling pathways. The components of these pathways can be grouped into functional axes.

Growth hormone axis

The growth hormone (GH)/insulin-like growth factor-1(IGF-1) axis is the key regulator of somatic growth in humans and its genes are plausible candidates to study the genetics of height variation.[105] Common polymorphisms of the GH1 gene have been associated with height variation and circulating IGF-1 levels.[106-110] Moreover, functional studies suggest that these polymorphisms influence transcription which may have an effect on circulating GH levels.[111] The IGF1 gene is one of the most studied genes in height, in particular the CA repeat in the promoter region of this gene. It has been associated with birth weight, adult height and circulating IGF-1 levels.[103, 112-116] A common deletion of exon 3 of the GHR gene is variably associated with increased growth velocity during the first year of GH treatment but not with increased height in the general population.[117] The -202 promoter polymorphism in the IGFBP3 gene has been correlated with circulating levels of IGFBP-3 and height variation in adults.[118] It was also associated with response to GH treatment in Dutch short children.[119]

Sex hormone axis

Longitudinal bone growth is not generated by the GH/IGF axis alone. Both gonadal steroids and growth factors of the skeletal axis greatly contribute to growth and maturation of long bones and the genes involved are good candidates to be associated with height variation. For instance, estrogens play a crucial role in the timing of cessation of longitudinal bone growth.[120] Estrogen receptor gene (ESR1) polymorphisms have been studied extensively in relation to bone mineral density (BMD) and have been associated with height variation.[121-123] Aromatase catalyses the rate-limiting step in the conversion of androgens to estrogens. Several studies have found an association of SNPs in the gene encoding aromatase (CYP19) in the genetic control of normal adult height, although others could not replicate the association.[124-126]

Skeletal axis

In the skeletal axis parathyroid hormone (and vitamin D are the principal regulators of bone mineralization. A tetranucleotide repeat (AAAG)n polymorphism in the promoter region of the PTH/PTHrP receptor gene is associated with promoter activity in vitro and bone mineral density and height variation in vivo.[127, 128] The VDR gene encodes a nuclear receptor for 1,25-dihydroxyvitamin D. In one study significant linkage was seen for a functional SNP in its gene that may be responsible for 34% of idiopathic short stature cases.[129] It has also been associated with height variation in the general population.[130]

Cell cycle

Recently genes involved in the cell cycle have been implicated as regulators of height variation. A SNP in the high mobility group-A2 (HMGA2) gene was robustly associated with adult height.[131-136] This is a strong biological candidate for influencing height because its homozygous deletion produces the dwarf *Pygmy* mutant in mice. In humans, each copy of the C allele of the SNP was associated with an increase of about 0.4 cm in height in the general population.[135]

Genome wide association analysis

Recent genome wide association analysis (GWAS) have shed new light on the genetic basis of *normal* human height variation: data were combined from several studies that ranged in size from 1.437 to 4.921 individuals with a *normal* height range and of European ancestry.[131, 132, 134] Across the three studies 54 genetic variants were robustly associated with height variation, with an impressive total of 40 previously unknown variants, many outside the expected biological pathways known to regulate growth. Later a Nature paper about a meta-analysis of GWA data from 46 studies comprising hundreds of thousands of individuals raised the number of relevant loci to at least 180.[137] However, effect sizes were small, on average ~0.4 to ~0.8 cm per 'increasing' allele between the two homozygous classes. This small effect size together with the stringent significance testing ($p<1x10^{-7}/5x10^{-8}$) explains why less than 10% of population height variation could be explained, in spite of the fact that ~63,000 individuals were included in these studies. It is expected that additional studies will raise the number of relevant loci well over one hundred. Further studies will possibly also elucidate whether interactions between alleles, either within or between loci, may explain part of the variation in height.

It should be recognized that the interpretation of these studies is still limited as there is no fine-mapping of the true causal loci usually not identified via GWAS. However, they convincingly confirm the polygenic nature of height and implicate genes that were never considered associated with this phenotype before, like Hedgehog signaling and basic cell cycle regulation, thus opening up new opportunities for further study. Although it has been shown that the phenotypic effects of genetic variants that were found in GWAS on *normal* height are rather small, requiring very large numbers of individuals being analyzed for obtaining statistically significant results, the expectation is that investigating people of extreme height and comparing the outcomes with those from a large population of normal-statured individuals will be promising to identify height genes.

Aims of the study

This thesis describes the results of several studies performed in constitutional tall men and women. Firstly, we studied the long-term effects of high-dose sex steroid treatment to reduce adult height of tall boys and girls. Secondly, we studied the genetic determinants of tall stature.

Part 1 - High-dose sex steroid treatment of constitutional tall stature

At present, only two studies have reported on long term fertility in estrogen treated tall women.[7,74] In the latest of these studies it was concluded that treatment reduces fertility. However, the assessment of fertility in this study was performed using a questionnaire and interview method and a causal relationship between treatment and reduced fertility could not definitely be established.[7] In addition, just one study has been performed in androgen treated tall men, which remained inconclusive.[74] For these reasons we initiated our studies. The aims of our retrospective cohort studies was to evaluate fertility and gonadal function in later life by means of a complete standardized andrological or gynecological assessment in three cohorts of Dutch tall men or women who did or did not receive high-dose sex steroid treatment in adolescence. In addition we evaluated whether there is a dose-response relationship between estrogen treatment of tall girls and fertility later in life.

Part 2 - Genetic determinants of constitutional tall stature

While polymorphisms in candidate genes of the GH/IGF-1 axis and other axes discussed above have been extensively studied in short stature, very few have attempted to study these genes in the extremely tall. We therefore studied common genetic variation in multiple candidate genes in a cohort of extremely tall Dutch individuals. The aim of this case-control study was to investigate if certain genotypes are more common in the tall and whether they are associated with height variation and circulating hormone levels.

With the introduction of GWAS the polygenic nature of height has been convincingly confirmed and more importantly genes were implicated that were never considered to be associated with this phenotype before, thus opening up new opportunities for further study. We, therefore, performed a GWAS in a cohort of extremely tall Dutch and compared the results with the Rotterdam Study GWAS on normal height. The aims of this study were to identify genetic loci associated with tall stature, to study the polygenic architecture of height and to discover novel genes involved.

Outline of the thesis

Chapter 1 gives a general introduction to the topics presented in this thesis.

Part 1 - High-dose sex steroid treatment of constitutional tall stature

Part 1 studies the long-term effects of high-dose sex steroid treatment. **Chapter 2** presents the long-term effects of high-dose estrogen treatment on fertility and ovarian function of constitutional tall women. **Chapter 3** studies whether any long-term effects of high-dose estrogen treatment are dose-dependent. **Chapter 4** presents the long-term effects of high-dose androgen treatment on fatherhood and testicular function of constitutional tall men.

Part 2 - Genetic determinants of constitutional tall stature

Part 2 studies the genetic determinants of constitutional tall stature**. Chapter 5** presents the association of common polymorphisms in candidate genes of the GH/IGF-1 axis with tall stature. In C**hapter 6** the association of genetic variation in other growth related candidate genes with tall stature is studied. **Chapter 7** present the results of a genome wide association study using extremely tall stature as trait.

Chapter 8 discusses the results of this thesis in relation to the current literature and evaluates the clinical implications of the study results. Chapter 9 summarizes the findings of the study in English and Dutch.

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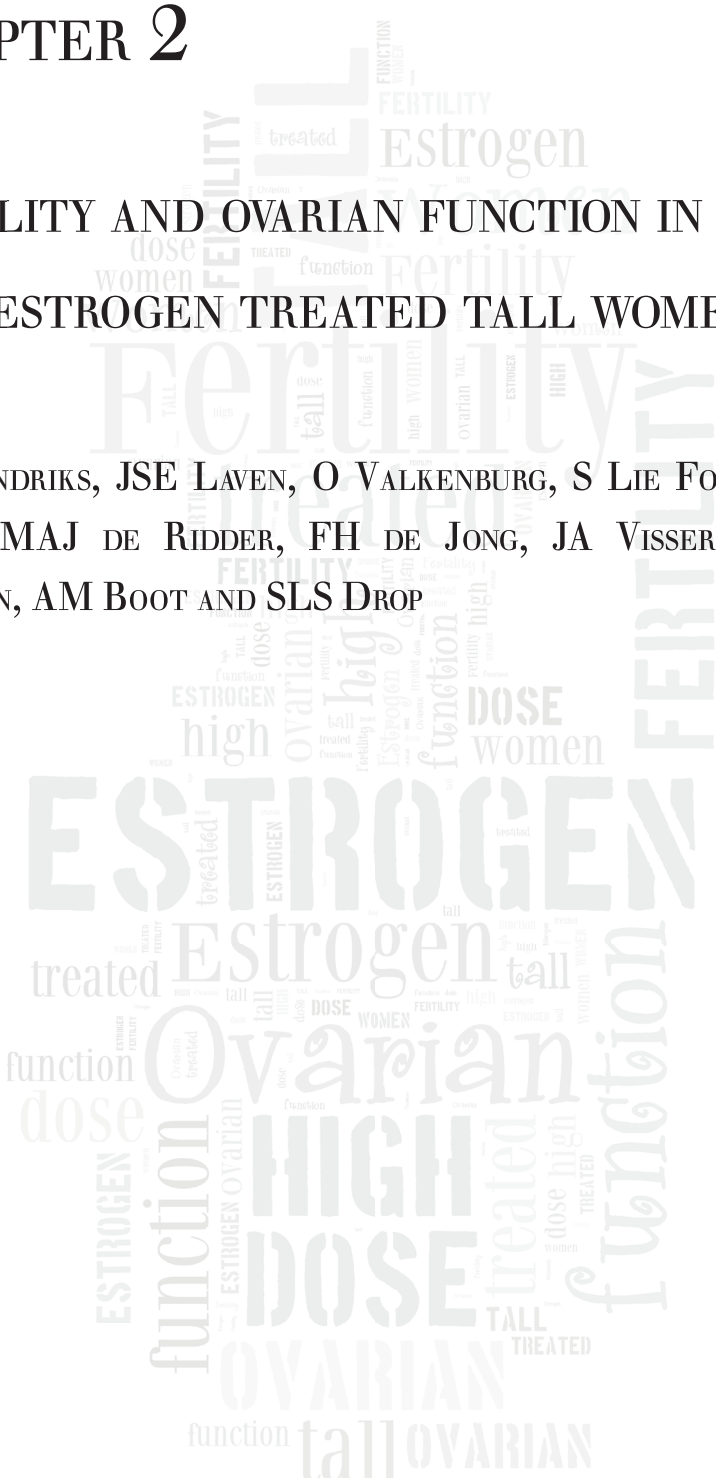
Part 1

High dose sex steroid treatment of constitutional tall stature

Chapter 2

Fertility and ovarian function in high dose estrogen treated tall women

AEJ Hendriks, JSE Laven, O Valkenburg, S Lie Fong, BCJM Fauser, MAJ de Ridder, FH de Jong, JA Visser, AM van Ginneken, AM Boot and SLS Drop



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Abstract

Background/Objective: High-dose estrogen treatment to reduce adult height of tall girls has been shown to interfere with fertility. Ovarian function has not been studied. We, therefore, evaluated fertility and ovarian function in tall women who did or did not receive such treatment in adolescence.

Methods: Retrospective cohort study of 413 tall women aged 23-48 years, of whom 239 women had been treated. A separate group of 126 fertile, normo-ovulatory volunteers aged 22-47 years served as controls.

Results: Fertility was assessed in 285 tall women (157 treated, 128 untreated) who had attempted to conceive. After adjustment for age, treated women were at increased risk of experiencing subfertility (OR=2.29, 95%CI 1.38-3.81) and of receiving infertility treatments (OR=3.44, 95%CI 1.76-6.73). Moreover, fecundity was notably affected, as treated women had significantly reduced odds of achieving at least one live birth (OR=0.26, 95%CI 0.13-0.52). Remarkably, duration of treatment was correlated with time to pregnancy (r=0.23, P=0.008).

Ovarian function was assessed in 174 tall women (119 treated, 55 untreated). Thirty-nine women (23%) exhibited a hypergonadotropic profile. After adjusting for age category, treated women had significantly higher odds of being diagnosed with imminent ovarian failure (OR=2.83, 95%CI 1.04-7.68). Serum FSH levels in these women were significantly increased, while antral follicle counts and serum AMH levels were decreased.

Conclusion: High-dose estrogen treated tall women are at risk of subfertility in later life. Their fecundity is significantly reduced. Treated women exhibit signs of accelerated ovarian ageing with concomitant follicle pool depletion, which may be the basis of the observed subfertility.

Introduction

From the late 1950s onwards, tall girls have been treated with sex steroids to reduce adult height.[1] This treatment has been widely used in Europe, Australia and the USA.[2, 3] In recent years, however, less adolescent girls have been treated, most likely due to greater social acceptance of tall female stature. The treatment of tall stature is based on the understanding that exposure to gonadal steroids leads to epiphyseal fusion of the long bones during pubertal development. In the Netherlands, tall stature among girls is clinically defined as an expected height of over 184 cm (2 standard deviations above the mean).[4] Risk for tall stature is typically assessed in girls at the age of 11-14 years. Interventions have been available for these girls based on psychosocial grounds.[5, 6] The most commonly used treatment is high doses of synthetic estrogens (100 or 200 µg/day ethinylestradiol (EE)) in combination with cyclic progestagens. The height reduction achieved varies between 2 and 10 cm, and is greater when the treatment is started at lower bone age.[7]

In the absence of randomized controlled trials, the true effectiveness of this treatment remains uncertain. Short-term side-effects of high-dose estrogen treatment for tall stature are, however, well documented and include: weight gain, acne, nausea, irregular menses, leg cramps, galactorrhea, benign breast disease, ovarian cysts, and thrombosis.[5] Regarding the long-term effects of treatment, only two studies have attempted to assess the impact of such treatment in adult women previously treated for tall stature. De Waal and colleagues reported in 1995 that, 10 years after treatment, menstrual cycle characteristics and reproductive outcome were equal between treated and untreated women.[2] However, a tendency towards an increased incidence of subfertility was observed in treated women when compared with the known baseline prevalence of 90% for normal fertility in the general female population.[2, 8] In 2004, Venn and colleagues reported that high-dose estrogen treatment in adolescence reduces fertility in later life.[1] The latter study revealed that treated tall women have an increased time to pregnancy and are at increased risk of subfertility compared with untreated tall women. They report that treated women were 40% less likely to conceive in any given menstrual cycle of unprotected intercourse. Since the assessment of fertility was performed using a questionnaire and interview method, a causal relationship between treatment for tall stature and reduced fertility could not definitely be established.

The aim of the current single center retrospective cohort study was to evaluate fertility and ovarian function in later life by means of a complete standardized gynecological

assessment in a large cohort of Dutch tall women who did or did not receive high-dose estrogen treatment in adolescence and to compare these data with those of a normal proven fertile cohort of age matched controls.

Methods

Subjects

We identified women who as young girls had sought medical attention for their tall stature at our institution between 1968 and 1998. All girls were evaluated at initial presentation by a pediatric endocrinologist. Skeletal age according to Greulich-Pyle was assessed by hand and wrist radiography to predict their adult height.[9] Women with a predicted height above +2 SD (97th percentile) were eligible to participate in the current study if an underlying disease as cause of their tall stature was excluded. This included girls who received estrogen treatment (200µg EE daily + cyclic 10mg progestin) in adolescence (treated group) and those who did not (untreated group). In general parents along with their daughters decided whether treatment was initiated. Common reasons for choosing not to have treatment were satisfaction with the predicted adult height, or uncertainty about possible side-effects. Girls choosing treatment had on average a higher predicted height than girls declining treatment.[7] Excluded were women with endocrine or metabolic disorders, chromosomal defects and primary or secondary growth disorders.

A cohort of 126 healthy normo-ovulatory fertile women of normal stature (aged 22-47 years) who had participated in previous studies on hormone levels in the normal menstrual cycle was used as the control group.[10, 11] These women had a regular menstrual cycle, did not use the oral contraceptive pill (OCP) and had previously given birth to at least one child and were hence proven fertile.

Data collection

Addresses of eligible women were traced using municipal registries and they were invited to participate by mail. Participants received a questionnaire assessing their personal and family history, relevant demographics, reproductive history and fertility problems. Two approaches were used to assess fertility outcome. Firstly, women were asked closed (yes/no) questions regarding fertility. Secondly, women were asked to estimate the number of months of unprotected intercourse before their first pregnancy. This time to first pregnancy (TTP) was divided into 4 categories: <6 months, 6-12 months, 1-2 years and

>2 years. Participants were also invited to visit the outpatient Reproductive Medicine unit of the Erasmus Medical Center to assess ovarian function. The study received ethical approval by the Erasmus Medical Center ethics committee and all participants provided written informed consent.

Clinical and endocrine examination

Subjects underwent a standardized examination between nine and eleven in the morning during the early follicular phase being the third, fourth or fifth day of the menstrual cycle. Women taking the oral contraceptive pill (OCP) were evaluated on the last day of the pill-free interval.[12] Transvaginal ultrasonography was performed, using a 6.5 MHz vaginal transducer (Hitachi Medical Corp., Tokyo, Japan), to assess ovarian volume and follicle count for both ovaries as previously described.[13, 14] Endocrine screening included serum assays for follicle stimulating hormone (FSH), luteinizing hormone (LH), SHBG, progesterone (fluorescence-based immunometric assays on Immulite 2000; Diagnostic Products Corp., Los Angeles, CA, USA), estradiol (E_2), testosterone (T) (coated tube RIA; Diagnostic Products), inhibin B (enzyme-immunometric assay; Serotec, Oxford, UK), and Anti-Müllerian hormone (AMH) (in-house ELISA, commercially available through Diagnostic Systems Laboratories, Webster, TX, USA).[15, 16] Intra/interassay coefficients of variation were <5/15% for LH, <3/8% for FSH, <5/7% for E_2, <3/5% for T, <4/5% for SHBG, <7/15% for inhibin B, and <4/5% for AMH. AMH had been measured in the controls by an Immunotech-Coulter assay. Values were adjusted to allow comparison with the in-house assay values.

Ovarian function

Subjects were classified into categories of ovarian function based on serum gonadotropin levels.[17, 18] Hypogonadotropic subjects presenting with oligomenorrhea and negligible endogenous estrogen activity, suggesting a disturbance at the hypothalamic-pituitary level, were classified as WHO-1 according to World Health Organization (WHO) recommendations.[18] Normogonadotropic subjects presenting with oligomenorrhea were classified as WHO-2. Those having features of either clinical or biochemical hyperandrogenism, or polycystic ovaries, were classified as polycystic ovary syndrome (PCOS) according to the Rotterdam consensus criteria.[19] Hypergonadotropic subjects were classified as WHO-3. To account for varying degrees of ovarian dysfunction due to follicle pool depletion these subjects were classified as either imminent ovarian failure (IOF) or premature ovarian failure (POF).[20] Criteria for IOF were FSH >10.0 IU/l on day 3-5 of

the menstrual cycle.[21] Our cut-off point of 10 IU/l FSH was calibrated to World Health Organization Standard 78/549.[22] In OCP users, IOF was defined as FSH >12.4 IU/l on day 7 of the pill-free interval. This cut-off point of 12.4 IU/l FSH was found to be the upper limit of the range in FSH on day 7 of the pill-free interval in healthy OCP using female volunteers.[12] POF was defined as FSH > 40.0 IU/l in women under 40 years of age.[23] Finally, subjects not fulfilling any of the criteria above were classified as normogonadotropic women.

Statistical analysis

To study the differences between treated and untreated women categorical variables were compared by means of χ^2 and Fisher's exact test. The linear by linear association χ^2 statistic was used to test for linear relationships in ordered categorical variables. For normally distributed continuous variables Student's t-test was used, and the Mann-Whitney U-test when non-normally distributed. Correlation of two continuous variables was studied using Pearson's correlation coefficient, while Spearman's correlation coefficient was used with ordinal variables. To adjust for age or age category, multiple binomial logistic regression was used to estimate adjusted odds ratios. In all analyses a two-tailed P-value of less than 0.05 was regarded to be statistically significant.

Analysis of time to first pregnancy (TTP) was done using the actuarial survival method with the event being the realization of a pregnancy for each time interval during which conception was attempted. Cumulative probabilities of conception were calculated by actuarial life-table analysis. The logrank test was used to test the null hypothesis of no difference between the groups in the probability of conception at any time point. Censoring was used for the following two conditions: *a*) TTP >24 months; and *b*) still having not conceived a pregnancy at the time of interview. For spontaneous pregnancies with <1 month of unprotected intercourse, TTP was recorded as 1 month. For the analysis of ovarian function, treated and untreated women were divided into two age categories: women up to the age of 40 years; and women over 40 years of age.

Actuarial life-table analysis was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). All other calculations were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL).

Results

Participants

We identified a cohort of 636 eligible subjects from the medical records at our department of pediatric endocrinology, of which 319 women were treated and 317 women remained untreated. Five hundred and eighty-five women could be traced and were invited to participate in the current study: 413 (71%) women (239 treated and 174 untreated) agreed to participate (Figure 1). Participants did not differ from non-participants with regard to age (36.3 vs. 35.6 yr, P=0.2), treatment regimen or adult height measured at last pediatric endocrinology outpatient visit (181.7 vs. 181.1 cm, P=0.6).

Table 1 shows the general characteristics of the participating treated and untreated women. Both groups were similar with regard to age, education and marital status. However, treated women were significantly taller than untreated women. Treatment was initiated at a mean (SD) age of 12.7 (1.5) years, mean treatment duration was 22.2 (9.3) months and mean period of follow-up was 23.4 (6.9) years after cessation of treatment.

Table 1. Characteristics of the study participants.

Characteristic	Treated	Untreated	P-value
n	239	174	
Age (yr)	36.1 (6.9)	36.5 (6.2)	0.55 [a]
Height (cm)	182.2 (3.8)	180.9 (4.3)	0.01 [a]
Weight (kg)	78.6 (13)	80.0 (15)	0.53 [a]
BMI	23.7 (3.8)	24.4 (4.5)	0.06 [a]
Marital status*			
Single	53 (22%)	28 (17%)	
Married or cohabitating	176 (75%)	139 (81%)	
Divorced or widowed	7 (3%)	4 (2%)	0.28 [b]
Highest education level**			
Low	22 (9%)	16 (9%)	
Medium	90 (39%)	51 (30%)	
High	121 (52%)	103 (61%)	0.19 [c]

Values are expressed as mean (SD) or number (percent). Differences between treated and untreated women were tested for significance by: [a] T-test; [b] χ^2-test; and [c] trend test. * missing: 3 treated & 3 untreated; ** missing: 6 treated & 4 untreated.

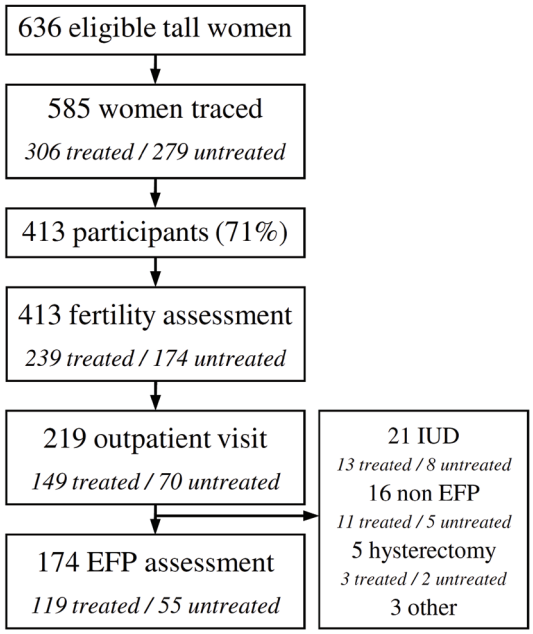


Figure 1. Participation flow diagram.

Abbreviations: EFP = early follicular phase; IUD = hormone excreting intrauterine device; non EFP = visit to our clinic during the mid- or late follicular or the luteal phase of their menstrual cycle; hysterectomy = previous history of hysterectomy.

Fertility

Fertility outcome of treated and untreated women is shown in Table 2. Of the 413 participants, 285 women had tried to conceive (157 treated (66%) and 128 untreated (74%) women). The resulting pregnancies had similar outcome regarding miscarriages. In treated women 17% had experienced one miscarriage and 10% two or more miscarriages, while in untreated women 18% had experienced one miscarriage and 6% two or more miscarriages (P=0.3). After adjustment for age, treated women had lower odds to conceive a pregnancy compared with untreated women (OR=0.22, 95%CI 0.09-0.55). Consequently, treated women were at increased risk of visiting a doctor for fertility problems (OR=2.29, 95%CI 1.38-3.81). Sixty percent of these visits resulted in a diagnosis, while in 40% no definite diagnosis could be made. Identified causes of reduced fertility included Fallopian tube abnormalities, endometriosis, ovulatory problems such as PCOS or a contributing male factor. Prevalences of these causes were not significantly different in the treated group compared with the untreated group. In line with these results treated women were at increased risk of receiving infertility treatments (OR=3.44, 95%CI 1.76-6.73). Moreover, fecundity was notably affected, as treated women had significantly reduced odds of achieving at least one live birth compared with untreated women (OR=0.26, 95%CI 0.13-0.52). The resulting involuntary childlessness in the treated group had been present for a median of 40 months.

Table 2. Fertility outcome in treated and untreated women.

Fertility	Treated	Untreated	OR# (95% CI)
n	239	174	
Attempt to conceive	157 (65.7%)	128 (73.6%)	0.71 (0.43-1.16)
Conceive a pregnancy	129 (82.2%)*	122 (95.3%)*	**0.22 (0.09-0.55)****
Doctors visit for fertility	68 (43.3%)*	32 (25.0%)*	**2.29 (1.38-3.81)****
Infertility treatments	44 (28.0%)*	13 (10.2%)*	**3.44 (1.76-6.73)****
Achieve live birth	110 (70.5%)*	113 (89.7%)*	**0.26 (0.13-0.52)****

Values are expressed as number (percent) or OR (95% CI). # Adjusted for current age. * Percentage of women who had attempted to conceive. ** P-value < 0.001.

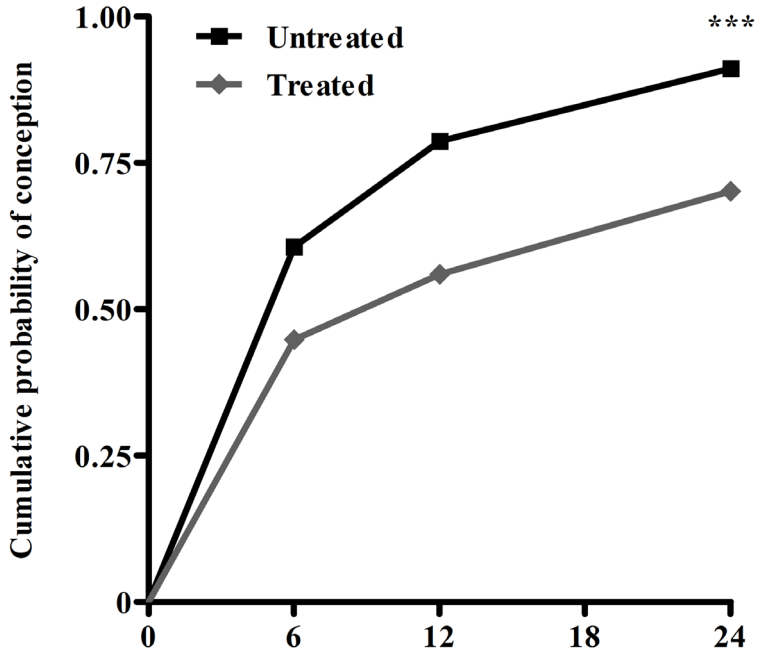
Detailed analysis of the time to first pregnancy in the group of women attempting to conceive showed that 79% (n=91) of the untreated women conceived their first pregnancy within the first 12 months of unprotected intercourse. In the treated group, however, time to first pregnancy was significantly increased with only 56% (n=61) of the women having conceived during the first year of unprotected intercourse (logrank test $p < 0.001$) (Figure 2).

Age at initiation of treatment was not correlated with time to first pregnancy (r= -0.03, P=0.8). In addition, we found no difference in mean age at initiation of treatment between treated women requiring infertility treatments or not (P=0.76), nor between treated women achieving a live birth or not (P=0.22). However, duration of treatment was significantly correlated with time to first pregnancy (r=0.23, P=0.008). Treated women with a time to first pregnancy exceeding 12 months had received treatment on average 3.2 months (95%CI 0.38-5.96 months, P=0.02) longer than women achieving their first pregnancy within 12 months of unprotected intercourse. Treatment duration was not associated with needing infertility treatments nor with achieving a live birth.

Ovarian function

Two hundred and nineteen women visited our outpatient clinic for early follicular phase assessment. For several reasons, 45 women visited our clinic while not in the early follicular phase and had to be excluded (Figure 1). Therefore, a total of 174 women, 119 treated and 55 untreated, were included in the analysis of ovarian function.

Early follicular phase assessment demonstrated 125 tall women to be normogonadotropic, of which 83 treated (70%) and 42 untreated (76%) women. No women presented with hypogonadotropic anovulation (WHO-1). PCOS was diagnosed in ten (6%) tall



Numbers at risk				
Untreated	128	50	24	12
Treated	157	84	62	39

Figure 2. Cumulative probability of conception of treated and untreated women.
*** logrank P-value < 0.001.

women, equally distributed among both groups. Although all PCOS women had oligomenorrhea and polycystic ovaries, only one had biochemical hyperandrogenism. We observed an unexpected high rate of hypergonadotropic tall women (Figure 3). While one treated woman was diagnosed with premature ovarian failure (POF), 38 (22%) women were diagnosed with imminent ovarian failure (IOF). Of these, 20 women were up to the age of 40 years, 17 were treated (16.5% of treated women up to the age of 40 years) and 3 untreated (7.5%), while 19 women were over 40 years, 12 were treated (75.0% of treated women over 40) and 7 untreated (46.7%). In a logistic regression model, while correcting for age category, treated women had significantly higher odds of being diagnosed with IOF (OR=2.83, 95%CI 1.04-7.68). In agreement with the diagnosis, women with IOF had significantly increased levels of FSH and LH, decreased levels of AMH and Inhibin B as well as lower antral follicle counts compared with normogonadotropic tall women (Table 3).

Finally, the results of the early follicular phase assessment in tall women were com-

pared with 126 normogonadotropic women who had been included as controls. Serum hormone levels and other ovarian parameters of normogonadotropic tall women were not different from values observed in the controls. While markers of ovarian function of tall women with IOF were significantly different from these normogonadotropic controls (Table 3). Age at initiation of treatment and treatment duration were not associated with outcome of early follicular phase assessment, nor correlated with antral follicle count or circulating hormone values.

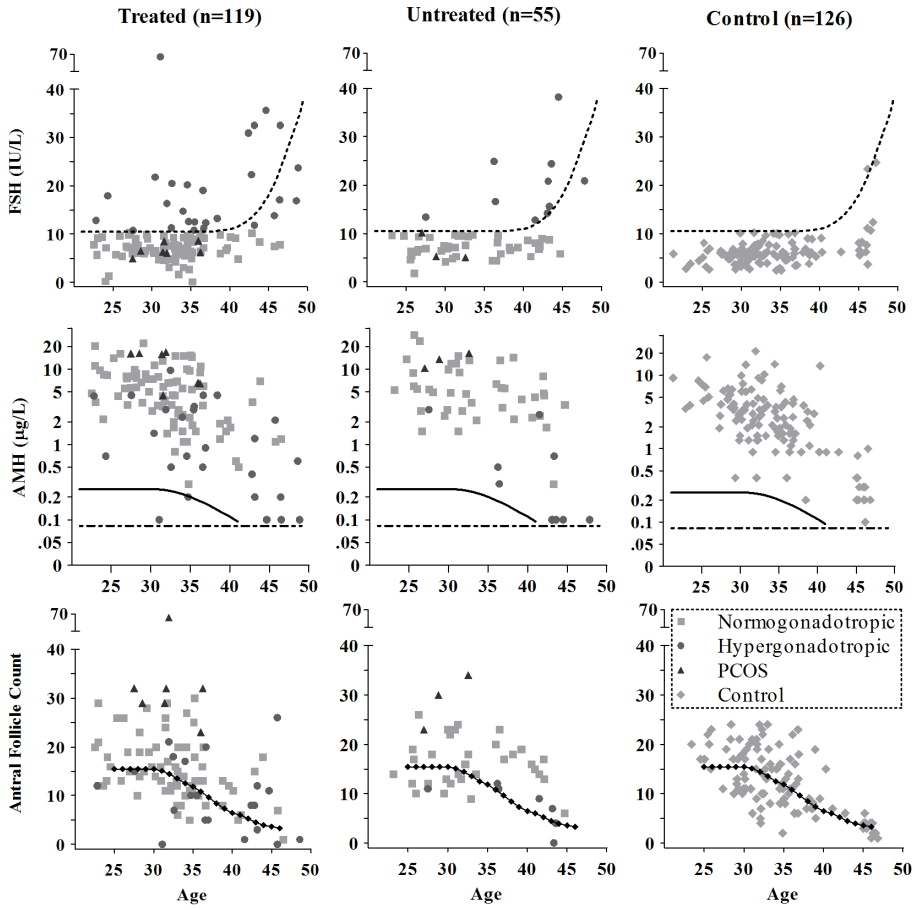


Figure 3. Individual hormone levels and antral follicle count in relation to age.

FSH levels, AMH levels (log scale) and total antral follicle count are presented by ovarian function category for tall treated and untreated women. Controls are presented seperately. In the FSH graphs the *dotted lines* indicate the p95 value for age.[16] In the AMH graphs the *solid lines* indicate the p5 value for age, the *dotted lines* indicate the menopausal threshold (0.086 µg/L).[23] In the antral follicle counts graph the *solid dotted lines* indicate the p50 value for age.[14]

Table 3. Hormone levels by age category in treated and untreated women categorized by ovarian function and in controls.

Subjects	n	Age (yr)	FSH (IU/L)	LH (IU/L)	AMH (µg/L)	InhB (ng/L)	E_2 (pmol/L)	T (nmol/L)	AFC
Age category ≤40 yr									
Treated									
Normogonadotropic	79	31.6 (4.5)	7.0 (2.5)	4.0 (2.0)	6.2 (4.7)	104.5 (54.8)	131.6 (111.4)	0.9 (0.5)	15.7 (6.1)
Hypergonadotropic	17	32.7 (4.4)	**17.7 (12.9)**[a,c]	**6.7 (4.0)**[b,d]	**2.6 (2.5)**[a,d]	107.7 (64.1)	161.7 (85.9)	0.9 (0.4)	**11.5 (6.4)**[b,d]
PCOS	7	31.9 (3.3)	6.7 (1.3)	**6.1 (1.7)**[b,d]	**11.8 (5.6)**[b,c]	94.6 (34.1)	100.9 (36.6)	**1.4 (0.9)**[b]	**34.4 (13.4)**[a,c]
Untreated									
Normogonadotropic	34	31.3 (4.7)	7.4 (2.4)	4.2 (3.2)	7.6 (6.3)	101.8 (53.0)	125.9 (106.5)	0.8 (0.5)	17.5 (6.5)
Hypergonadotropic	3	33.4 (5.1)	**18.3 (5.9)**[b,d]	4.5 (0.4)	**1.2 (1.5)**[b,d]	83.7 (28.0)	164.7 (58.9)	0.7 (0.3)	**11.3 (0.6)**[b,d]
PCOS	3	29.5 (2.8)	7.2 (3.5)	**8.3 (2.5)**[b,d]	**13.4 (3.0)**[b,c]	223.7 (236.8)	224.0 (161.7)	**1.6 (0.2)**[b]	**28.5 (7.8)**[b,c]
Controls	110	32.2 (4.1)	6.2 (2.5)	3.3 (1.5)*	4.0 (3.5)	101.5 (40.3)	166.5 (71.1)	1.1 (0.5)*	14.9 (7.0)
Age category >40 yr									
Treated									
Normogonadotropic	4	44.7 (1.7)	8.4 (1.1)	4.3 (1.5)	3.2 (2.7)	83.3 (31.6)	159.5 (91.5)	1.1 (0.5)	10.3 (7.7)
Hypergonadotropic	12	44.9 (2.4)	**34.9 (27.6)**[b,c]	**13.9 (11.6)**[b]	**0.4 (0.6)**[b]	**24.8 (20.4)**[b,c]	**100.7 (67.1)**[c]	0.8 (0.5)	7.7 (8.1)
Untreated									
Normogonadotropic	8	42.3 (1.1)	7.3 (1.3)	3.0 (1.5)	3.7 (2.4)	116.3 (72.6)	139.9 (99.3)	0.8 (0.5)	13.0 (4.2)
Hypergonadotropic	7	43.8 (2.0)	**21.0 (8.7)**[a,c]	**7.0 (4.7)**[b]	**0.5 (0.9)**[b]	**43.3 (26.3)**[b,d]	**73.7 (44.9)**[c]	0.9 (0.4)	**5.0 (3.9)**[b]
Controls	16	45.3 (1.5)	10.1 (6.1)	-	0.4 (0.3)	88.2 (62.6)	299.3 (169.0)	-	3.7 (1.7)

Values are expressed as mean (SD). Significant values are given in *bold*. [a] P-value < 0.001 & [b] P-value < 0.05 compared with normogonadotropic women of the same treatment category. [c] P-value < 0.001 & [d] P-value < 0.05 compared with controls. * n = 41. InhB, inhibin B; AFC, antral follicle count

Discussion

We evaluated fertility and ovarian function in tall women who did or did not receive high-dose estrogen treatment in adolescence. Our results indicate that treated women experience more difficulties getting pregnant compared with untreated women and more often receive infertility treatments. We show for the first time that abnormal serum levels of hormones related to the hypothalamus-pituitary-gonadal axis, especially FSH, may be involved in the observed subfertility.

First we studied fertility of treated women, which was significantly reduced compared with untreated women. Fifty-six percent of treated women conceived their first pregnancy within 12 months of unprotected intercourse. As a consequence, 43% of treated women visited a doctor because of fertility problems and 28% required some form of infertility treatment. More importantly, we observed a significantly reduced chance of achieving a live birth. At the time of study almost one third of the treated women were suffering from involuntary childlessness for a median of 40 months. This is unexpected in light of earlier findings indicating only a slight reduction in the probability of eventually having a live birth.[1] This may be explained by the fact that we studied fertility only in women who had attempted to conceive, which we believe better represents the women at risk of involuntary childlessness. Our time to pregnancy data is self reported and may be confounded by recall bias. However, we believe that our conclusions are not affected by such bias because we also assessed fertility based on data such as having received infertility treatments which is not prone to recall bias and showed similar results.

We also studied the effects of treatment within treated women only. We found that while age at initiation of treatment was not associated with outcome, duration of treatment was significantly correlated with time to pregnancy. Women with a time to first pregnancy of more than 12 months had on average been treated for 3 months longer. Although the effect of oral contraceptives on subsequent fertility has not been extensively studied, one study has reported an effect of estrogen dose on conception delay.[24] Recent studies did not find such an association, possibly because low dosage estrogen pills were used.[25] Because of no variation in dosage in our population we were unable to study the effect of estrogen dose more specifically.

Next, we analyzed ovarian function to study possible causes of the reduced fertility. Ovarian function was categorized based on serum gonadotropin levels. We observed an increased frequency of women with a hypergonadotropic profile. Our principal finding is that treated women are at increased risk of being diagnosed with IOF compared with

untreated women. To account for normal changes in ovarian function in the late reproductive stages, treated and untreated women were divided into two age categories for the analysis of ovarian function.[21] Taking these age categories into account, the odds of IOF diagnosis in treated women was almost threefold higher than in untreated women. Although ovarian function was primarily categorized based on serum FSH levels, the diagnosis of IOF was also supported by other parameters.[23] We observed significantly decreased antral follicle counts and serum AMH levels in women with IOF as compared with normogonadotropic tall women. Serum AMH is currently the best marker for primordial follicle pool size because in the ovary it is expressed in granulose cells of follicles that have undergone recruitment but have not yet been selected for dominance.[15, 26, 27] In addition, AMH plays an important role in regulating folliculogenesis as it is involved in determining the individual FSH threshold of early antral follicles.[28, 29] In addition, we believe some other possible pathologies, such as PCOS, can now be excluded as a potential cause of the observed infertility because of prevalence levels similar to the estimated population frequency.[30]

Finally, we compared our results to a cohort of healthy fertile controls. Parameters of ovarian function were comparable between normogonadotropic tall women and these controls. Comparison with hypergonadotropic women confirmed that parameters of ovarian function in these women are indicative of accelerated follicle pool depletion.

The results of our study do not only validate earlier epidemiological findings from an Australian study, but may also provide physicians with clinically useful information aiding in the diagnosis and treatment of estrogen treated tall women with fertility problems.[1] To our best knowledge this is the first report establishing ovarian dysfunction in these women. It seems that follicle dynamics have changed in that respect that a considerable number of these women seem to suffer from accelerated follicle loss being reflected by increased serum FSH levels along with decreased AMH levels as well as low antral follicle counts. Hence it seems that tall women who have been treated with estrogens in the past are prone to lose their reproductive capacity earlier in life and they should be counseled accordingly.

In conclusion, we evaluated fertility and ovarian function in later life of tall women who did or did not receive high-dose estrogen treatment in adolescence. We found that estrogen treated women experienced more difficulties conceiving and more often received medical treatment for infertility compared with untreated women. Treated women had a decreased chance of achieving at least one live birth. We observed a possible dose-response relationship as duration of treatment was correlated with time to pregnancy.

Finally, we showed that treated women were at increased risk of being diagnosed with IOF. They exhibit signs of accelerated ovarian ageing with concomitant follicle pool depletion, which may be the basis of the observed subfertility. However, the mechanism behind this accelerated follicle loss by high-dose estrogen treatment remains unknown and requires future research.

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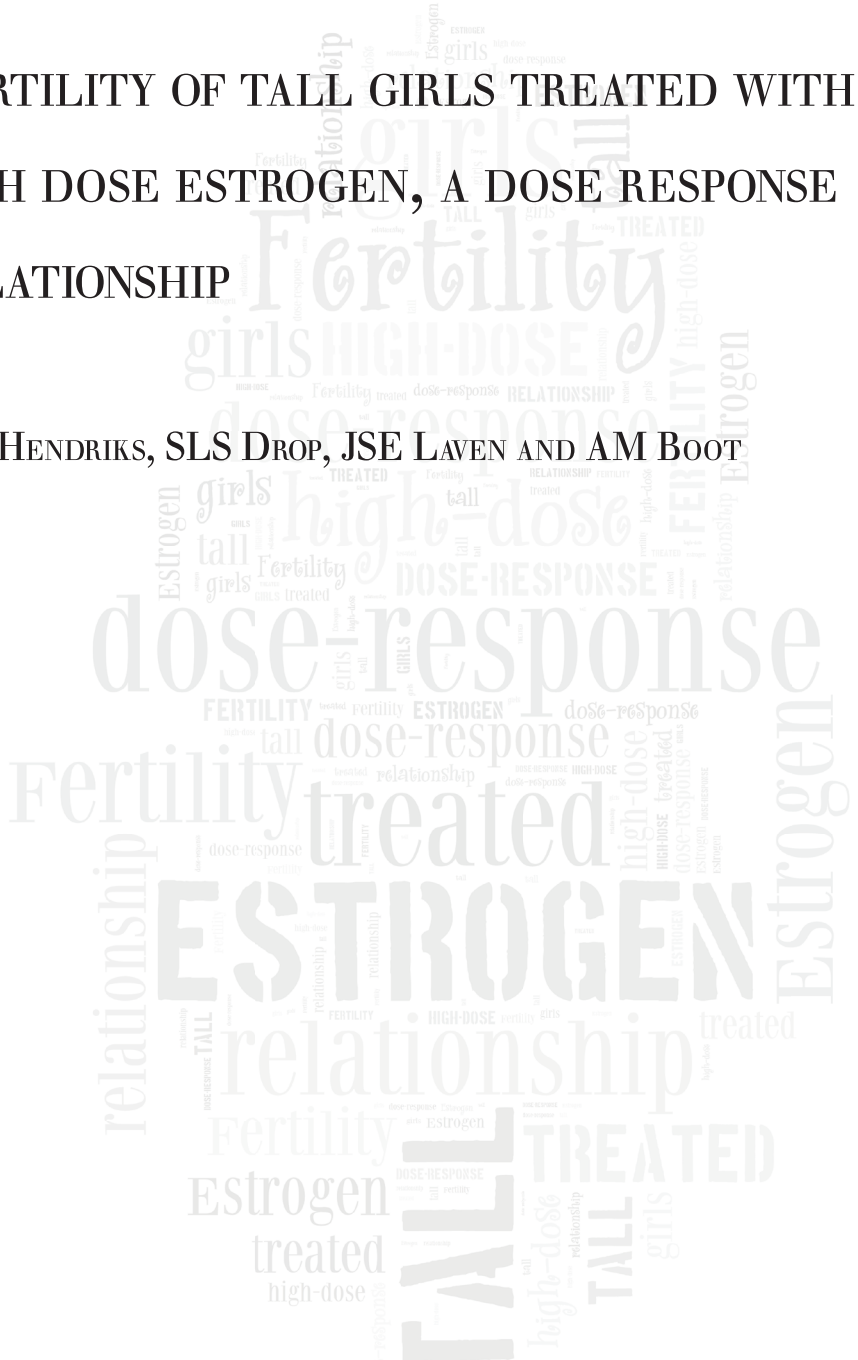
Chapter 3

Fertility of tall girls treated with high dose estrogen, a dose response relationship

AEJ Hendriks, SLS Drop, JSE Laven and AM Boot



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Abstract

Context: High-dose estrogen treatment to reduce adult height of tall girls increases their risk for infertility in later life.

Objective: To study the effect of estrogen dose on fertility outcome of these women.
Design/Setting: Retrospective cohort study of University hospital patients.

Patients: We studied 125 tall women aged 20-42 years, of whom 52 women had been treated with 100 µg and 43 women with 200 µg of ethinylestradiol (EE) in adolescence.

Main outcome: Time to first pregnancy, treatment for infertility and live birth rate.

Results: Time to first pregnancy was increased in treated women. Of untreated women 80% conceived within one year versus 69% in women treated with 100 µg EE and 59% in women treated with 200 µg EE. This trend of increased time to pregnancy with increasing estrogen dose was significant (logrank trend test P=0.01). Compared with untreated women, fecundability was reduced in women treated with both 100 µg EE (HR=0.42, 95%CI 0.19-0.95) and 200 µg EE (HR=0.30, 95%CI 0.13-0.72). We also observed a significant trend in the incidence of treatment for infertility with increased estrogen dose (p=0.04). Fecundity was affected in women treated with 200 µg EE, who had reduced odds of achieving at least one live birth (OR=0.13, 95%CI 0.02-0.81), but not in women treated with 100 µg EE.

Conclusions: We report a dose-response relationship between fertility in later life and estrogen dose used for the treatment of tall stature in adolescent girls, a higher estrogen dose is associated with increased infertility.

Introduction

High-dose estrogen treatment to reduce adult height of tall girls has been widely used.[1, 2] Recent studies have shown that tall women treated with high-dose estrogen in adolescence are at increased risk of infertility in later life and that their fecundity is reduced.[3, 4] These treated women exhibit signs of primary ovarian insufficiency with concomitant early follicle pool depletion.[3] The mechanism behind the observed fertility problems and accelerated follicle loss associated with high-dose estrogen treatment is still unknown. In addition, no such association has been reported on low dose estrogen treatment used for contraception.[5] This raises the questions whether there is a dose-response relationship. Thus far the reported infertility was associated with an ethinylestradiol (EE) dose of 200 μg/day, however over the years varying dosages of EE have been used. While in the 1960s most practitioners used 500 μg EE; in the 1970s 200 μg was used and in later years 100 μg EE was shown to be sufficient.[6, 7]

At the department of pediatric endocrinology of the University Medical Center Groningen two dosages of EE have been used for the treatment of tall stature; 100 μg/day and 200 μg/day. In the late eighties girls here were treated with 200 μg/day until a large study in the beginning of the 1990s showed similar effectiveness of 100 μg/day after which in the early nineties girls were also treated with the latter dose.[7] We therefore initiated the current study to evaluate whether there is a dose-response relationship between estrogen treatment of tall girls and fertility later in life.

Methods

Subjects

We identified women who during childhood had sought medical attention for their tall stature at the department of pediatric endocrinology of the University Medical Center Groningen between 1979 and 1999. All evaluations at initial presentation had been performed by pediatric endocrinologists who assessed skeletal age according to Greulich-Pyle using hand and wrist radiography to predict adult heights.[8] Women with a predicted height above 97th percentile according to Dutch standards were eligible to participate in the current study if an underlying disease as cause of their tall stature was excluded.[9] They included girls who received estrogen treatment of either 100 μg EE (treated 100)

or 200 µg EE (treated 200) daily (+ cyclic 10mg progestin) in adolescence and girls who did not (untreated group). In general parents along with their daughters decided whether treatment was initiated or not. Common reasons for choosing not to have treatment were satisfaction with the predicted adult height, or uncertainty about possible side-effects. Excluded were women with endocrine or metabolic disorders, chromosomal defects and primary or secondary growth disorders.

Data collection

Eligible women were traced using municipal registries and invited by mail to participate. Participants received a questionnaire assessing their personal and family history as well as relevant demographics. They were invited to visit the outpatient clinic of the University Medical Center Groningen. Clinical examination included a standardized interview to evaluate reproductive history and fertility problems. Two approaches were used to assess fertility outcome. Firstly, we asked closed (yes/no) questions regarding fertility. Secondly, we asked to estimate the number of months of unprotected intercourse before their first pregnancy was established, either spontaneous or planned. Height was measured using a SECA 225 stadiometer. Target height was calculated from mid-parental height corrected for secular trend according to Dutch standards,[9, 10] The study received ethical approval by the institutional medical ethics review boards of the University Medical Center Groningen and the Erasmus Medical Center and all participants provided written informed consent.

Statistical analysis

To study the differences between the three treatment categories the linear by linear association chi-square statistic was used to test for linear relationships of ordered categorical variables. For normally distributed continuous variables one-way analysis of variance with Bonferroni correction was used. For ordered categorical variables the Kruskal-Wallis one-way analysis of variance was used. Correlation of two continuous variables was studied using Pearson's correlation coefficient. Multiple binomial logistic regression was used to adjust for the confounders age, body mass index (BMI) and smoking. Exact logistic regression was used when expected counts in cells of the two-by-three tables were less than five. In all analyses a two-tailed P-value of less than 0.05 was regarded to be statistically significant.

Analysis of time to first pregnancy (TTP) was done using the Kaplan-Meier (KM) survival method to compare periods of not achieving a pregnancy between the three

treatment categories. Cumulative probabilities of conception were calculated by KM life-table analysis. The logrank trend test was used to test the null hypothesis of no difference between the treatment categories in the probability of conception at any time point. Censoring was used for the following two conditions: *a*) TTP >24 months; and *b*) still having not conceived a pregnancy at the time of interview. For spontaneous pregnancies with <1 month of unprotected intercourse, TTP was recorded as 1 month. Cox proportional hazard model with TTP as time variable was used to account for possible confounders. Variables were entered into the model and retained in a backward stepwise manner based on the likelihood ratio if their presence significantly improved the fit or if their presence in the model substantially modified the estimate of the treatment effect. Log-minus-log plots were made per treatment category to inspect possible deviations from the proportional hazard assumption. The resulting hazard ratio (HR) represents the fecundability of treated subjects compared with untreated subjects. Exact logistic regression was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). All other calculations were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL).

Results

Participants

From the patient records of the department of pediatric endocrinology at the University Medical Center Groningen we identified 222 eligible tall women. We were able to trace 219 (99%) women who were invited to participate in our study, 137 of these women had been treated with high-dose estrogen (76 with 100 µg EE and 61 with 200 µg EE). In total 125 women (57%) agreed to participate, this included 52 women (68% of the original cohort) treated with 100 µg EE and 43 women (70%) treated with 200 µg EE. Non-participating women were slightly younger than participating women (29.3 vs. 30.1 years, P=0.03) but were not different with regard to predicted adult height (187.0 vs. 187.7 cm, P=0.1).

Table 1 shows the general characteristics of the participating women by treatment categories. Treated and untreated women were mostly similar with regard to age, anthropometrics, education and marital status. However, women treated with 200 µg EE were older than the other women.

Table 1. Characteristics of the participating women.

Characteristic	Untreated	Treated (100)	Treated (200)
n	30	52	43
Age (years)	28.8 (2.0)	29.2 (2.6)	31.8 (4.1) [a]
Height (cm)	183.5 (4.1)	183.6 (3.3)	183.7 (3.2)
Weight (kg)	82.1 (12.3)	78.3 (15.2)	79.6 (11.6)
BMI	24.4 (3.5)	23.2 (4.4)	23.6 (3.5)
Marital status*			
Single	8 (27%)	10 (20%)	9 (21%)
Married or living together	21 (70%)	41 (80%)	34 (79%)
Divorced or widowed	1 (3%)	0 (0%)	0 (0%)
Highest education level			
Low	2 (7%)	3 (6%)	2 (5%)
Medium	9 (30%)	15 (29%)	17 (39%)
High	19 (63%)	34 (65%)	24 (56%)

Values are expressed as mean (SD) or number (percent). [a] $P<0.05$ compared with both untreated and treated (100) women. * missing: 1 treated (100) woman.

Table 2 shows the clinical parameters of the women when they as girls first presented at our department of pediatric endocrinology. Age, bone age and target height did not differ between treated and untreated women. However treated women were taller at presentation and had a higher predicted height than untreated women. Tanner breast stage and pubic hair stage at presentation did not differ between treated and untreated women (Kruskal-Wallis P=0.84 and P=0.52) nor did the number of girls who had experienced menarche (P=0.68). Table 2 also shows the treatment specifics. Treatment details were similar for both treatment categories, except that the mean period of follow-up after cessation of treatment was longer in women treated with 200 µg EE, reflecting that treatment with 100 µg EE is a more recent therapy. All treated girls were followed until regular menstrual cycles after cessation of the treatment. Anosmia in participants or their family members was not reported. In our study population treatment had on average been initiated in the year 1989 (range 1983-1999) in women treated with 200 µg EE and in the year 1993 (range 1986-1996) in women treated with 100 µg EE.

Table 2. Clinical parameters at first presentation and treatment specifics.

Clinical parameters	Untreated	Treated (100)	Treated (200)
n	30	52	43
At first presentation*			
Age (years)	12.6 (1.5)	12.9 (1.1)	12.8 (1.2)
Bone age (years)	12.7 (1.2)	12.4 (0.5)	12.3 (0.6)
Height (cm)	173.9 (6.5)	177.8 (3.3)[a]	177.5 (4.4)[a]
Predicted height (cm)	184.5 (3.2)	188.8 (2.4)[a]	189.4 (3.0)[a]
Target height (cm)	182.0 (5.9)	181.6 (4.6)	182.8 (4.2)
Tanner breast stage**			
Stage 1 & 2	8 (30%)	6 (13%)	4 (11%)
Stage 3	3 (11%)	16 (34%)	16 (46%)
Stage 4 & 5	16 (59%)	25 (53%)	15 (43%)
Tanner pubic hair stage***			
Stage 1 & 2	6 (22%)	9 (21%)	8 (24%)
Stage 3	8 (30%)	21 (49%)	15 (44%)
Stage 4 & 5	13 (48%)	13 (30%)	11 (32%)
Menarche*	10 (33%)	13 (27%)	9 (25%)
Treatment specifics			
Age at start (years)	-	12.9 (1.2)	12.8 (1.2)
Duration (months)	-	23.6 (7.8)	24.1 (6.3)
Follow-up (years)	-	15.9 (1.9)	19.0 (3.8)[b]

Values are expressed as mean (SD) or number (percent). [a] P<0.01 compared with untreated; [b] P<0.05 compared with treated (100). * missing: 4 treated (100) and 7 treated (200). ** missing: 3 untreated, 5 treated (100) and 8 treated (200). *** missing: 3 untreated, 9 treated (100) and 9 treated (200)

Time to pregnancy

Of the 125 participating women, 71 women had attempted to conceive a pregnancy. Fourteen of these women were untreated (47% of untreated women), 25 were treated with 100 µg EE (48%) and 32 were treated with 200 µg EE (74%). Results of the time to first pregnancy analysis in women who had attempted to conceive are shown in Figure 1.

Time to pregnancy was significantly increased in treated women compared with untreated women. While 80% (n=11) of the untreated women conceived in the first year, only 69% (n=17) of the women treated with 100 µg EE and 59% (n=19) of the women treated with 200 µg EE had conceived their first pregnancy within one year. The observed trend of increased time to pregnancy with increasing estrogen dose was statistically significant (logrank trend test P=0.01).

While accounting for several possible confounders in a discrete Cox model we were able to calculate fecundability for each dose. The model was adjusted for age, BMI and smoking. The curves for the three treatment categories in the log-minus-log plots ran parallel, indicating that the proportional hazards assumption was met. Compared with untreated women, fecundability was reduced in both women treated with 100 µg EE (HR=0.42, 95%CI 0.19-0.95, P=0.04) and women treated with 200 µg EE (HR=0.30, 95%CI 0.13-0.72, P=0.007). Neither age at initiation of treatment nor duration of treatment were correlated with time to first pregnancy in treated women.

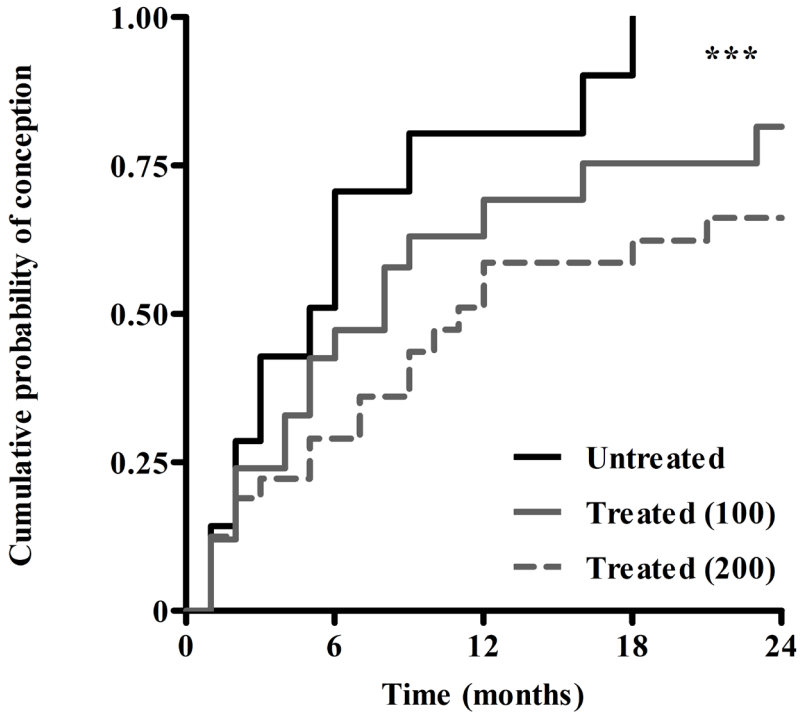

Figure 1. Kaplan-Meier curves, estimating cumulative conception probabilities for the first attempted pregnancy.

*** logrank trend test p-value = 0.01.

Fertility outcome

Figure 2 shows the fertility outcome of the women who had attempted to conceive. Treated women were more likely to report fertility problems than untreated women were. Although both groups in general had been equally able to conceive a pregnancy, treated women were more likely to have needed 12 months or more to achieve that pregnancy. As a consequence, treated women had more often visited a doctor because of fertility problems and more often received infertility treatments. We observed a significant trend of increasing fertility problems with increasing estrogen dose; i.e. women treated with 200 µg EE experienced more fertility problems than women treated with 100 µg EE, who in turn experienced more fertility problems than untreated women.

Because women treated with 200 µg EE were older and had longer follow-up time than women treated with 100 µg EE we corrected for age in an exact logistic regression model. The effect sizes were not significantly influenced by adding age, nor BMI or smoking to the model. In addition, we performed a sensitivity analysis to see if differential follow-up could explain the observed difference in fertility outcome between women treated with 100 µg EE and women treated with 200 µg EE. We assumed that participating women, both treated with 100 and 200 µg EE, who had not attempted to conceive had overall fertility outcome comparable to women treated with 200 µg EE. When this hypothetical group of treated women who had not attempted to conceive was added to the analysis, the significant trends of increasing fertility problems with increas-

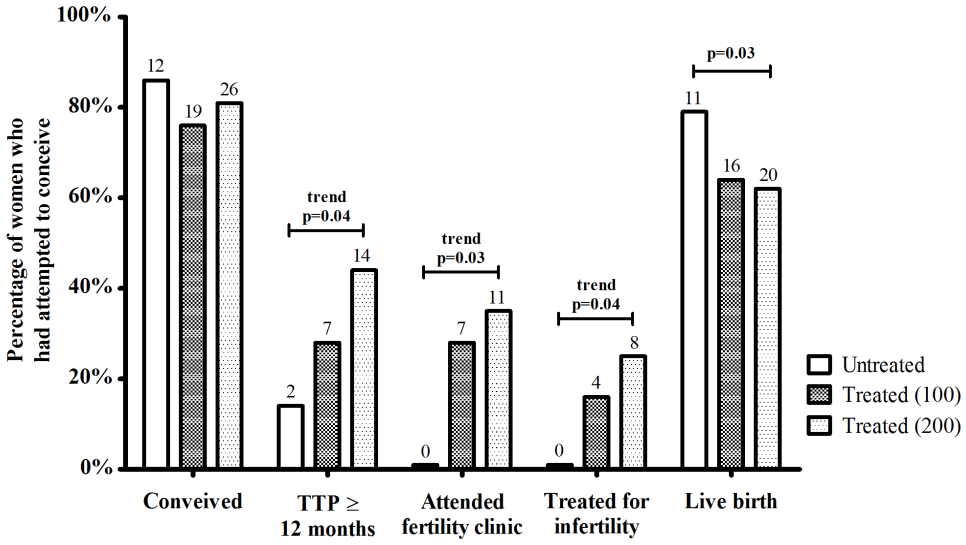

Figure 2. Fertility outcome by treatment category.

ing estrogen dose remained (trend P<0.05).

Women treated with 200 µg EE had significantly reduced odds of achieving at least one live birth compared with untreated women (OR=0.13, 95%CI 0.02-0.81, P=0.03). Although the percentage of women treated with 100 µg EE who had achieved a live birth was similar to the percentage of women treated with 200 µg EE, the difference with untreated women was not statistically significant. The median duration of involuntary childlessness in women treated with 200 µg was 25 months. There were no differences between the treatment categories and the risk of miscarriages. In treated women who had visited a doctor because of fertility problems 56% of these visits had resulted in a diagnosis. Identified causes of reduced fertility included Fallopian tube abnormalities, endometriosis or ovulatory problems such as PCOS. Prevalence of these causes were not significantly different between both treatment groups. As for the partners of these women, a low prevalence of a contributing male factor was reported (5%) and mean height of the partner was similar in all groups (untreated 187.9 vs. treated (100) 189.4 vs. treated (200) 187.1 cm, P=0.49). There were no differences in mean age at initiation of treatment or mean duration of treatment between treated women requiring infertility treatments or not, nor between treated women achieving a live birth or not.

Discussion

It has been shown that high-dose estrogen treatment to reduce adult height of tall girls increases their risk for infertility in later life.[3, 4] Here we studied the effect of estrogen dose on fertility outcome of these women. We compared women who received no treatment to women who received either 100 µg EE or 200 µg EE. Our study confirms that tall women treated with high-dose estrogen have an increased time to pregnancy and experience more fertility problems compared with untreated women. We demonstrate for the first time that the association between estrogen treatment and the observed infertility is dose-dependent.

As previously shown, we also observed fecundity to be reduced in women treated with 200 µg EE as they had significantly reduced chances of achieving at least one live birth. At the time of study one third of these women were suffering from involuntary childlessness for a median of 25 months. Based on our results it seems probable that women treated with 100 µg EE are also at risk of reduced fecundity. However, currently we did not observe a significantly reduced live birth rate, possibly due to a relatively small sample size.

While only a few studies have reported on fertility outcome after high-dose estrogen treatment for tall stature, multiple studies have been performed on the long-term effects of low-dose estrogens used for contraceptive treatment. In the 1960s several reports suggested that oral contraceptive pill (OCP) use may cause secondary amenorrhea, however this was not established by later studies.[5, 11, 12] Interestingly in these early papers estrogen doses equal to 50-100 μg of EE were given, however due to a lack of proper study design no conclusions could be drawn based on these observations. In the late 1970s and early 1980s several studies reported on fertility after discontinuation of OCPs. These studies have demonstrated some delay in the time to pregnancy in previous OCP users.[13, 14] However, this impairment was typically seen in the early months after discontinuation of OCP use while 12-month pregnancy rates were within the normal range.[5] Albeit that a few early studies on estrogen doses equal to 50 μg EE reported pregnancy rates of 75% in the first year, which is lower than the generally accepted fertility rates of 85%.[15] Only one study has reported on the effect of estrogen dose on fertility after cessation of OCPs. They observed consistently longer conception delays in women discontinuing OCPs containing ≥ 50 μg of estrogen compared with women who had used < 50 μg of estrogen.[16] In addition, one other study has reported that OCP users in the lower weight percentiles have longer conception delays suggesting a possible dose effect.[17] These studies are in line with our results of a dose-dependent increased time to pregnancy in women treated with high-dose estrogen.

While human studies on the effects of treatment with estrogens have mostly focused on OCP users, animal studies have focused on environmental exposure to EE as an endocrine-disruptor and on the effects of Diethylstilbestrol (DES). In rodents both in utero and postnatal exposure to EE or DES produces permanent adverse effects on the developing female reproductive system.[18-20] Animal studies on in utero exposure to DES have shown disruption at the follicle level. In DES-exposed mice reduced numbers of primordial follicles and of oocytes after ovulation induction have been found.[19, 21, 22] Neonatal exposure to DES in lambs reduces the primordial follicle pool by stimulating their initial recruitment, resulting in increased numbers of atretic follicles.[23] Finally, DES induces transient changes in gene expression during gestation, these changes could be involved in follicle development, rate of atresia, or patterns of secretion or metabolism of steroid hormones.[24] These animal studies suggest that pharmacological doses of estrogens may influence fertility in many ways and at various time points. This knowledge, although difficult to extrapolate, may help in better understanding the mechanism behind the observed infertility in tall women treated with high-dose estrogen.

Apart from the dosage used, high-dose estrogen treatment to reduce adult height also differs from low-dose contraceptive treatment with regard to regimen (continuous vs. cyclic), timing (prepubertal vs. peri/post-pubertal) and duration (1-2 years vs. several months to many years). A recent study on return to fertility after continuous-use of OCPs for up to 1 year established a normal 12-month pregnancy rate of 81% and concluded that there was no delay in fertility.[25] In addition, several studies have shown that there is no evidence that increased duration of OCP use delays subsequent fertility.[5, 26]

At first presentation treated women were taller and had higher predicted heights than untreated women. Therefore, the possibility of an association between tall stature and reduced fertility needs to be considered. We found no difference between treated and untreated women with respect to clinical parameters representing gonadal function at first presentation such as Tanner stage and age at menarche. In the available literature height is negatively correlated with reproductive success in Western society, however this is attributed to sexual selection and the chance of finding a mate rather than the fertility of these women which has not been studied in detail.[27] Finally, the height difference between untreated and treated women equals 1 percentile (98th vs. 99th percentile) according to Dutch standards.[9] From a population perspective it seems unlikely that this could explain 15-25% reduction in achieving a first pregnancy in the first year.

The possibility of partial hypogonadotropic hypogonadism (HH) in these women needs to be considered as it is associated with both tall stature and reduced fertility. However, HH is a secondary growth disorder and its growth pattern is distinctively different from constitutional tall stature. In HH growth during childhood is unremarkable and tall stature does not become evident until the teenage years when growth continues because of lack of epiphyseal closure.[28] Constitutional tall stature is characterized by accelerated growth velocity in early childhood and tall stature becomes apparent at the age of 3 to 4 years.[6] Although we excluded women with secondary growth disorders, we cannot fully exclude the possibility of partial HH in some women as serum gonadotropin and sex steroid concentrations were not measured at initial presentation.

Previously it has been shown that a considerable number of tall women treated with high-dose estrogen in adolescence suffer from primary ovarian insufficiency with concomitant early follicle pool depletion diagnosed by increased serum FSH levels, decreased serum AMH levels and low antral follicle counts.[3] Although the mechanism behind this accelerated follicle loss observed in these women remains unknown, based on our results we conclude that estrogen may play a key dose-dependent role. This is supported by a study on in utero exposure of women to DES, who reported an earlier age

at menopause with cumulating doses of DES.[29] We hypothesize that the effects of high-dose estrogen could be directly at the follicle level or indirectly through other intra-ovarian regulatory hormones such as IGF-1 or AMH. Several studies have reported on these effects of estrogen, however none have studied the effects of supra-physiological levels of estrogen. Future research is needed to test these hypotheses. Studies on physiological levels of estrogen have shown that human granulosa cells are a site of estrogen reception, while it is still uncertain whether the human oocyte is also estrogen responsive.[30] Among the local intrafollicular actions of estrogen is its responsibility for facilitating the differentiation of granulosa cells, including the induction of receptor systems for FSH and LH and it can influence post-receptor mechanisms.[31] Studies on estrogen depleted ovaries have shown that folliculogenesis halts in the antral stage causing infertility due to the inability to ovulate.[31] Animal studies have shown that IGF-1 is required for reproduction. It has been suggested that IGF-1 promotes fertility by limiting the recruitment of primordial follicles in the growing pool thus conserving the resting pool.[32] Interestingly, it has been shown that serum IGF-1 levels are greatly reduced during high-dose estrogen treatment.[33] One study, in fact, has shown that serum IGF-1 levels are lower in girls receiving 200 µg EE compared with 100 µg EE after 12 months of high-dose estrogen treatment.[34] In addition, in boys treated with high-dose androgen neither an effect on IGF-1 levels is seen, nor any effects on later fatherhood.[33, 35]

Women treated with 200 µg EE had started treatment in earlier years and as a result had a longer duration of follow-up and were older at the time of study. We, therefore, cannot fully exclude the possibility of bias due to differential follow-up. While this is corrected for by the use of the time to event analysis in the calculation of time to first pregnancy, it is possible that with similar follow-up time women treated with 100 µg EE would have had overall fertility outcome similar to women treated with 200 µg EE. However, this seems unlikely as correcting for age and the use of a sensitivity analysis in the calculation of fertility outcome did not significantly influence our results.

In conclusion, we report a dose-response relationship between fertility in later life and estrogen dose used for the treatment of tall stature in adolescent girls. Treated women had an increased time to pregnancy and more often sought medical attention for infertility. Of these, women treated with 200 µg of EE significantly more often experienced fertility problems than women treated with 100 µg. Our results suggest an important role of estrogen dose on fertility outcome and a possible lead towards explaining the mechanism behind the loss of fertility in tall women treated with high-dose estrogen in adolescence.

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Chapter 4

Fatherhood in tall men treated with high dose sex steroids during adolescence

AEJ Hendriks, WPA Boellaard, NJ van Casteren, JC Romijn, FH de Jong, AM Boot and SLS Drop

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Abstract

Background/Objective: Sex steroid treatment to reduce adult height of tall boys has been available since the 1950s. In women, it has been shown to interfere with fertility. In men, no such data are available. We, therefore, evaluated fertility and gonadal function in tall men who did or did not receive high-dose androgen treatment in adolescence.

Methods: Retrospective cohort study of 116 tall men, of whom 60 had been treated. Reproductive and gonadal function was assessed by standardized interview, semen analysis, endocrine parameters, ultrasound imaging and fatherhood. Mean age at treatment commencement was 14.2 years and mean follow-up was 21.2 years.

Results: Sixty-six men, 36 treated and 30 untreated, had attempted to achieve fatherhood. The probability of conceiving their first pregnancy within one year was similar in treated and untreated men (26 vs. 24, Breslow P=0.8). Eleven treated and 13 untreated men presented with a left-sided varicocele (P=0.5). Testicular volume, sperm quality and serum LH, FSH, and inhibin B levels were comparable between treated and untreated men. However, treated men had significantly reduced serum T levels, adjusted for known confounders (mean (SD) 13.3 (1.8) vs. 15.2 (1.9) nmol/l, P=0.005). In addition, testicular volume and serum inhibin B and FSH levels in treated men were significantly correlated with age at treatment commencement.

Conclusion: At a mean follow-up of 21 years after high-dose androgen treatment we conclude that fatherhood and semen quality in tall treated men are not affected. Serum testosterone levels, however, are reduced in androgen treated men. Future research is required to determine if declining testosterone levels may become clinically relevant for these men as they age.

Introduction

High-dose sex steroid treatment to reduce adult height of tall boys has been widely used.[1-3] Possibly due to greater social acceptance of tall stature, fewer adolescent boys are treated these days. The treatment of tall stature is based on the understanding that exposure to gonadal steroids leads to epiphyseal fusion of the long bones during pubertal development. In the Netherlands, treatment has been available based on psychosocial grounds for boys with a predicted height over the 97th percentile (198 cm).[4, 5] Commonly used treatment is an intramuscular injected preparation of testosterone ester mixtures (Sustanon '250'®) in a dose of 250 mg per week for, on average, a period of 1.5 years.[5]

High doses of androgens are known to greatly reduce sperm production, which is reversible in adult men although it sometimes may take years until full recovery.[6, 7] However, because in the maturing gonads the initiation of spermatogenesis is stimulated by hormones of the hypothalamic-pituitary-gonadal axis, treatment given during puberty may have lasting effects on pituitary-gonadal functioning.[8] Therefore, several studies have looked at possible side-effects of high-dose androgen treatment for tall stature. Short-term side-effects are well documented and include: weight gain, acne, gynecomastia, muscle ache and edema.[5] A few studies have reported on long-term fertility and reproductive function after a follow-up of up to ten years. One study found marginally higher serum follicle stimulating hormone levels and lower serum luteinizing hormone levels in androgen treated boys compared to untreated tall boys at an average follow-up of ten years.[8] None have found significant effects on sperm quality or fertility. In contrast, recent studies with over 20 years of follow-up of high-dose sex steroid treated tall women have shown reduced fertility, which is very relevant to the many treated young women who are still planning to have children.[9] We initiated the current study because there have been no such long-term follow-up studies in tall treated men. The aim of this single center retrospective cohort study was to evaluate fertility and testicular function in a cohort of tall Dutch men who did or did not receive high-dose androgen treatment in adolescence.

Methods

Subjects

From our records, we identified men who attended our clinic between 1968 and 1998 for evaluation of tall stature. All evaluations at initial presentation had been performed by pediatric endocrinologists who assessed skeletal age according to Greulich-Pyle using hand and wrist radiography to predict adult heights.[10] Men with a predicted height above the 97th percentile were eligible to participate in the current study if an underlying disease as cause of their tall stature was excluded. Participants included men who received intramuscular testosterone treatment (250 mg Testosterone Esters a week) in adolescence (treated group) and those who did not receive treatment (untreated group). Common reasons for choosing not to have treatment were satisfaction with the predicted adult height or uncertainty about possible side-effects. Approximately half of these men had previously participated in the study by de Waal et al.[11] Men with endocrine or metabolic disorders, chromosomal defects and primary or secondary growth disorders were excluded.

Eligible men were traced using municipal registries and invited by mail to participate. Participants received a questionnaire assessing their personal and family history as well as relevant demographics. Participants were invited to visit the outpatient Andrology clinic of the Erasmus Medical Center. The study received ethical approval of the Erasmus Medical Center ethics committee, and all participants provided written informed consent.

Clinical and endocrine examination

Subjects underwent a standardized examination between one and four in the afternoon. Clinical examination included a standardized interview that encompassed reproductive history, fertility problems and sexual difficulties. Two approaches were used to assess fertility outcome. Firstly, men were asked yes/no questions regarding fertility. Secondly, men were asked to estimate the number of months of unprotected intercourse before their first pregnancy, either spontaneous or planned.

Height was measured using a SECA 225 stadiometer. Testicular ultrasounds were performed, using a 12 MHz transducer (Toshiba Nemio 20) equipped with color flow imaging to assess testicular volume, varicocele grade and other abnormalities. Varicocele was diagnosed when veins in the pampiniform plexus had a minimum resting diameter

of 3 mm or an increase in venous diameter beyond 3 mm with the Valsalva maneuver. Varicoceles were graded according to the degree of reflux identified by color Doppler ultrasound: grade 1 = little reflux (<2 sec.) with Valsalva maneuver; grade 2 = clear reflux (>2 sec.) with Valsalva maneuver; and grade 3 = spontaneous venous reflux.[12, 13]

Semen analysis was performed according to WHO guidelines by an experienced technician.[14] All semen samples were obtained at the hospital by masturbation. Sperm concentration was determined in duplicate in an improved Neubauer counting chamber, after appropriate dilution of the sample. Total sperm count was calculated from the data of sperm concentration and sample volume. Sperm motility was assessed at 37°C on duplicate slides and expressed as percentage of grade a, b, c and d.

Endocrine screening included serum assays for follicle stimulating hormone (FSH), luteinizing hormone (LH), SHBG (fluorescence-based immunometric assays on Immulite 2000; Siemens, Los Angeles, CA, USA), testosterone (T) (coated tube RIA; Siemens), inhibin B (enzyme-immunometric assay; Serotec, Oxford, UK), and Anti-Müllerian hormone (AMH) (in-house ELISA, commercially available through Diagnostic Systems Laboratories, Webster, TX, USA).[15] Intra/interassay coefficients of variation were for LH <5/7%, FSH <3/8%, T <3/5%, SHBG <4/5%, inhibin B <7/15%, and AMH <4/5%. Non-SHBG-bound testosterone (T non-SHBG) was calculated using the method described by Sodergard et al.[16] assuming a fixed albumin level of 40 g/l. The formulas for these calculations have been described earlier.[17]

Statistical analysis

To study the differences between treated and untreated men, categorical variables were compared using the χ^2 and Fisher's exact test. The linear-by-linear association χ^2 statistic was used to test for linear relationships in ordered categorical variables. For normally and non-normally distributed continuous variables, Student's t-test and the Mann-Whitney U-test were used, respectively. To adjust for age, multiple binomial logistic regression was used to estimate adjusted odds ratios. In all analyses, a two-tailed P-value of less than 0.05 was regarded to be statistically significant.

Analysis of time to first pregnancy (TTP) was done using the Kaplan-Meier (KM) survival method to compare periods of not conceiving a pregnancy between treated and untreated men. Cumulative probabilities of conception were calculated by KM life-table analysis. The Breslow test was used to test the null hypothesis of no difference between the groups in the probability of conception at any time point. Censoring was used for the following two conditions: *a*) TTP >12 months; and *b*) still having not conceived

a pregnancy at the time of interview. For spontaneous pregnancies with <1 month of unprotected intercourse, TTP was recorded as 1 month. Cox proportional hazard model was used to account for possible confounders. Variables were entered into the model and retained in a backward stepwise manner based on the likelihood ratio if their presence significantly improved the fit or if their presence in the model substantially modified the estimate of the treatment effect.

Due to following a non-Gaussian distribution all hormone values were log-transformed. Multiple regression modeling was used to study gonadal parameters and test for significant associations with androgen treatment. Age, BMI and smoking are known confounders and were added to all models.[18, 19] Varicocele was added to the models analyzing semen parameters, because of a significant association observed in our study. Longitudinal follow-up was analyzed using two-way repeated measures analysis of variance (ANOVA). Serum hormone levels were log-transformed and total sperm count was rank-transformed because of non-normality after log transformation. All calculations were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL).

Results

Participants

From our patient records at the department of pediatric endocrinology we identified 400 eligible subjects, of whom 377 (94%) could be traced and 116 (31%) agreed to participate. One hundred fifty-three men had been treated with high-dose testosterone in the past, and 60 of them (39%) were included in this study. The untreated group consisted of 56 men. Men who did not participate in our study were slightly younger (33.7 vs. 35.5 yr, P=0.02) than participants but were not different with regard to adult height (198.7 vs. 197.2 cm, P=0.1) or treatment regimen.

Table 1 shows the general characteristics of the participating treated and untreated men. Both groups were similar except that treated men, despite having received growth suppressive treatment, were significantly taller than untreated men. Mean (SD) age at treatment commencement was 14.2 (1.3) years, mean treatment duration was 15.6 (5.7) months and mean period of follow-up was 21.2 (5.2) years.

Table 1. Characteristics of the study participants.

Characteristic	Treated	Untreated	P-value
n	60	56	
Age (yr)	35.3 (5.5)	35.7 (6.8)	0.69 [a]
Height (cm)	198.2 (3.9)	195.9 (5.5)	0.01 [a]
Weight (kg)	100.8 (15)	101.2 (16)	0.88 [a]
BMI	25.6 (3.6)	26.3 (3.7)	0.30 [a]
Marital status			
Single	10 (17%)	13 (23%)	
Married or cohabitating	47 (78%)	43 (77%)	
Divorced or widowed	3 (5%)	0 (0%)	0.18 [b]
Highest education level achieved*			
Low education level	8 (13%)	5 (9%)	
Medium education level	20 (34%)	28 (51%)	
High education level	32 (53%)	22 (40%)	0.47 [c]

Differences between treated and untreated men were tested for significance by the [a] T-test, [b] χ^2-test and [c] trend test. Values are mean (SD) or n (%). * one untreated missing.

Fatherhood

Of the 116 participants, 66 men had attempted to achieve fatherhood (36 treated and 30 untreated men). The resulting pregnancies had similar outcome regarding miscarriages. The probability of conceiving their first pregnancy within one year was similar in treated and untreated men (26 (72%) vs. 24 (79%), Breslow P=0.8)(Figure 1). Accounting for several possible confounders in the discrete Cox model did not significantly change the estimate of the treatment effect on time to first pregnancy. In addition, indicated by the need to see a doctor and/or receiving fertility treatment for difficulties becoming pregnant, treated tall men were as likely as untreated tall men to experience reduced fertility: eight (22.2%) and six men (20.0%), respectively. Of these, six treated men (16.7%) and two untreated men (6.7%) had advanced to receive fertility treatment, but this was not significantly different between both groups (OR=2.85, 95%CI 0.53-15.4). Both male and female factors were involved in causing fertility problems, which were equally distributed among treated and untreated men. In addition, mean age and height of female

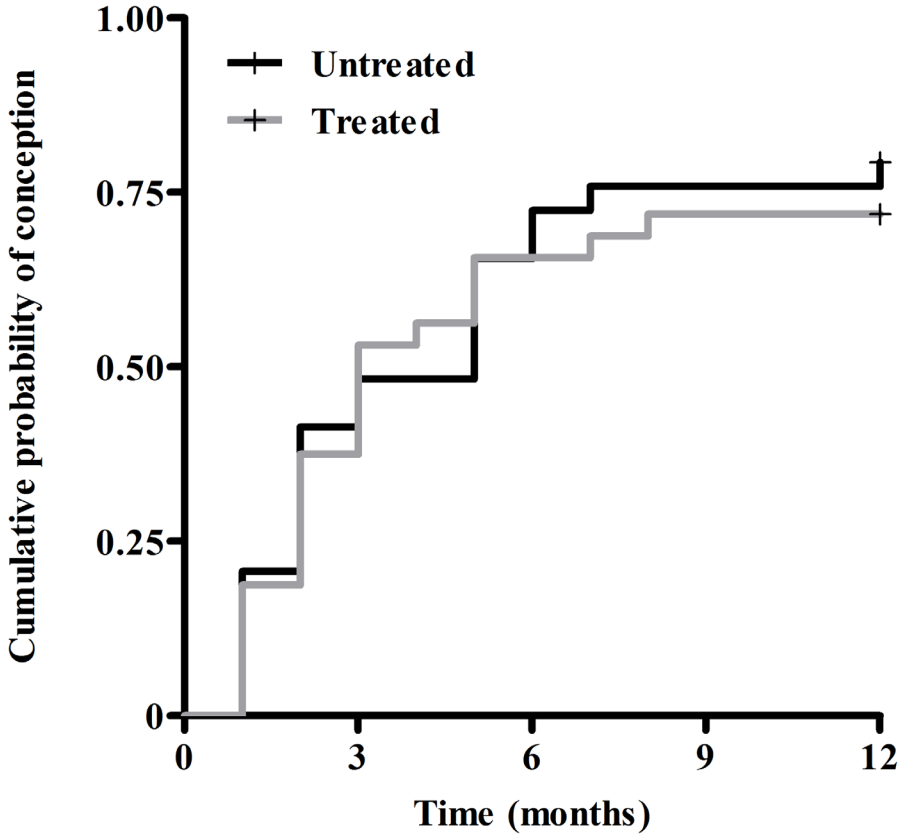

Figure 1. Cumulative probability of conception of treated and untreated men.

Breslow P-value = 0.8.

partners did not differ between the groups. Most men and their partners had achieved at least one live birth in both treated and untreated groups (32 (88.9%) vs. 26 (86.7%), OR=1.27, 95%CI 0.28-5.68). Partners of two men were currently pregnant with their first pregnancy. Three treated (8%) and three untreated (10%) men had not achieved a first pregnancy at the time of interview with a median (range) of 60 months (28-168 months) of attempting to achieve fatherhood. Age at treatment commencement was not correlated with time to first pregnancy (r=-0.02, P=0.9). In addition, we found no difference in mean age at treatment commencement in treated men with impaired or normal fertility (P=0.8).

Testicular examination and semen analysis

Several potential confounding factors that may influence semen quality were taken into account (Table 2). Ten men had chosen for a vasectomy after achieving at least one live

birth. In treated men the decision for vasectomy was not related to timing and duration of treatment. At physical examination and testicular ultrasound, 11 treated (18%) and 13 untreated men (23%) presented with a varicocele, all were left-sided. Neither prevalence nor severity of varicocele differed between treated and untreated men. In the treated group there were more current smokers than in the untreated group. Two men, one treated and one untreated, were excluded from further analyses because of a previous history of chemotherapy (M.Hodgkin, Glioblastoma).

Table 2. Confounding factors of semen quality.

Confounding factor	Treated	Untreated	P-value
n	60	56	
Varicocele (left-sided)	11 (18%)	13 (23%)	0.52 [a]
Grade 1	4 (36%)*	7 (54%)*	
Grade 2	5 (46%)*	4 (31%)*	
Grade 3	2 (18%)*	2 (15%)*	0.51 [b]
Currently smoking	17 (28%)	7 (13%)	0.04 [a]
Venereal disease	2 (3%)	3 (5%)	0.59 [a]
Cryptorchism	5 (8%)	3 (5%)	0.53 [a]
Vasectomy	6 (10%)	4 (7%)	0.59 [a]

Differences between treated and untreated men were tested for significance by the [a] χ^2-test and [b] trend test. Values are n (%). * Percentage of men with varicocele.

Mean testicular volume at the time of follow-up did not differ between treated and untreated men (Table 3). Further ultrasound examination revealed no significant abnormalities or differences between both groups. Adult mean testicular volume was moderately correlated with sperm concentration in both groups (r=0.26, P=0.009). In treated men, mean testicular volume was significantly correlated with age at treatment commencement (r=0.29, P=0.03) (Figure 2) but not with treatment duration. In addition, in all tall men, adult testicular volume was correlated with serum FSH and inhibin B levels (r=-0.43 and r=0.54, P<0.001) and was borderline significantly correlated with serum T levels (r=0.19, P=0.049).

Semen analysis could not be performed in 12 men because 10 were vasectomized and 2 refused. In the resulting 102 men, we found no significant differences in semen parameters between 52 treated and 50 untreated men, which is summarized in Table 3.

Table 3. Results of testicular ultrasound and semen analysis.

Measurement	Treated	Untreated	P-value
Testicular ultrasound (n)	59	55	
Testicular volume (ml)	15.8 (3.9)	16.1 (3.6)	0.33 [a]
Epididymal diameter (mm)	9.0 (1.7)	9.3 (1.7)	0.83 [a]
Soft testicular consistency	5 (9%)	1 (2%)	0.21 [b]
Intratesticular microcalcifications	3 (5%)	4 (7%)	0.63 [b]
Epididymal cysts	6 (10%)	11 (20%)	0.15 [b]
Semen analysis (n)	52	50	
Semen volume (ml)	4.2 (1.0-9.8)	4.0 (1.8-11.2)	0.76 [c]
Sperm concentration (10^6/ml)	47.0 (2.0-316)	49.5 (0.2-213)	0.67 [d]
Total sperm count (10^6)	188.7 (4.2-882)	166.9 (0.6-880)	0.72 [d]
Progressive motility (%)	50 (4-82)	53 (9-91)	0.23 [d]
Oligozoospermia	9 (18%)	13 (25%)	0.39 [b]
Asthenozoospermia	16 (32%)	21 (40%)	0.38 [b]

Differences between treated and untreated men were tested for significance by the [a] multiple linear regression analysis with correction for vasectomy, [b] χ^2-test, [c] T-test and [d] multiple linear regression analysis on log transformed outcome variables with correction for age, BMI, and the presence of a varicocele. Values are mean (SD) or n (%).

In total, nine treated (18%) and 13 untreated (25%) men had oligozoospermia (sperm concentration <20*10^6/ml), while 16 (32%) and 21 (40%) men had asthenozoospermia (number of grade a motile sperm <25% and of grade a+b <50%). Semen quality was not correlated with age at treatment commencement nor with treatment duration.

In tall men, either treated or untreated, the presence of a varicocele significantly predicted the risk of developing oligozoospermia (OR=4.7, 95%CI 1.5-14.2, P<0.01) in a logistic regression model correcting for age and BMI. Moreover, borderline significance was reached for varicocele (OR=2.6, 95%CI 0.95-7.0, P=0.062) in the prediction of asthenozoospermia. In addition, men with varicocele had a higher risk of needing fertility treatment (OR=5.3, 95%CI 1.12-25.3, P=0.044), but the chance of achieving at least one live birth was not significantly affected (OR=0.7, 95%CI 0.13-3.96, P=0.65).

Endocrine function

Multiple regression modeling was used to analyze the associations of androgen treat-

ment with serum hormone levels of the hypothalamic-pituitary-gonadal axis while correcting for several known confounders. First, we compared treated men to untreated men. Results of this analysis are shown in Table 4. Androgen treatment did not have an effect on the major hormones involved in Sertoli cell function: after adjustment for age, BMI and smoking, androgen treatment was not significantly associated with serum FSH and inhibin B levels. However, we did observe an association between androgen treatment and serum AMH levels, with lower levels observed in treated men.

Moreover, multiple linear regression analysis showed that androgen treatment in adolescence has an effect on hormones involved in Leydig cell function later in life. Serum testosterone levels were significantly lower in treated compared to untreated men after adjustment for age, BMI and smoking. Mean (SD) adjusted testosterone levels, were 13.3 (1.8) nmol/L in treated men compared to 15.2 (1.9) nmol/L in untreated men (P=0.005). Serum LH and SHBG levels were not influenced by androgen treatment. However, using the same model, non-SHBG-bound testosterone was also reduced in treated men. Serum T and LH levels were significantly correlated in both groups (r=0.3, P=0.03).

Table 4. Multiple regression analysis of factors influencing gonadal parameters.

Dependent variable	Age (yr)		BMI (kg/m^2)		Smoking		Treatment		Adj. R^2
	β	p	β	p	β	p	β	p	
Sertoli cell function									
FSH[1]	0.004	n.s.	0.004	n.s.	0.018	n.s.	0.002	n.s.	0.14
Inhibin B[1]	0.001	n.s.	-0.010	**0.009**	-0.013	n.s.	-0.009	n.s.	0.41
AMH[1]	-0.011	**0.007**	-0.003	n.s.	0.048	n.s.	-0.091	**0.049**	0.07
Leydig cell function									
LH[1]	0.001	n.s.	0.001	n.s.	-0.045	n.s.	-0.025	n.s.	0.01
T [1]	0.001	n.s.	-0.009	**0.009**	0.106	**0.001**	-0.068	**0.005**	0.14
SHBG[1]	0.009	**0.001**	-0.020	**0.001**	0.063	0.104	-0.052	0.087	0.16
T n-SHBG[1]	-0.004	**0.042**	-0.003	n.s.	0.091	**0.001**	-0.054	**0.012**	0.14

Significant values are given in bold. n.s.: not significant and p > 0.10, β: regression coefficient, p: p-value. Number of missing data: BMI; n=1 (weight not measured) [1] Log transformed

Second, we studied the effect of treatment within treated men only. Results of this analysis are shown in Figure 2. We found a significant negative correlation between age at treatment commencement and serum FSH levels during follow-up (r=-0.39, P=0.004) (Figure 2). Similarly, serum inhibin B levels were positively correlated with age at treatment commencement (r=0.29, P=0.029). Moreover, in a linear regression model, age at treatment commencement was significantly associated with serum FSH (P=0.002) and inhibin B (P=0.003) levels after adjustment for age, BMI and smoking. No correlations between LH or T and age at treatment commencement were observed.

Longitudinal follow-up

A subgroup of 36 men (23 treated and 13 untreated) had participated 15 years ago in the study by de Waal, allowing us to study possible longitudinal effects that would be missed in our cross-sectional design.[8] Eleven men previously diagnosed with varicocele participated in the current study. Five of them presented again with a left-sided varicocele of the same grade and one man had progressed from grade 2 to grade 3. Another five men, who were previously diagnosed with a grade 0 varicocele using Doppler stethoscope, were not diagnosed with varicocele using ultrasound in the present study.

A two-way repeated measures ANOVA revealed a significant interaction of time with treatment for total sperm count (F(1,29)=5.25, P=0.03), indicating that in treated men the total sperm count over time had significantly decreased (mean decrease = $57*10^6$ spermatozoa) compared to untreated men. In addition, inhibin B showed a similar time dependent treatment effect with serum levels decreasing faster in treated men (mean decrease = 49 ng/L, F(1,14)=5.44, P=0.04). No time dependent effects of treatment on

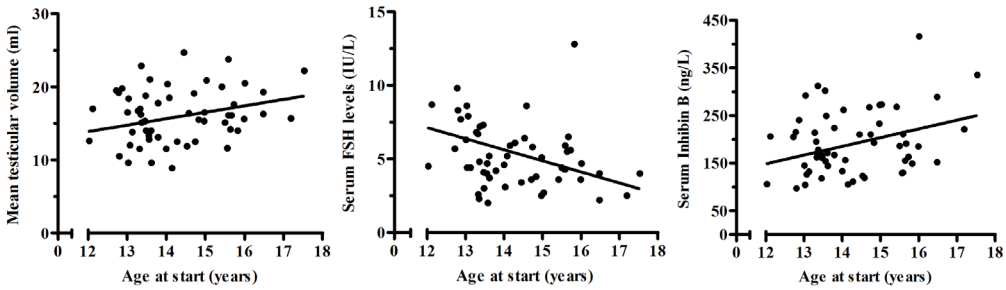

Figure 2. Correlations of testicular volume and serum hormone levels with age at treatment commencement in androgen treated men.

Mean testicular volume: r=0.29, P=0.03, FSH: r=-0.39, P=0.004, inhibin B: r=0.29, P=0. 029.

other serum hormone levels were observed. Although over time serum LH and FSH had significantly increased while T levels had decreased, these changes were independent of treatment.

Discussion

We evaluated fertility and testicular function in tall men who did or did not receive high-dose androgen treatment in adolescence. Our results show that there is no long-term impact on fatherhood in treated men. Time to first pregnancy did not differ between treated and untreated men and the number of men that had experienced subfertility was similar in both groups. Most importantly, over 85% percent of treated and untreated men who had attempted to achieve fatherhood had achieved at least one live birth. These results contrast with recent findings of increased risk for subfertility in later life in high-dose estrogen treated tall girls.[9]

Testicular ultrasound examination showed no major abnormalities in treated men. Testicular volume and epididymal diameter did not differ between treated and untreated men. We found an overall prevalence of varicocele of 20% in tall men, which is half of the previously reported 40%.[8] This discrepancy is likely caused by our use of more sensitive imaging methods, as confirmed by our longitudinal follow-up where none of the low grade varicoceles detected by Doppler stethoscope were judged to be varicoceles using ultrasound imaging. In fact, low specificity of the Doppler stethoscope has been reported before.[20, 21] The observed 20% prevalence of varicocele in tall men is higher than the known population prevalence of 11%.[22] Therefore, our study supports the hypothesis of previous studies that increased height is a risk factor for varicocele.[23, 24]

In line with the fertility results, semen parameters were comparable in androgen treated and untreated tall men. This suggests that no severe long-term complications of high-dose androgen treatment on spermatogenesis are apparent after 21 years of follow-up, which is in agreement with findings of previous studies performed directly after cessation of treatment or after a few years of follow-up.[2, 11] In contrast, in the subgroup analysis we show that in treated men total sperm count and inhibin B levels decline more rapidly when followed longitudinally. Longer follow-up is necessary to clarify whether this decline will continue and result in disproportionately lower semen parameters in treated men as they age. This may have implications for treated men that wish to father a child at a more advanced age. However, the mean age of the population currently studied is 35 years old, which corresponds to the average age at which Dutch men father their

children.[25] We therefore believe that semen quality as measured in our study is a good predictor for chances of fatherhood at the age when the average man wishes to father children.

Overall, hormones involved in Sertoli cell function were not affected by androgen treatment, except for AMH, which was significantly decreased in treated men. One study has shown that lower AMH levels are seen in men with testicular dysfunction, while another study found no such relationship.[26, 27] The true relationship between serum AMH and male fertility remains to be established, but currently AMH seems inferior to FSH as a marker of sperm production.[28] Our results contrast with earlier findings of marginally increased FSH levels in treated men.[8] This association may have been lost due to the age-related increase of FSH as seen in our longitudinal follow-up and in a previous study.[29] In addition, while Sertoli cell function was not affected in comparison to untreated men, within treated men we observed decreased testicular volume and inhibin B levels and increased FSH levels in those treated earlier. Thus, the older the boy at the time of treatment, the more favorable are parameters associated with Sertoli cell function. However, age at treatment commencement was not correlated with semen parameters or fatherhood.

Leydig cell function was significantly affected by androgen treatment. In treated men both serum testosterone and non-SHBG-bound testosterone levels were significantly reduced compared to untreated men. This association of androgen treatment with circulating T levels was independent of LH, as serum LH levels were not associated with treatment. We hypothesize that the decreased T levels may be caused by reduced Leydig cell growth during puberty and suboptimal functioning of the Leydig cells in later life. In humans, there are three waves of Leydig cell growth: the third is during puberty when growth is strictly under the control of luteinizing hormone.[30] High-dose androgen treatment in tall boys is given during puberty and suppresses the hypothalamic-pituitary-gonadal axis.[1, 2] This results in low levels of LH during the third wave of Leydig cell growth. It is therefore not unlikely that Leydig cell growth and function would be impaired in these men. This is supported by our findings of decreased testicular volume when treatment is started at an earlier age as well as the reduced T levels observed in treated men. Although Leydig cells make up less than 20% of the testicular volume, we and others have observed a significant correlation between testicular volume and serum T levels.[31]

Published normal ranges of serum T in healthy men are typically above 10.4 nmol/L. Levels below 8.7 nmol/L are considered unequivocal hypogonadism, while values be-

low 12.0 nmol/L have been associated with mild sexual dysfunctioning.[32-34] A recent study using 140 healthy controls of average height with a median age of 32 years old found mean T levels of 16.0 nmol/L.[35] This is comparable to our observed T levels in untreated men. However, T levels in the androgen treated men, albeit still within the normal range, were significantly lower and approach clinically relevant cut-off values. Since T levels will continue to decline as these men age, we believe our results may be of clinical relevance to androgen treated tall men as they grow older. Especially in light of a recent cohort study of men over 65 years old, which found low levels of non-SHBG-bound T to be associated with worse baseline frailty status.[36] In addition, LH levels may only rise as soon as clinically relevant low serum T levels are observed. Therefore, future studies are required to fully elucidate the effects of treatment on LH and T levels.

Thirty-one percent of the eligible men participated in our study, we therefore cannot fully exclude the possibility of selection bias. It is possible that men with reproductive problems may have been more interested in participating. In addition, our sample was older than the source population. However, we believe this may increase our power to detect differences in fatherhood as more men may have reached the age at which they wish to father children.

In conclusion, we evaluated fertility and testicular function in later life of tall men who did or did not receive high-dose androgen treatment in adolescence. We found no long-term impact of this treatment on fatherhood or semen quality. Testosterone production, however, is reduced in androgen treated men. Future research is required to determine if declining testosterone levels may become clinically relevant for these men as they age.

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Part 2

Genetic determinants of constitutional tall stature

Chapter 5

Common polymorphisms in the GH/IGF-1 axis contribute to growth in extremely tall subjects

AEJ Hendriks, MR Brown, AM Boot, BA Oostra, FH de Jong, SLS Drop and JS Parks

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Abstract

Context/Objective: The growth hormone (GH)/insulin-like growth factor-1(IGF-1) axis is the key regulator of somatic growth in humans and its genes are plausible candidates to study the genetics of height variation. Here, we studied polymorphic variation in the GH/IGF-1 axis in the Dutch extremely tall.

Methods: Case-control study of 166 tall cases with height >2 SDS and 206 controls with normally distributed height <2 SDS. Excluded were subjects with endocrine disorders or growth syndromes. We analyzed genomic DNA at 7 common polymorphisms in the GH1, GH receptor (GHR), IGF1 and IGFBP3 genes.

Results: The association of the GH-1 1663 SNP with tall stature approached statistical significance, with the T-allele more present in the tall (allele frequency (AF): 0.44 vs. 0.36; p=0.084). Moreover, haplotype frequencies at this locus were significantly different between cases and controls, with the GGT haplotype most commonly seen in cases (p=0.01). Allele frequencies of GHR polymorphisms were not different. For the IGF-1 CA-repeat we observed a higher frequency of homozygous 192-bp carriers among tall males compared to control males (AF: 0.62 vs. 0.55; p=0.02). The IGFBP-3 -202 C-allele occurred more frequently in cases than in controls (AF: 0.58 vs. 0.50; p=0.002). Within cases, those carrying one or two copies of the -202 C-allele were significantly taller than AA genotype carriers (AC, p=0.028 and CC, p=0.009). Serum IGFBP-3 levels were highest in AA genotype carriers, the -202 SNP explained 5.8% of the variation.

Conclusion: Polymorphic variation in the GH1, IGF1 and IGFBP3 genes is associated with extremely tall stature. In particular, the IGFBP-3 -202 SNP is not only associated with being very tall but also with height variation within the tall.

Introduction

Human growth and adult height are considered highly heritable polygenic traits that reflect the input of multiple genes interacting with environmental factors such as nutrition. Estimates of heritability for stature are high ($h^2 \sim 0.80–0.90$).[1, 2] Constitutionally tall stature (CTS) is a condition characterized by a normal pattern of childhood growth and a final adult height of more than two standard deviations above the mean of the normal population. In the Netherlands, CTS is currently defined as a final adult height of over 184 cm in women and over 198 cm in men.[3] In CTS mean birth length is at or above the 75th percentile (0.7 SDS) and tall stature becomes evident at the age of 3-4 years.[4] Growth velocity is accelerated in early childhood but slows down after 4-5 years of age when the growth curve starts to parallel the normal curve.[5] No apparent abnormalities are present at physical examination which makes it possible to distinguish CTS from primary or secondary excessive growth syndromes.[4] In CTS, usually one or both parents are tall; thus, genetic factors play a key etiological role.

The growth hormone (GH)/insulin-like growth factor-1(IGF-1) axis is the key regulator of somatic growth in humans and its genes are plausible candidates to study the genetics of height variation. Common polymorphisms of the GH1 gene have been associated with height variation and circulating IGF-1 levels.[6-10] Moreover, functional studies suggest that these polymorphisms influence transcription which may have an effect on circulating GH levels.[11] The IGF1 gene is one of the most studied genes in height, the CA repeat in the promoter region of this gene in particular. It has been associated with birth weight, adult height and circulating IGF-1 levels.[12-17] A common deletion of exon 3 of the GHR gene is variably associated with increased growth velocity during the first year of GH treatment but not with increased height in the general population.[18] The -202 promoter polymorphism in the IGFBP3 gene has been correlated with circulating levels of IGFBP-3 and height variation in adults.[19] It was also associated with response to GH treatment in short or growth hormone deficient children.[20, 21]

Sampling at the extremes of a quantitative trait while using common controls has been shown to maximize statistical power as much of the information is provided by individuals in the tails of the distribution.[22] While the genes of the GH/IGF-1 axis discussed above have been extensively studied in the very short, very few authors have attempted to study these genes in the extremely tall. One study reported on variations in genes of the GH/IGF-1 axis in tall stature and found no consistent variations.[23] However, their tall cohort, with heights between the 90th-95th percentiles of the respective height

distribution, stood only slightly taller (< 0.5 SDS) than the average Dutch population. We therefore studied common genetic variation in genes of the GH/IGF-1 axis (GH-1, GHR, IGF-1 and IGFBP-3) in the extremely tall Dutch with heights beyond the 97th percentile.

Methods

Subjects

From our records, we identified former patients who attended our clinic for evaluation of tall stature. Eligible subjects were traced using municipal registries and invited by mail to participate. We also identified several tall families through advertisement in the members' magazine of the Dutch advocacy club for tall people (Klub voor Lange Mensen). Subjects eligible for participation fulfilled the following inclusion criteria: 1) height SDS above +2 SD according to Dutch standards[3]; 2) Caucasian and of Dutch ancestry, defined as being born to Dutch parents who themselves were born in the Netherlands. Subjects with endocrine or metabolic disorders, or with primary or secondary growth disorders were excluded.

A cohort of Caucasian young, healthy adults of Dutch ancestry, recruited as part of the Peak Bone Mass (PBM) study, with a normally distributed height of less than +2 SD served as controls.[24] The study received Erasmus MC ethics committee approval and participants gave written informed consent.

Clinical and endocrine parameters

Participants were invited to visit the outpatient clinic of the Erasmus Medical Center. Height was measured using a stadiometer (SECA 225; SECA, Hamburg, Germany), and expressed as SDS according to Dutch standards.[3] A questionnaire concerning each subject's medical and family history and demographic information was completed.

Serum IGF-1 and IGFBP-3 levels were measured in one laboratory using an automated chemi-luminescence immunometric assay (Immulite-2000, Siemens-DPC, Los Angeles, CA, USA). The intra-assay coefficients of variation (CVs) were < 4% and the interassay CVs were < 10%. Serum levels of total IGF-I and IGFBP-3 were expressed as SDS to adjust for age and sex, based on the respective normative data which has been published previously.[25]

Genotyping

Genomic DNA was extracted from venous blood using standard methods. Genotyping was performed in duplicate using published primers and protocols, short descriptions are given below for polymorphisms in the GH-1[6-11], GHR[18, 26-29], IGF-1[12-17] and IGFBP-3[19, 20] gene.

A 2.7-kb specific GH-1 fragment was amplified from genomic DNA with oligonucleotide primers 5'-AGCCCCAGCAATGCTCAGGGA-3' (forward) and 5'-GGAGGGGTCACAGGGATGCCA-3' (reverse) and used as template for a nested polymerase chain reaction (PCR) with primers 5'-TGCTCACAACCCCCACAATC-3' (forward) and 5'-CCCTTCTCTCCCACTGTTGC-3' (reverse) that amplify a 438bp product of the GH1 gene including the SNPs at -308 (rs1811081) and -301 (rs2011732). Cycling parameters for the initial reaction were 94°C for 2 min; 30 cycles of 45 sec at 94°C, 30 sec at 61°C and 3 min at 72°C; 72°C for 2 min. Cycling parameters of the nested reaction were 94°C for 2 min; 30 cycles of 45 sec at 94°C, 30 sec at 58°C and 45 sec at 72°C; 72°C for 2 min. The PCR product was digested with 5U of Cac8-I and Fnu4H (New England Biolabs (NEB), Ipswich, MA, USA) for the -308 (rs1811081) and -301 (rs2011732) SNP respectively for 14 h at 37°C. Digestion products were visualized on 8% polyacrylamide gel (29:1 acrylamide:bisacrylamide) and stained using silver staining.

For the GHR del 3 polymorphism DNA was amplified by PCR using a multiplex strategy (5'-CCTGGATTAACACTTTGCAGACTC-3' (reverse)) to selectively amplify the alleles containing (5'-TGTGCTGGTCTGTTGGTCTG-3' (forward)) or missing exon 3 (5'-AGTCGTTCCTGGGACAGAGA-3' (forward)). Cycling parameters were 94°C for 5 min; 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 90 sec at 72°C; 72°C for 7 min. Amplification products were visualized on 1.7% agarose gel and stained with ethidium bromide.

Genotypes of the GH-1 intron 4 base 1663 SNP (rs2665802) and the GHR exon 10 L544I SNP (rs6180) were determined with Taqman allelic discrimination assay (Applied Biosystems (ABI), Foster City, CA, USA), using Assay-by-Design service. PCR amplification and post-PCR fluorescence measurement was performed on an ABI Taqman 7500.

For the IGF-1 CA-repeat PCR was performed using primers designed to amplify the polymorphic cytosine–adenine (CA) repeat 1 kb upstream of the human IGF1 gene. Forward primer 5′-ACCACTCTGGGAGAAGGGTA-3′ and reverse primer 5′-GCTAGCCAGCTGGTGTTATT-3′ were used. PCR cycling parameters were 94°C for 2 min; 35 cycles of 45 sec at 94°C, 15 sec at 55°C, and 30 sec at 72°C; 72°C for 10 min.

Forward primers were labelled with FAM, to determine the size of PCR products by autosequencer (ABI 3130, POP 7, filter set D, 36 cm column, peak height between 100 and 2000, ABI). The size of the PCR products was determined by comparison with an internal LIZ-labelled size standard (ABI). As in previous studies, IGF-1 genotypes were categorized in the following categories based on their 192-bp allele: homozygous 192-bp (wild-type), heterozygous 192-bp and homozygous non-carrier 192-bp.[13]

DNA for genotyping of the IGFBP-3 -202 SNP (rs2854744) was amplified by PCR using 5'-CCACGAGGTACACACGAATG-3' (forward) and 5'-AGCCGCAGTGCTC-GCATCTGG-3' (reverse) primers. Cycling parameters were 94°C for 5 min; 35 cycles of 1 min at 96°C, 1 min at 64°C and 1 min at 72°C; 72°C for 5 min. The PCR product was digested with 10U of BsiHKA-I (NEB) for 14 h at 65°C. Digestion products were visualized on 8% polyacrylamide gel (29:1) and stained using silver staining.

Statistical analysis

Genotype distributions for significant departure from the Hardy-Weinberg equilibrium were calculated using the chi-square test. The inclusion of families may increase type 1 error. This increase is relatively low in small pedigrees of sib pairs and nuclear families compared to larger pedigrees. In our analysis we accounted for family structure to keep type 1 error rates at the nominal 0.05 alpha level.[30] A pairwise kinship matrix specifying the degree of relatedness between each pair of individuals was used in the analysis to account for the relatedness among family members. Association testing for polymorphisms was performed using a variance components model that allows for relatedness in estimating the regression coefficients.[30] Statistical significance was determined using a score test as implemented in genABEL for R statistical software package.[31-33] The primary statistical inference was the additive genetic model.

Haplotype reconstruction was performed for the GH-1 and GHR polymorphisms and haplotype frequencies were computed using Phase 2.1 software package.[34, 35] Differences in the global distribution of haplotypes between cases and controls were assessed with a PAC-likelihood permutation test implemented in Phase. After an interim analysis in 116 cases and 103 controls 3 SNPs were excluded from further study due to limited resources and a low probability ($p>0.2$) of being associated with tall stature.

Differences in serum hormone SD-scores between cases and controls were calculated with Student's t-test. Pearson's correlation coefficient was used to measure the degree of linear correlation between two variables. The Kolmogorov-Smirnov test of normality was used to test for deviations from the normal distribution. Analysis of variance was

used to compare the height and serum hormone SD-scores according to genotype. A general linear model was employed to estimate the percent variation in IGFBP-3 SDS that can be explained by genotype.

A sample size of 166 cases and 206 controls achieved 80% power to detect an effect size of 0.10 or larger using a 2 degrees of freedom chi-square test with an alpha level of .05. In addition, a total sample of 166 cases achieved 80% power to detect an association with 0.2 height SDS and 0.3 IGFBP-3 SDS using an F test with an α level of .05. A two-tailed P-value of < 0.05 was regarded to be statistically significant. All calculations were performed using R statistical software package (R Foundation for Statistical Computing, Vienna, Austria) and SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

General characteristics

We included 166 tall stature cases and 206 controls. Cases had a mean age of 40.7 ± 12.3 years and controls of 25.3 ± 6.4 years. Nineteen families participated in the study. In total 46 subjects were related; 22 siblings, 18 parents/children, and 6 avuncular relatives. Characteristics of the cases and controls are shown in Table 1. Height in controls

Table 1. Characteristics of the study participants.

Characteristic	Cases		Controls	
	Men	Women	Men	Women
n (%)	68 (41%)	98 (59%)	55 (27%)	151 (73%)
Age (yr)	41.9 (14.4)	39.9 (10.6)	26.8 (9.9)	24.8 (4.4)
Height (cm)	201.7 (5.3)	183.6 (4.7)	181.5 (5.2)	170.0 (6.0)
Height SDS	2.96 (0.76)	2.34 (0.72)	-0.30 (0.81)	-0.09 (0.94)
Weight (kg)	108.0 (15.1)	84.7 (16.2)	79.8 (12.2)	67.7 (9.8)
BMI	26.5 (3.5)	25.1 (4.6)	24.2 (3.7)	23.5 (3.2)
IGF-1 SDS	-0.13 (0.26)	-0.21 (0.22)	0.07 (0.18)	0.07 (0.17)
IGFBP-3 SDS	-0.77 (1.43)	-1.05 (1.21)	0.22 (0.86)	0.42 (0.90)

Values are mean (SD).

was normally distributed around -0.3 SDS in men and -0.09 in women. In line with the study design, cases were taller than controls and their height was normally distributed around 2.96 SDS in men and 2.34 SDS in women.

IGF-1 SDS was significantly lower in cases compared to controls (mean (SD) = -0.17 (0.24) vs. 0.07 (0.17), p<0.001), as was IGFBP-3 SDS (-0.93 (1.31) vs. 0.37 (0.89), p<0.001). IGF-1 and IGFBP-3 levels were strongly correlated (r=0.63, p=0.003) in both cases and controls.

Association with tall stature

Polymorphic variation at the seven loci was common. Table 2 presents the genotype frequencies and allele frequencies in cases and controls. All genotype distributions were in Hardy-Weinberg equilibrium (HWE). Several polymorphisms showed significant differences in genotype frequencies between cases and controls, the strength of the associations of the seven polymorphisms is shown in Table 2. Results of the haplotype analysis are shown in Table 3.

Allele frequencies of polymorphisms in the GHR gene were not significantly different between cases and controls, nor were differences in observed haplotype frequencies. The association of the GH-1 SNP at position 1663 (rs2665802) with tall stature approached statistical significance, with the T-allele more present in the tall (allele frequency (AF): 0.44 vs. 0.36; p=0.084). In addition, haplotype analysis revealed that haplotype frequencies of GH-1 SNPs were significantly different between cases and con-

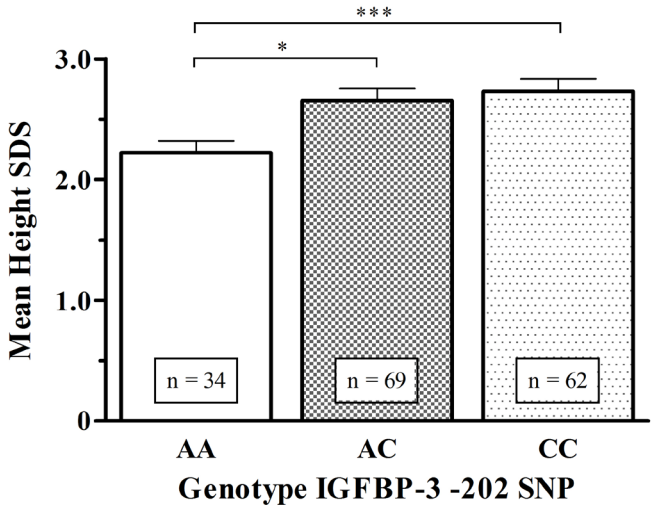

Figure 1. Mean height SDS in 165 tall subjects separated by IGFBP-3 genotype at the -202 locus.
* p < 0.05, *** p < 0.01

trols, with the GGT haplotype being more commonly seen in cases (p=0.01) (Table 3). The IGFBP-3 -202 C-allele (rs2854744) was significantly more present in the tall cohort compared to controls (0.58 vs. 0.50; p=0.002).

Table 2. Genotype frequencies of seven polymorphisms in the GH/IGF-1 axis and association with tall stature.

Polymorphism	**Chr**	**Sample**	**HWE**	**MAF**	**Genotype**			**p**
GH-1 [a]					**GG**	**GT**	**TT**	
SNP -308	17	Case	0.62	0.25	65	45	6	n.s.
rs1811081 G > T		Control	0.75	0.30	51	42	10	
GH-1 [a]					**GG**	**GT**	**TT**	
SNP -301	17	Case	0.62	0.25	65	45	6	n.s.
rs2011732 G > T		Control	0.45	0.29	54	39	10	
GH-1					**AA**	**AT**	**TT**	
SNP 1663	17	Case	0.55	0.44	54	78	34	**0.084**
rs2665802 A > T		Control	0.69	0.36	82	98	26	
GHR [a]					**3+3+**	**3+d3**	**d3d3**	
Exon 3 deletion	5	Case	0.36	0.30	55	52	8	n.s.
3+ > d3		Control	0.65	0.30	52	41	10	
GHR					**AA**	**AC**	**CC**	
SNP I544L	5	Case	0.95	0.44	54	79	33	n.s.
rs6180 A > C		Control	0.52	0.47	59	101	46	
IGFBP-3					**AA**	**AC**	**CC**	
SNP -202	7	Case	0.08	0.58	34	69	62	**0.002**
rs2854744 A > C		Control	0.48	0.50	48	108	50	
IGF-1					**192/192**	**192/ ***	*** / ***	
CA-repeat	12	Case	0.07	0.35	76	64	26	0.28
192 > *		Control	0.64	0.37	83	93	30	

[a] for these polymorphisms 116 cases and 103 controls were genotyped. Abbreviations: Chr = Chromosome, HWE = Hardy-Weinberg equilibrium, MAF = Minor Allele Frequency, 3+ = full exon 3, d3 = deleted exon 3, 192 = carrier of the 192bp allele, * = non-carrier of the 192bp allele. Significant p-values are given in bold.

Table 3. Haplotype frequencies of GH-1 and GHR polymorphisms.

Haplotype [a]	Frequency [d]		p-value
	Cases	Controls	
GH-1 [b]			
GGA	0.270	0.368	**0.01**
GGT	0.483	0.343	
TTA	0.245	0.287	
GHR [c]			
3+A	0.302	0.253	0.15
3+C	0.391	0.463	
d3A	0.304	0.276	
d3C	0.001	0.005	

[a] polymorphisms in physical order along the chromosome, [b] SNP -308, SNP -301, SNP 1663, [c] Exon 3 deletion, SNP I544L, [d] in 116 cases and 103 controls. Abbreviations: 3+ = full exon 3, d3 = deleted exon 3. Significant p-values are given in bold.

Table 4. Genotype frequencies of the IGF-1 CA-repeat stratified by gender.

IGF-1 CA-repeat	Genotype			p-value
Men	**192/192**	**192/ ***	*** / ***	
Cases	30	24	14	**0.02**
Controls	16	28	11	
Women	**192/192**	**192/ ***	*** / ***	
Cases	46	40	12	n.s.
Controls	67	65	19	

Abbreviations: 192 = carrier of the 192bp allele, * = non-carrier of the 192bp allele. Significant p-values are given in bold.

The estimated minor allele effect was 0.11 under an additive model. Overall, the IGF-1 CA-repeat was not associated with tall stature. However, when stratified by gender we observed a lower frequency of heterozygous and non-carriers of the 192-bp allele among tall males compared to control males (p=0.02) (Table 4). Stratification by gender did not change the results of the other polymorphisms.

In addition to studying the association of the polymorphisms in cases of tall stature compared to controls of normal stature, we studied whether the polymorphisms were associated with adult height variation. We found the IGFBP-3 -202 SNP (rs2854744) to

be significantly associated with variation in height within the tall. Tall cases with one or two copies of the C-allele were significantly taller than the tall cases carrying the AA genotype (0.43 SDS; p=0.028 and 0.50 SDS; p=0.009, respectively) (Figure 1). No such association with height variation was observed in the controls. The IGF-1 CA-repeat and the other GH/IGF-1 axis polymorphisms studied were not associated with height variation within cases or controls.

Association with serum hormone levels

As depicted in Figure 2, serum IGFBP-3 levels were highest in tall stature cases and in controls carrying the AA genotype of the IGFBP-3 -202 SNP (rs2854744). In controls levels decreased significantly in a stepwise manner in subjects carrying one or two copies of the C allele. Using a general linear model, the percent variation in IGFBP-3 SDS that could be explained by the IGFBP-3 -202 SNP was 5.8% (p=0.002). In cases the C allele was also associated with lower IGFBP-3 levels, however levels were not different between heterozygous and homozygous carriers. Variation in IGF-I levels was not associated with the IGFBP-3 -202 SNP.

Circulating IGF-1 levels did not differ for the various genotypes of the IGF-1 CA-repeat within cases or controls. Moreover, IGFBP-3 levels were not affected by the genotype at this polymorphic marker.

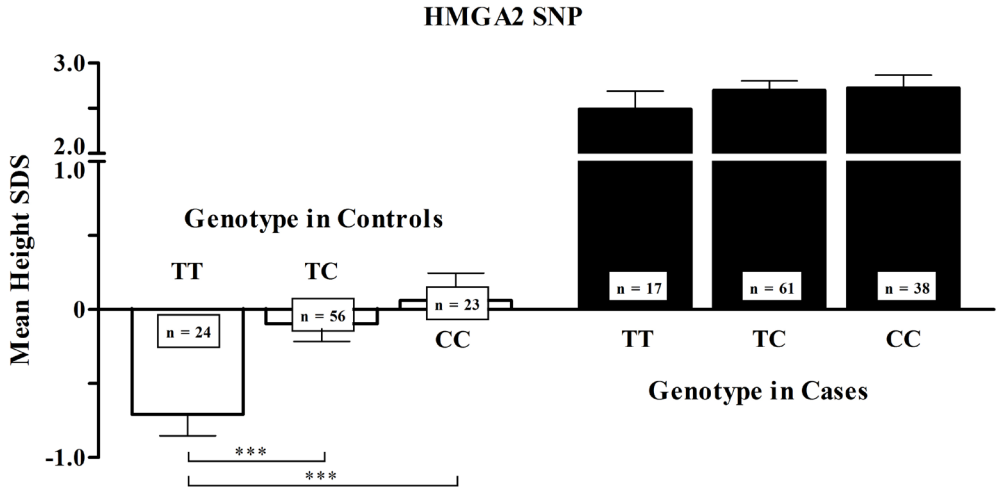

Figure 2. Mean serum IGFBP-3 SDS in 165 tall subjects and 206 controls separated by IGFBP-3 genotype at the -202 locus.

* $p < 0.05$, *** $p < 0.01$

Discussion

We performed a case-control study to assess the role of polymorphic variation in the GH/IGF-1 axis in the regulation of extreme tall stature. We found significant associations of some of the studied polymorphisms with height variation and circulating levels of hormones of the GH/IGF-1 axis. The IGFBP-3 -202 promoter polymorphism has been shown extensively to have an important role in regulating IGFBP-3 levels in both adults and children.[19, 20] Functional studies have shown that the -202 SNP can differentially affect transcription of reporter genes depending on the genotype.[19] It may exert its effects through nearby upstream stimulatory factor binding sites.[36] The -202 SNP has also been associated with height variation, one study observed the CC genotype to be more prevalent among taller individuals.[19] In a cohort of short SGA children the -202 SNP was not associated with short stature. However, in conjuncture with a C-allele at the -185 position, it was associated with increased growth response to GH treatment.[20]

In our cases of tall stature the IGFBP-3 -202 C-allele occurred more frequently than in controls of normal height. The allele frequency observed in controls was comparable with the frequency found in other studies of Caucasian subjects. Remarkably, the C-allele was also associated with increased height within the cases. Carriers of one or two copies of the C-allele were on average almost 0.5 SDS taller. Polymorphic variation explained 5.8 percent of age and gender adjusted circulating IGFBP-3 levels. In line with previous studies, IGFBP-3 levels in controls were highest in carriers of the AA genotype and declined in a stepwise manner per C-allele. In cases, however, both carriers of the AC genotype and carriers of the CC genotype had similar decreased IGFBP-3 levels compared to the AA genotype. We hypothesize that the observed dominant effect of the C allele on circulating IGFBP-3 levels in cases may be related to GH levels. It has been shown before in GH deficient subjects that the effect of the IGFBP-3 SNP may be dependent on GH action.[21] It has also been shown that GH levels in tall stature subjects show great diversity with a number of tall cases secreting lower than normal levels of GH while maintaining normal IGF-1 levels.[37] However, neither GH nor other possibly involved posttranslational variables such as acid labile subunit were measured in our study.

The IGF-1 CA-repeat promoter polymorphism has been shown to influence IGF-1 levels, growth early in life and adult height. Several studies have reported that the absence of the 192-bp allele is associated with a decreased adult height.[15, 17] Rietveld *et al.* have shown this association to be gender specific.[15] These authors found that male ho-

mozygous carriers of the 192-bp allele were taller than non-carriers, while in females no such relationship was found. Studies on the influence of the IGF-1 CA-repeat on growth early in life, however, show the 192-bp allele to be associated with reduced growth. A recent cohort study found non-carriers of the 192-bp allele to have an increased growth rate from mid-pregnancy to early infancy.[13] Another study has shown that the absence of the 192-bp allele was an independent risk factor for accelerated weight gain in the first year of life.[38] These contrasting associations of the IGF-1 CA-repeat with early growth and adult height are fascinating and require larger studies on growth in early childhood. Especially growth of constitutionally tall children would be of interest since a key feature of constitutionally tall stature is accelerated growth in the first years of life.

In our study, we observed a gender specific association of the CA-repeat with tall stature in adults. As in earlier studies, carrying the 192-bp allele was associated with tall stature specifically in males. In boys, height is regulated by IGF-1 longer than in girls, whose growth spurt is stunted earlier with the appearance of estradiol. This may lead to genetically determined gender differences in height. It has also been suggested that there may be a prepubertal gender difference in IGF-1 sensitivity which may explain the observed effect of 192-bp allele.[15] It has been shown in a large population based sample that there is an optimum in circulating IGF-I levels for the 192-bp allele.[15] We did not observe this association with IGF-1 levels in our population. We hypothesize that the association of the 192-bp allele with tall stature is related to (pre)pubertal growth and IGF-I gene expression. It has to be noted that the frequency of the 192-bp allele in control males was lower than previously reported in Dutch controls, however lower frequencies have been seen before in other cohorts.[12, 17] Therefore, replication of these findings is necessary to confirm the role of the 192-bp allele in tall stature.

We observed significantly decreased IGF-1 and IGFBP-3 levels in the cases compared to the controls. In CTS children, relatively high levels of IGF-I have been measured, although other authors found no differences in IGF-1 and IGFBP-3 levels with controls.[39, 40] Several studies in adults have also reported on the association of anthropometric measures such as height and IGF-1 levels. Most studies reported an increase of IGF-1 levels with increasing height.[41, 42] One study, however, showed that the individuals in the tallest tertile as a child had the lowest IGF-1 levels as an adult.[43] Another study showed IGF-1 and IGFBP-3 levels to be decreased in the highest quartile of height, albeit not statistically significant.[44] We belief our findings in the extremely tall are novel and require future research, especially in light of the associations of IGF-1 and IGFBP-3 levels with the risk of cardiovascular disease and cancer.

We observed a significant association between tall stature and haplotype at the GH1 gene. While we show that a T at SNP 1663 is borderline significantly associated with tall stature, together with G and G at SNPs -308 and -301 this haplotype is significantly more present in our cases. It has been shown that having G and G gives full in vitro promoter activity while T and T results in roughly 50% activity.[11] Until recently the GH1 gene had not been robustly associated with height variation, however data from large GWAS using dense arrays now clearly implicate this gene in affecting stature as do our results in the very tall.[45, 46]

We did not observe an associated with the GHR gene. However, our coverage of this gene is limited. Future dense array analysis is needed to study the role of the GHR and other genes in tall stature as is recently supported by a large genome-wide association study (GWAS).[45] Also, our decision to not genotype all samples for these SNPs, because of low interim association with tall stature, may have resulted in false negative results.

Initially, GWAS had yielded 47 genetic loci robustly associated with height variation.[47-49] However, effect sizes were small and therefore approximately five percent of the population height variation could be explained. Recently, this number was expanded to 180 loci explaining approximately 10% of the height variation.[45] The strength of GWAS is their unbiased approach to examine common variants across the entire genome. Their weakness is that large numbers of subjects are needed to reach statistical significance and identified SNPs are often not the actual causal variant. While GWAS brings new pathways to light, the percentage of variation explained still remains rather small. In comparison, candidate gene analysis is appropriate for pursuing an interesting gene based on knowledge of biological pathways in relatively small samples of a specific phenotype. It's disadvantage, however, is that it is a hypothesis driven approach which lacks gene finding abilities and is often not replicated when tested in other cohorts. This approach has merit in cohorts of a specific and accurately measurable phenotype, such as the very extreme manifestations of traits, i.e. the very short or very tall. We hypothesize that the extremes represent either a sum of all the low penetrance common variants and that new variants are therefore more easily found at the extremes or that it is caused by a few moderately penetrant rare variants, which may cluster at the extremes.

We believe that combining reports from GWAS and candidate gene studies will lead to identifying new SNPs and genes involved in height variation. While initially the genes of the GH/IGF-1 axis were not implicated by GWAS, the recent use of more dense arrays and direct genotyping of previously reported uncommon variants has resulted in significant associations with loci near to or containing genes of the GH/IGF-1 axis.[45, 46] Future

research should use these methods to study the genes of the GH/IGF-1 axis, especially IGF-1 which is repeatedly implicated and biologically plausible but not yet unequivocally confirmed to be associated with height variation. In addition, genes containing common variants with small effect sizes may also harbor rare variants with larger effects. Sequencing genomic regions identified by common variants, such as the IGFBP3 gene studied here, may identify these rare variants. Especially if extreme phenotypes are used as these seem to more often represent effects of loss-of-function alleles.[50]

In conclusion, we observed two common polymorphisms in the IGF1 and IGFBP3 genes and haplotype at the GH1 gene to be associated with extremely tall stature in a Dutch cohort. In particular, the IGFBP-3 -202 SNP is not only associated with being very tall but also with serum IGFBP-3 levels and with height variation within the tall cases.

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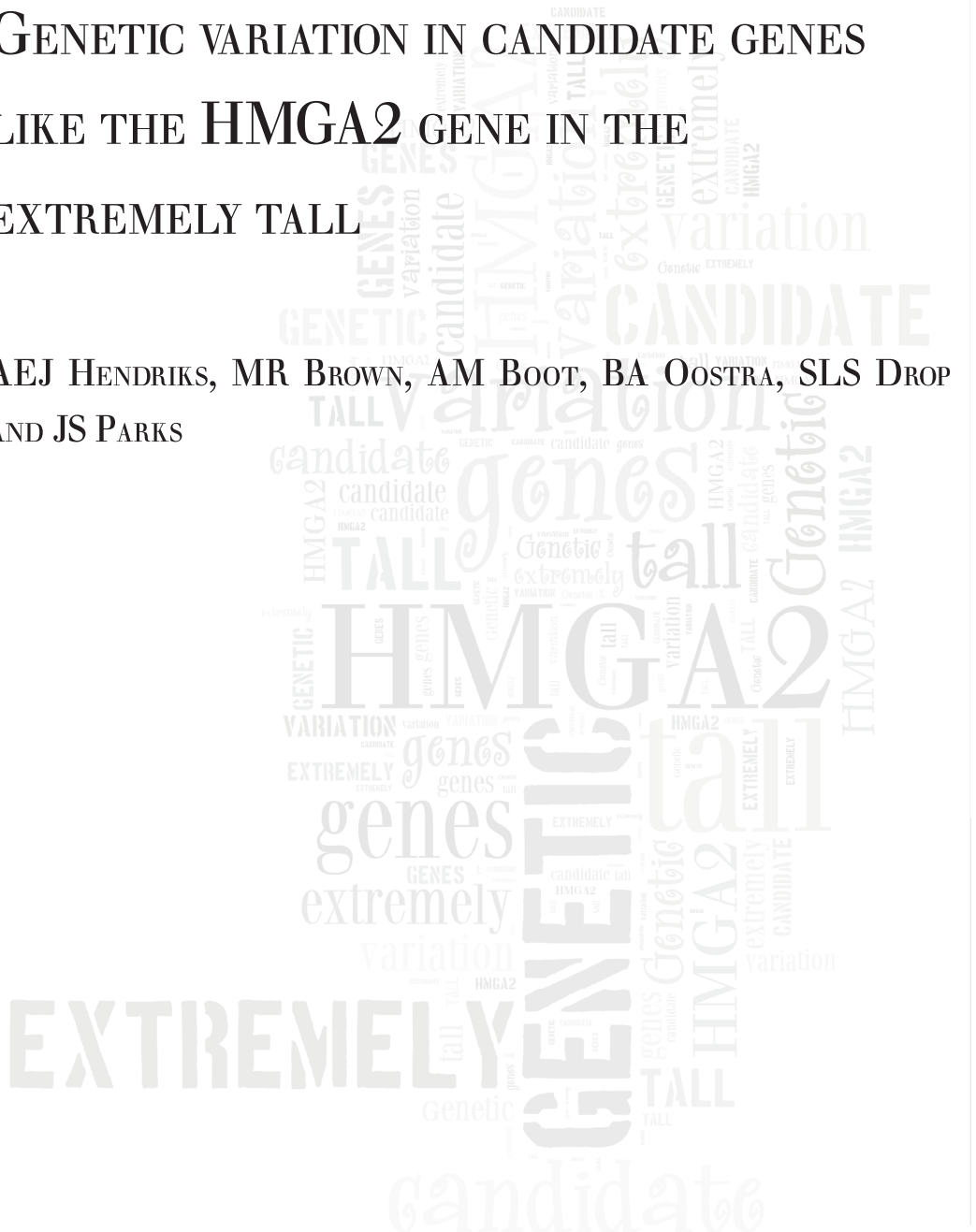
Chapter 6

Genetic variation in candidate genes like the HMGA2 gene in the extremely tall

AEJ Hendriks, MR Brown, AM Boot, BA Oostra, SLS Drop and JS Parks



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Abstract

Background/Aims: Genetic variation in several candidate genes has been associated with short stature. Recently a high mobility group-A2 (HMGA2) gene SNP was robustly associated with height in the general population. Few have attempted to study these genes in extremely tall stature. We therefore studied common genetic variation in candidate genes for height in the Dutch extremely tall.

Methods: We included 116 constitutionally tall cases with height >2 SD and 103 controls with normally distributed height <2 SD. We genotyped 10 common polymorphisms previously associated with height variation.

Results: The HMGA2 gene SNP was significantly associated with tall stature. Using a logistic regression model we calculated that carrying the HMGA2 (rs1042725) C-allele significantly increased the odds of being a case (OR=1.53, 95%CI 1.02-2.28, p=0.03). In addition, controls with one or two copies of the C-allele were significantly taller than controls carrying the TT genotype (TC, mean (SD): +0.61 (0.21) SD; p=0.004 and CC: +0.77 (0.25) SD; p=0.003).

Conclusion: Our study shows that a common polymorphism in the HMGA2 gene is not only associated with height variation in the general population but also plays an important role at one of the extremes of the height distribution.

Introduction

Human growth and adult height are highly heritable ($h^2 \sim 0.80–0.90$) polygenic traits.[1, 2] Initially the study of genetics of height mainly focused on short stature, with many possible candidate genes identified. Notably, however, few genes were reproducibly associated with height variation. In recent years, focus has shifted to genome wide association studies in large populations of average height. While multiple genetic loci have been robustly associated with height variation, effects sizes were small and cumulatively explained approximately five percent of the population height variation. Additional strategies are therefore necessary. Sampling at the extremes of a quantitative trait while using common controls has been shown to maximize statistical power as much of the information is provided by individuals in the tails of the distribution.[3]

Constitutionally tall stature (CTS) is characterized by accelerated growth in early childhood and a adult height of more than two standard deviations (SD) above the mean of the normal population.[4] In the Netherlands, CTS is currently defined as a final adult height of over 184 cm in women and over 198 cm in men.[5] In CTS, usually one or both parents are tall; thus, genetic factors play a key etiological role.

Gonadal steroids and growth factors of the skeletal axis greatly contribute to growth and maturation of long bones and the genes involved are good candidates to be associated with height variation. Estrogens play a crucial role in the timing of cessation of longitudinal bone growth.[6] Estrogen receptor alpha gene (ESR1) polymorphisms have been studied extensively in relation to bone mineral density and have been associated with height variation.[7-9] Aromatase catalyses the rate-limiting step in the conversion of androgens to estrogens. Several studies have found an association of single nucleotide polymorphisms (SNPs) in the gene encoding aromatase (CYP19) in the genetic control of normal adult height.[10, 11]

In the skeletal axis parathyroid hormone (PTH) and vitamin D are the principal regulators of bone mineralization. The VDR gene encodes a nuclear receptor for 1,25-dihydroxyvitamin D. One study observed significant linkage for a functional SNP in its gene that may be responsible for 34% of idiopathic short stature cases.[12] It has also been associated with height variation in the general population.[13]

Recently genes involved in the cell cycle are implicated as regulators of height variation. A SNP in the high mobility group-A2 (HMGA2) gene was robustly associated with adult height. This is a strong biological candidate for influencing height because its homozygous deletion produces the dwarf *Pygmy* mutant in mice. In humans, each copy

of the C allele of the SNP was associated with an increase of about 0.4 cm in height in the general population.[14]

Very few have attempted to study these candidate genes in the extremely tall. We therefore studied common genetic variation in candidate genes for height in the Dutch extremely tall.

Methods

Subjects

From our records, we identified former patients who attended our clinic for evaluation of tall stature. Eligible subjects were traced using municipal registries and invited by mail to participate. We also identified several tall families through advertisement in the members' magazine of the Dutch advocacy club for tall people (Klub voor Lange Mensen). Subjects eligible for participation fulfilled the following inclusion criteria: 1) height standard deviation score (SDS) above +2 SD according to Dutch standards[5]; 2) Caucasian. Subjects with endocrine or metabolic disorders, chromosomal defects and primary or secondary growth disorders were excluded. Subjects treated with steroids to reduce adult height were eligible to participate if they fulfilled the inclusion criteria.

A cohort of Dutch Caucasian young, healthy adults, recruited as part of the Peak Bone Mass (PBM) study, with a normally distributed height of less than 2 SDS served as controls.[15] The study received Erasmus MC ethics committee approval and participants gave written informed consent.

Clinical parameters

Participants were invited to visit the outpatient clinic of the Erasmus Medical Center. Height was measured using a stadiometer (SECA 225; SECA, Hamburg, Germany), and expressed as SDS according to Dutch standards.[5] A questionnaire concerning each subject's medical and family history and demographic information was completed.

Genotyping

Genomic DNA was extracted from venous blood using standard methods. We genotyped 10 candidate gene polymorphisms using published primers and protocols. Brief descriptions are given below for polymorphisms in the genes for luteinizing hormone-beta

(LHB)[16], cytochrome P450c17α (CYP17)[17], CYP19[10, 11], ESR1[7-9], PTH/PTHrP receptor (PTHR1)[18, 19], VDR[12, 13], collagen type Iα1 (COLIα1)[20, 21], catechol-*O*-methyltransferase (COMT)[22, 23], HMGA2[14], peroxisome proliferators-activated receptor γ 3 (PPARγ3).[24] All assays were run in duplicate to detect possible genotyping error.

Genomic DNA for genotyping of the SNP's was amplified by polymerase chain reaction (PCR) using forward and reverse oligonucleotide primers. Cycling parameters were optimized based on previous publications. The PCR products were digested with digestion enzymes. Digestion products were stained and visualized on gels. See Table 1 for SNP specific genotyping parameters.

Genotypes of the HMGA2 SNP (rs1042725) were determined with Taqman allelic discrimination assay (Applied Biosystems (ABI), Foster City, CA, USA), using Assay-by-Design service. PCR amplification and post-PCR fluorescence measurement was performed on an ABI Taqman 7500.

Statistical analysis

Genotype distributions for significant departure from the Hardy-Weinberg equilibrium were calculated using the chi-square test. The inclusion of families may increase type 1 error. This increase is relatively low in small pedigrees of sib pairs and nuclear families compared to larger pedigrees. In our analysis we accounted for family structure to keep type 1 error rates at the nominal 0.05 alpha level.[25] A pairwise kinship matrix specifying the degree of relatedness between each pair of individuals was used in the analysis to account for the relatedness among family members. Association testing for polymorphisms was performed using a variance components model that allows for relatedness in estimating the regression coefficients.[25] Statistical significance was determined using a score test as implemented in genABEL for R statistical software package.[26-28] The primary statistical inference was the additive genetic model.

The inclusion of cases who had received steroids to reduce adult height may cause us to underestimate effect sizes in cases. However, we have chosen to not correct for this possible treatment effect because of the uncertainty in height prediction models making any estimate of such an effect inaccurate. We thus use current height only, moreover because in our case-control study design it does not affect our primary objective of comparing cases to controls.

Analysis of variance was used to compare height SDS according to genotype. A general linear model was employed to estimate the percent variation in height SDS that can be explained by genotype. Logistic regression was used to estimate odds ratios.

Table 1. Genotyping parameters.

Polymorphism	Primers (forward and reverse)	PCR Cycle	Digestion	Visualization
COMT SNP 158	5'-CCTTTTTCCAGGTCTGACAA-3'	95°C 2m; 30x 30s 95°C, 30s	5U CviAII[a]	Sequencer [b]
rs4680	5'-CATCACCATCGAGATCAACC-3'	57°C & 30s 68°C; 68°C 2m	6h 25°C	FAM labeled
PTHR1	5'-AAATAACAGTCCTGCGCGC-3'	95°C 2m; 30x 45s 95°C, 30s		
Repeat $(AAAG)_n$	5'-GTGCAGAGCTGCGTCAGG-3'	60°C & 45s 68°C; 68°C 2m		
PTHR1	5'-GAAGCCACAGCTCCCATTTC-3'	95°C 2m; 30x 45s 95°C, 30s		Sequencer [b]
Nested reaction	5'-GCCTCGGAGCGAAGAAATC-3'	56°C & 30s 68°C; 68°C 2m		VIC labeled
LHβ SNP 15	5'-GAAGCAGTGTCCTTGTCCCA-3'	95°C 3m; 30x 1m 94°C, 45s	4U FokI [a]	6% PAG
rs1800447	5'-GAAGAGGAGGCCTGAGAGTT-3'	65°C & 1m 72°C; 72°C 2m	6h 37°C	Silver staining
CYP17 SNP -34	5'-CATTCGCACTCTGGAGTC-3'	94°C 2m; 30x 45s 94°C, 15s	10U MspA1I[a]	6% PAG
rs743572	5'-GGCTCTTGGGGTACTTG-3'	55°C & 45s 72°C; 72°C 2m	6h 37°C	Silver staining
CYP19 SNP exon 3	5'-TGCAACTACTACCACCGGGT-3' [c]	94°C 2m; 30x 45s 94°C, 15s	10U DraIII[a]	8% PAG
rs700518	5'-AGAAACAAAGACATCAAGATTC-3'	50°C & 30s 72°C; 72°C 2m	6h 37°C	Silver staining
ESR1 SNP Pvu II	5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3'	94°C 2m; 30x 30s 94°C, 40s	10U PvuII[a]	1.2% AG
rs2234693	5'-TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3'	61°C & 90s 72°C; 72°C 2m	6h 37°C	EB staining
PPARγ3 SNP -681	5'-TCATGTAGGTAAGACTGTGTAGAA-3'	94°C 2m; 30x 45s 95°C, 15s	5U CviAII[a]	8% PAG
rs10865710	5'-TGGCAAAAACGATCCTTAG-3'	51°C & 30s 72°C; 72°C 2m	6h 25°C	Silver staining
VDR SNP exon 2	5'-AGCTGGCCCTGGCACTGAC-3'	95°C 3m; 30x 60s 94°C, 30s	4U FokI[a]	6% PAG
rs2228570	5'-ATGGAAACACCTTGCTTCTTCTCC-3'	59°C & 45s 72°C; 72°C 2m	6h 37°C	Silver staining
COLIα1	5'-TAACTTCTGGACTATTTGCGGACTTTTTGG-3'	94°C 3m; 40x 50s 94°C, 10s	3U MscI[a]	2.8% AG
rs1800012	5'-GTCCAGCCCTCATCCTGGCC-3' [c]	62°C [d] & 15s 72°C; 72°C 5m	6h 37°C	EB staining

[a] New England Biolabs; [b] Autosequencer ABI 3130, POP 7, filter set D, 36 cm column, peak height between 100 and 2000, comparison with internal LIZ-size standard; [c] 1-bp mismatch for restriction site; [d] ramping 1°C per 10s to 72°C; PAG = polyacrylamide gel (29:1), AG = agarose gel, EB = ethidium bromide.

A sample size of 116 cases and 103 controls achieved 85% power to detect an odds ratio of 1.5 or larger using a logistic regression with an alpha level of .05. In addition, a total sample of 103 controls achieved 90% power to detect an association with 0.3 height SDS using an F test with an alpha level of .05. A two-tailed P-value of < 0.05 was regarded to be statistically significant. All calculations were performed using R statistical software package (R Foundation for Statistical Computing, Vienna, Austria) and SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

One-hundred sixteen tall stature cases and 103 controls participated in the study. The general characteristics of the study population are shown in Table 2. Cases had a mean age of 39.9 ± 12.8 years and controls of 26.4 ± 8.1 years. Seventeen families were included in the study. In total 38 participants were related; 17 siblings, 21 parents/children. In cases height was normally distributed around 3.0 SDS in men and 2.4 SDS in women and in controls around -0.35 SDS in men and -0.03 SDS in women.

Polymorphic variation was common at all loci. Genotype frequencies and allele frequencies in cases and controls are presented in Table 3. All genotype distributions were in Hardy-Weinberg equilibrium (HWE), except for the CYP19 SNP (rs700518), where in cases more heterozygotes were observed than expected under HWE.

Table 2. Characteristics of the study participants.

Characteristic	Cases		Controls	
	Men	Women	Men	Women
n	47 (40%)	69 (60%)	31 (30%)	72 (70%)
Age (yr)	40.9 (14.9)	39.1 (11.2)	29.7 (12.2)	24.9 (4.8)
Height (cm)	202.2 (5.1)	183.9 (4.7)	180.9 (4.9)	170.3 (6.2)
Height SDS	3.03 (0.80)	2.38 (0.76)	-0.35 (0.83)	-0.03 (0.98)
Weight (kg)	110.6 (15.5)	84.5 (16.8)	83.3 (13.4)	69.2 (10.6)
BMI	27.1 (3.7)	24.9 (4.8)	25.5 (3.9)	23.9 (3.4)

Values are mean (SD).

Table 3. Genotype frequencies of ten candidate gene polymorphisms and association with tall stature.

Polymorphism	Chr	Sample	HWE	MAF	Genotype			p
COMT					**AA**	**AG**	**GG**	
SNP 158 (rs4680)	22	Case	0.69	0.43	36	58	20	n.s.
A > G		Control	0.84	0.45	30	51	20	
HMGA2					**TT**	**TC**	**CC**	
SNP (rs1042725)	12	Case	0.48	0.59	17	61	38	**0.03**
T > C		Control	0.43	0.50	24	56	23	
PPARγ3					**CC**	**CG**	**GG**	
SNP -681 (rs10865710)	3	Case	0.09	0.27	57	53	5	n.s.
C > G		Control	0.94	0.28	52	40	8	
LHβ					**TT**	**TC**	**CC**	
SNP 15 (rs1800447)	19	Case	0.88	0.10	94	21	1	n.s.
T > C		Control	0.49	0.06	90	13	0	
CYP17					**TT**	**TC**	**CC**	
SNP -34 (rs743572)	10	Case	0.72	0.36	45	54	14	n.s.
T > C		Control	0.48	0.36	40	51	12	
CYP19					**GG**	**GA**	**AA**	
SNP exon 3 (rs700518)	15	Case	**0.01**	0.48	23	71	19	n.s.
G > A		Control	0.62	0.49	28	49	26	
ESR1					**CC**	**CT**	**TT**	
SNP Pvu II (rs2234693)	6	Case	0.78	0.50	28	59	28	n.s.
C > T		Control	0.92	0.45	31	50	21	
PTHR1[a]					**5/5**	**5/6**	**6/6**	
Repeat $(AAAG)_n$	3	Case	0.72	0,19	67	32	3	n.s.
$(AAAG)_5 > (AAAG)_6$		Control	0.79	0,19	62	29	4	
VDR					**CC**	**CT**	**TT**	
SNP exon 2 (rs2228570)	12	Case	0.44	0.40	44	51	20	n.s.
C > T		Control	0.70	0.39	37	51	15	
COLIα1					**GG**	**GT**	**TT**	
SNP (rs1800012)	17	Case	0.27	0.15	85	26	4	n.s.
G > T		Control	0.81	0.18	68	31	3	

Table 3 continued. [a] 12 cases and 7 controls had different lengths of the (AAAG)n repeat. Abbreviations: Chr = Chromosome, HWE = Hardy-Weinberg equilibrium, MAF = Minor Allele Frequency. Significant p-values are given in bold.

For the HMGA2 SNP (rs1042725) we observed a significant association with adult height variation. The HMGA2 C-allele was significantly more frequent in cases than in controls under an additive model (0.59 vs. 0.50; p=0.03). Using a logistic regression model we calculated that carrying the C-allele significantly increased the odds of being a case (odds ratio = 1.53, 95%CI 1.02-2.28, p=0.03). Additionally, controls with one or two copies of the C-allele were significantly taller than controls carrying the TT genotype (TC, mean (SD): +0.61 (0.21) SDS; 95%CI 0.2-1.0; p=0.004 and CC: +0.77 (0.25) SDS; 95%CI 0.3-1.3; p=0.003) (Figure 1). Using a general linear model, the percent variation in height SDS in controls that could be explained by the HMGA2 SNP was 7.8% (p=0.002). In cases the association of the HMGA2 SNP with height variation followed a similar pattern as in controls, where carriers of the C-allele were taller than TT carriers. However, this difference was not statistically significant. As shown in Table 3 no association with tall stature was observed for the other studied SNPs.

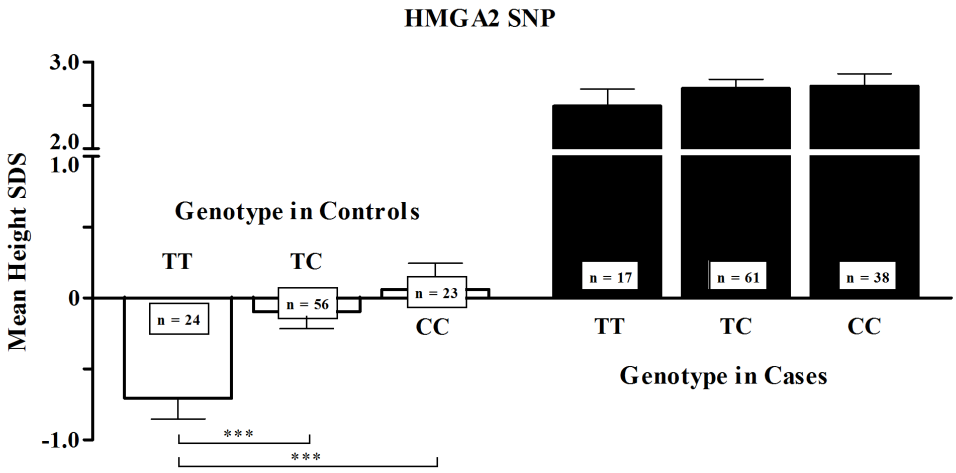

Figure 1. Mean height SDS by HMGA2 SNP genotype in 116 cases and 103 controls.

*** $p < 0.01$

Discussion

Many have studied the association of variation in height with common polymorphisms in candidate genes. Few have looked at the extremely tall. In this case-control study we assessed the role of these known polymorphisms in the regulation of extreme tall stature. We show that the recently described HMGA2 SNP is significantly associated with being extremely tall and with adult height variation.

The HMGA2 SNP was the first common variant to be strongly associated with adult and childhood height using genome-wide association data.[14] It has since been robustly associated with height variation in the general population.[29-31] Here, we replicate these previous studies as in our normal height controls the ones carrying the C-allele were significantly taller than those carrying only T-alleles. The C-allele frequency we observed in controls (0.50) was similar to previously reported allele frequencies (0.48-0.54), as was the direction of the allele effect.[14] In the current study the effect size of one or two copies of the C-allele was an increase in adult height of 0.61-0.77 SDS in controls. This is equivalent to 4-5 cm, whereas in previous studies the effect-size was around 0.4-0.9 cm.[14, 32] Possible explanations could be the Dutch ethnicity or homogeneity of our control population. However, this cannot fully explain the observed effect size as then we would have expected similar effects in the Rotterdam Study, which also is a homogeneous Dutch population.[33] It is likely that our effect size is inflated because of a relatively small sample size resulting in large confidence intervals due to lack of precision. Replication in other homogeneous cohorts or larger Dutch samples is needed to know the true effect size of the HMGA2 SNP.

One study has shown the HMGA2 SNP to increase the risk of being tall, however their tall cohort was only slightly taller (< 0.5 SDS) than the average Dutch population.[14] Therefore, it was worth evaluating whether the association holds in the extremely tall Dutch. In this study, we demonstrate a significant association of the HMGA2 C-allele with being extremely tall. However, due to the smaller range in height variation in cases, which were selected on being at the far right end of the normal distribution, our study did not have the power to demonstrate an effect of this SNP on height variation within cases. In future studies, a larger number of cases would be needed to achieve the power necessary to detect significant differences in height among the genotypes of the HMGA2 SNP at the extremes of the distribution.

Our replication of the association of the HMGA2 C-allele with increased odds of being tall suggests that this common SNP, that is robustly associated with height variation

in the general population, may also play an important role in the regulation of extreme tall stature. However, it remains to be established whether the extremes of the distribution, such as tall stature, represent the sum of all low penetrance common variants or that they are caused by a few moderately penetrant rare variants. The observed association of the HMGA2 SNP with extreme tall stature supports the former hypothesis.

In conclusion, we have demonstrated that a common polymorphism in the HMGA2 gene is associated with extreme tall stature in the Dutch. Our study shows that this gene is not only associated with height variation in the general population but also plays an important role at one of the extremes of the height distribution. Additional research is necessary to determine whether newly emerging polymorphisms robustly associated with height variation cluster at the extremes.

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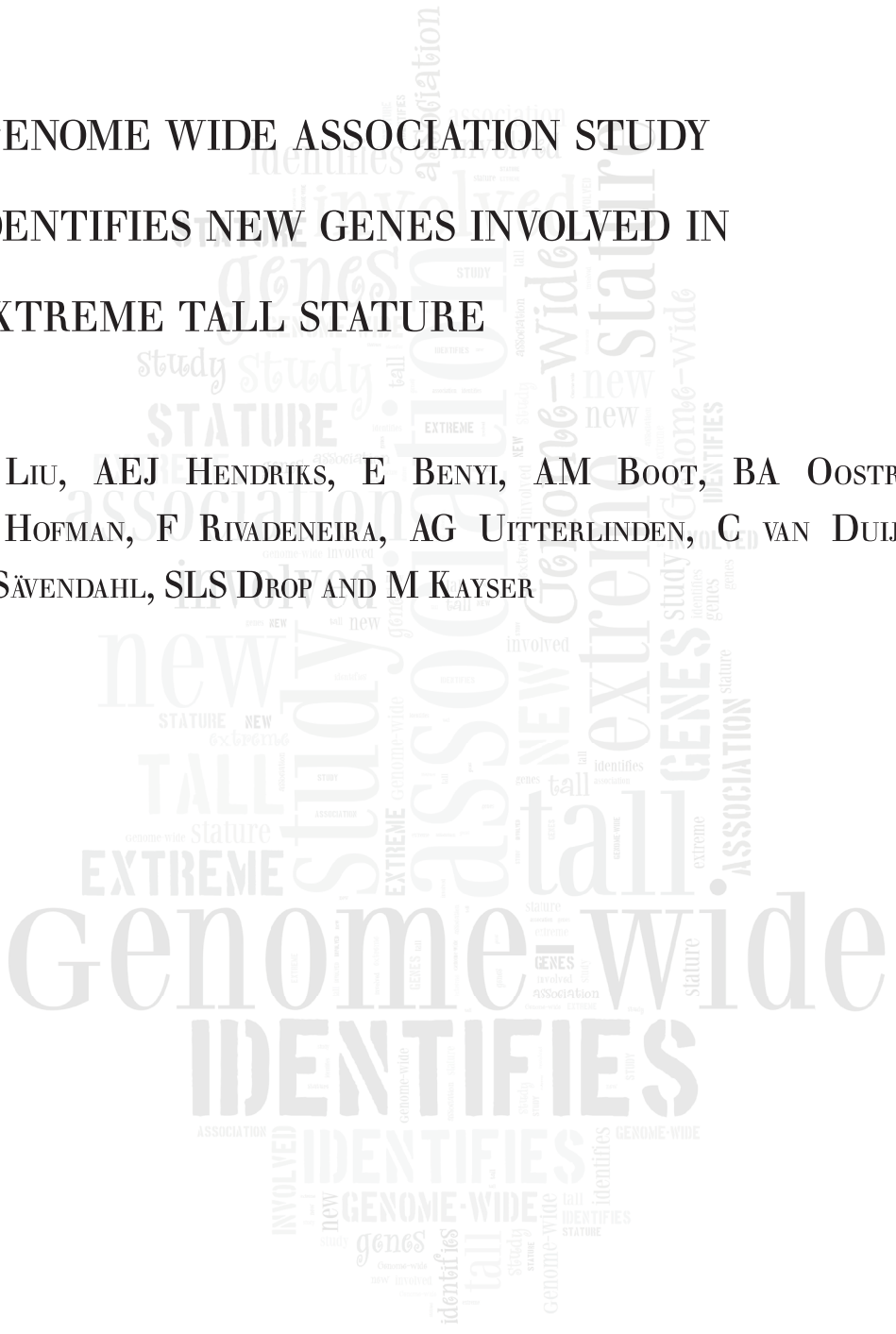
Chapter 7

Genome wide association study identifies new genes involved in extreme tall stature

F Liu, AEJ Hendriks, E Benyi, AM Boot, BA Oostra, A Hofman, F Rivadeneira, AG Uitterlinden, C van Duijn, L Sävendahl, SLS Drop and M Kayser



Submitted

Abstract

Context/Objective: Adult height is a highly heritable and classic polygenic trait. Genome-wide association (GWA) studies have identified over 180 loci robustly associated with height variation. These studies explain however only 10% of the phenotypic variation. Sampling at the extremes of a quantitative trait while using common controls has been shown to maximize statistical power. Here we study genome-wide polymorphic variation in Dutch extremely tall individuals.

Methods: Case-control study of 484 tall cases in the discovery cohort and 336 tall cases in the replication cohort with heights >1.88 SDS. Excluded were subjects with endocrine disorders or growth syndromes. We included 1029 participants of the Rotterdam Study with average height as controls. Cases and controls were genotyped using Illumina Human610Quad array.

Results: First we replicated the 180 single nucleotide polymorphisms (SNP) previously reported to be associated with normal height variation, of which 84 SNPs (46.7%) were nominally significantly associated with the extreme tall phenotype in our cohort ($P < 0.05$). The most significant P value was obtained for rs11259936 in the ADAMTSL3 gene on chromosome 15q25.2 ($P = 2.95\times10^{-6}$). In the GWA analysis 3 loci were identified with at least one SNP showing P values $< 10^{-6}$. These loci were 6q24.1, 15q25.2 containing the ADAMTSL3 gene, and 20q11.22 containing the RBM39 gene. We replicated the association observed at 6q24.1 and 20q11.22 ($P < 0.05$). Among these, the association at 20q11.22 was the most significantly replicated (rs2425073, $P = 6.09\times10^{-6}$). This was the only locus reaching a genome-wide significance level in a combined analysis including all 820 extremely tall individuals as cases (allelic OR for G allele = 1.63, 95%CI: 1.38-1.92, $P = 8\times10^{-9}$).

Conclusions: We have identified 3 loci to be associated with the extreme tall phenotype. These loci were 15q25.2 containing the known growth gene ADAMTSL3, 20q11.22 containing the gene RBM39 that codes for a transcriptional coactivator of sex steroid hormone receptors, and 6q24.1 containing 2 pseudogenes which has not been associated with height variation before.

Introduction

Human growth and adult height are considered highly heritable ($h^2 \sim 0.80–0.90$) polygenic traits.[1-7] Due to its quantitative nature and its easy and accurate measurability, stature has long served as reference phenotype in genetic studies of complex traits.[8-10] Notably, however, over the last decades only a few genes were reproducibly associated with height variation in the general population.[11-13] Recently, genome-wide association studies (GWAS) have brought a change by identifying multiple common variants which are associated with adult height. Initially three GWAS comprising tens of thousands of individuals with a normal height range and of European ancestry found 54 genetic variants to be robustly associated with normal height variation.[14-16] This included an impressive total of 40 previously unknown variants, many outside the expected biological pathways known to regulate growth. Later a Nature paper about a meta-analysis of GWA data from 46 studies comprising hundreds of thousands of individuals raised the number of relevant loci to at least 180.[17] These studies convincingly confirm the polygenic nature of height and implicate genes that were never considered associated with this phenotype before, like Hedgehog signaling and basic cell cycle regulation, thus opening up new opportunities for further study. However, in spite of a large number of loci identified, these genetic variants together only explained up to about 10% of the gender and age adjusted height variance. Additional strategies are therefore necessary in search of the missing heritability of adult height and other human complex traits. Sampling at the extremes of a quantitative trait while using common controls has been shown to maximize statistical power as much of the information is provided by individuals in the tails of the distribution.[18-20] While in recent years genetic determinants of height have been extensively studied, few groups have studied these genes in extremely tall individuals. Here we perform a GWAS of adult height in a case-control study including Dutch extremely tall individuals compared with Dutch individuals of average height.

Methods

Extreme Height cohort

As cases we collected 500 unrelated extremely tall Dutch individuals. From the records of the division of pediatric endocrinology at Erasmus Medical Center - Sophia Children's Hospital, we identified former patients who attended this clinic for evaluation of tall stature. Eligible subjects were traced using municipal registries and invited by mail to participate. We also identified several healthy tall individuals through advertisement in local specialized shops, sports centers and institutions of higher education in Rotterdam. Subjects eligible for participation fulfilled the following inclusion criteria: 1) adult height standard deviation score (SDS) above +1.88 SD according to Dutch standards [21]; 2) Dutch European ancestry, defined as being born to Dutch parents who themselves were born in the Netherlands. Subjects with endocrine or metabolic disorders, or primary or secondary growth disorders were excluded. Subjects treated with high-dose sex steroids to reduce adult height were eligible to participate if they fulfilled the inclusion criteria. Participants were invited to visit the outpatient clinic of the Erasmus Medical Center. Height was measured using a stadiometer (SECA 225; SECA, Hamburg, Germany), and expressed as SDS according to Dutch standards.[21] Genomic DNA was extracted from venous blood using standard methods. The Medical Ethics Committee of the Erasmus Medical Center approved the study protocol and all participants provided written informed consent.

Rotterdam Study cohort

The Rotterdam Study (RS) is a population-based prospective study including a main cohort and two extension.[22-24] The study cohort now includes ~15,000 participants from Rotterdam. The participants were all examined in detail at baseline. Collection and purification of DNA have been described in detail previously.[25] The Medical Ethics Committee of the Erasmus Medical Center approved the study protocol, and all participants provided written informed consent. The current study included a total of 2,082 RS participants from the second extension of the cohort. Most of the RS participants were considered as controls in the GWAS. Because oversampling of controls does not provide a noticeable gain in statistical power, all individuals from RS cohort falling in the upper middle range of the height distribution (165-180 cm for females and 180-195 cm for males) were excluded (N=964). This large gap between cases and controls minimizes

the potential misclassification of case control status due to the effects of environmental factors such as age, sex, and any unknown factors.

Dutch replication cohort

We additionally collected 186 extremely tall Dutch individuals as cases for the purpose of replication of our GWAS findings. From the records of the division of pediatric endocrinology at the Beatrix Children's Hospital of the University Medical Center Groningen, we identified former patients who attended this clinic for evaluation of tall stature. Eligible subjects were invited by mail to participate. We also identified several healthy tall individuals through advertisement in local sports centers and institutions of higher education in Groningen. Inclusion and exclusion criteria were the same as described above. Participants were invited to visit the outpatient clinic of the University Medical Center Groningen. Height was measured using a stadiometer (SECA 225; SECA, Hamburg, Germany), and expressed as SDS according to Dutch standards.[21] Genomic DNA was extracted from venous blood using standard methods. The Medical Ethics Committee of the University Medical Center Groningen approved the study protocol and all participants provided written informed consent.

Swedish replication cohort

Finally a cohort of 150 extremely tall Swedish individuals was collected as cases also for the purpose of replication of our GWAS findings. We identified former patients of the Pediatric Endocrine Clinic at Astrid Lindgren Children's Hospital of the Karolinska University Hospital, who had attended this clinic for evaluation of tall stature. Inclusion and exclusion criteria were the same as described above. Eligible subjects were invited by mail to participate. Participants were requested to submit a cheek swab sample for DNA testing. A cheek swab kit including detailed instructions of the standard sampling procedure was included in the original mailing. Adult height was self reported. The Medical Ethics Committee of the Karolinska University Hospital approved the study protocol and all participants provided written informed consent.

Genotyping and quality control

The Extreme Height cohort was genotyped using Illumina Human610Quad array (with 620,901 markers, both SNPs and CNV probes). The total number of Extreme Height samples that we processed was 500 samples. The controls of the Extreme Height study were Rotterdam Study III (RS-III) samples, which were genotyped using the same array. We first conducted standard SNP QC separately in each data set. The two data sets were

then merged on Genome Studio software v2010 and QCed together. The standard QC induced the analysis of gender, call rate, heterozygosity and homozygosity, and family relationship. Hardy-Weinberg equilibrium (HWE) was not included in QC. Principal components were derived using multidimensional scaling analysis of the 1-IBS matrix. The potential population stratification was checked by plotting the first 2 principal components. We compared the SNP call rate by cohort status and excluded 10,396 SNPs with a significant difference ($P < 0.05$). This QC step is important to reduce potential false positive findings particularly for our study design since people with extreme tall phenotype were genotyped in a separate batch. The final data set included 2477 individuals (462 from Extreme Height, 2015 from RS-III) and 528,618 SNPs. Genotypes were imputed to 2,543,887 SNPs using MACH [26] based upon phased autosomal chromosomes of the HapMap CEU Phase II panel (release 22, build 36). Pair-wise identity by state (IBS) matrix between individuals was recalculated by using a subset of pruned markers that are in approximate linkage equilibrium.

Power estimation

The statistical power was estimated using the method of Fleiss, Tytun, and Ury implemented in the bpower function of the Hmisc library in R (http://www.r-project.org).[27] The input parameters included alpha = 5×10^{-8}, number of cases, number of controls, OR, and p, which was calculated based on MAF under dominant or recessive models assuming HWE.

Validation

We first attempted to replicate the 180 "proband" SNPs ascertained from the Supplementary Table 1 of the Nature paper for association with the extreme tall phenotype in our case-control samples.[17] Forty nine out of the 180 "proband" SNPs were directly genotyped on our chip, and the remaining SNPs were imputed according to HapMap CEU reference samples with acceptable imputation qualities. The association analysis was based on logistic regression where allelic ORs were derived. We consider $P < 0.05$ as statistically significant in this analysis.

GWA analysis

The effects of sex and age were adjusted for when the extreme tall individuals were collected based on their height standard deviation score, we therefore did not adjust for these factors in the GWA analysis. Single SNP association was based on a score test of the additive effect of the minor allele and the $\chi 2$ value with 1df was derived. The dis-

tribution of observed P values was inspected using Q-Q plots against the P values from the null $\chi 2$ distribution with 1df. An inflation factor was estimated at 1.0 and not further considered. P values smaller than 5×10^{-8} were considered to be genome-wide significant for the single SNP analysis. All significant SNPs were further examined using logistic regression, where sex, age and the first 4 principal components from MDS analysis were adjusted for as covariates. GWA analyses were conducted using R library GenABEL v1.4-3.[28] Haplotype and LD analysis were conducted for the regions of interest using Haploview v4.1.[29] Replication analysis was conducted in the same logistic regression model using R.

Results

Participants

The discovery cohort consisted of 484 Extreme Height cases, 300 females and 184 males, and 1029 Rotterdam Study controls, 568 females and 461 males. Figure 1 shows the height distribution of the cases and controls. Cases had a height of over +1.88 SDS corresponding to a height taller than 180 cm in females and 195 cm in males. Controls were shorter than 165 cm as females and 180 cm as males.

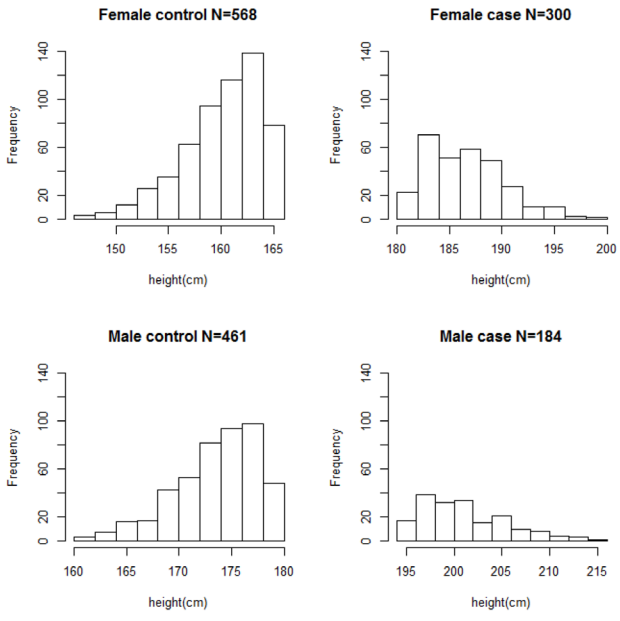


Figure 1. Height distribution in cases and controls included in the initial GWAS and prediction analysis.

Table 1. All SNPs associated with extreme tall phenotype in the discovery cohort at $P < 1 \times 10^{-6}$.

				Controls			Cases				
SNP	**Chr**	**Position**	**Alt**	**Eff**	**HWE**	**fEff**	**fEff**	**OR**	**Low**	**Up**	**P**
All samples				N = 1029				N = 484			
ATP5F1P6, LOC100129554											
rs1936208	6q24.1	140007078	T	C	0.46	0.43	0.50	1.48	1.27	1.73	8.50E-07
rs1936209	6q24.1	140007353	A	G	0.46	0.43	0.50	1.48	1.27	1.73	8.50E-07
ADAMTSL3											
rs979543	15q25.2	82143568	A	C	0.48	0.19	0.14	0.58	0.47	0.72	7.56E-07
rs2585049	15q25.2	82143877	T	G	0.48	0.19	0.14	0.58	0.47	0.72	7.56E-07
rs2554383	15q25.2	82147320	G	A	0.44	0.19	0.14	0.58	0.46	0.72	5.89E-07
rs1477593	15q25.2	82154049	A	G	0.44	0.19	0.14	0.58	0.47	0.72	9.49E-07
rs4843152	15q25.2	82154546	C	T	0.44	0.19	0.14	0.58	0.47	0.72	9.49E-07
rs2562776	15q25.2	82159167	A	G	0.61	0.19	0.14	0.58	0.47	0.72	8.87E-07
RBM39											
rs2425073	20q11.22	33757113	A	G	0.38	0.20	0.25	1.62	1.34	1.96	8.13E-07
rs2425105	20q11.22	33776248	C	T	0.35	0.20	0.25	1.61	1.33	1.94	9.30E-07
rs6060582	20q11.22	33776711	C	T	0.35	0.20	0.25	1.61	1.33	1.94	9.30E-07

Abbreviations: Chr, chromosome; Alt, Eff, the alternative allele and the effective allele; fEff, the frequency of the effective allele; OR, odds ratio; Low, Up, lower and upper bound of the 95% confidence interval.

Table 1 continued.

				Controls			Cases				
SNP	**Chr**	**Position**	**Alt**	**Eff**	**HWE**	**fEff**	**fEff**	**OR**	**Low**	**Up**	**P**
Female				N = 568			N = 300				
GRAMD1B											
rs2852845	11q24.1	122934385	G	A	0.20	0.24	0.14	0.48	0.37	0.64	3.70E-07
Male				N = 461			N = 184				
SYT10, ALG10											
rs814669	12p11.1	33993726	C	T	0.67	0.34	0.51	1.98	1.53	2.56	1.58E-07
rs1844526	12p11.1	33994047	G	A	0.67	0.34	0.51	1.98	1.53	2.56	1.58E-07
rs11053014	12p11.1	33994786	G	A	0.67	0.34	0.51	1.98	1.53	2.56	1.58E-07
rs11052922	12p11.1	33848743	A	G	0.67	0.35	0.51	2.01	1.55	2.61	1.70E-07
rs1826144	12p11.1	33985539	A	G	0.60	0.34	0.51	1.95	1.52	2.52	2.18E-07
rs1912773	12p11.1	33988668	T	C	0.60	0.34	0.51	1.95	1.52	2.52	2.18E-07
rs12818637	12p11.1	33640297	A	G	0.53	0.33	0.48	1.98	1.53	2.56	2.37E-07
rs10844654	12p11.1	33649701	G	A	0.59	0.32	0.48	1.99	1.53	2.58	2.53E-07
rs4086956	12p11.1	33996314	A	T	0.52	0.34	0.50	1.96	1.52	2.53	2.50E-07
rs11052897	12p11.1	33810748	T	A	0.53	0.34	0.50	1.99	1.53	2.59	2.66E-07

Abbreviations: Chr, chromosome; Alt, Eff, the alternative allele and the effective allele; fEff, the frequency of the effective allele; OR, odds ratio; Low, Up, lower and upper bound of the 95% confidence interval.

A GWAS power calculation showed this case-control sample provides good power (> 80%) to detect an allelic OR > 2.5 for dominant alleles with frequencies > 10% at the genome-wide significant threshold ($P < 5\times10^{-8}$) (Figure S1A). This sample is still useful to detect dominant alleles having low frequency (> 2%) but large effects (preferably allelic ORs > 5.0). On the other hand, the power to detect recessive alleles is much lower. Recessive alleles with frequencies < 10% are nearly undetectable regardless to their effect sizes (Figure S1B), and the ones with frequencies > 10% are detectable only if they have large effects (preferably allelic OR > 5.0). Regardless to the genetic model, this sample provides limited power to detect allelic ORs smaller than 2.0.

Validation

For the 180 "proband" SNPs selected from the Supplementary Table 1 of the Nature paper, 84 (46.7%) were nominally significantly associated with the extreme tall phenotype in all samples, males, or females ($P < 0.05$, Table S1).[17] The most significant P value was obtained for rs11259936 in ADAMTSL3 on chromosome 15q25.2 ($P = 2.95\times10^{-6}$).

Genome wide association study

We next conducted a standard case-control GWAS for extreme tall phenotype, which did not reveal any genome-wide significant association at the traditional threshold of 5×10^{-8} (Figure S2). Three loci were identified with at least one SNP showing P values < 10^{-6} (Table 1). These loci were 6q24.1, 15q25.2 containing ADAMTSL3, and 20q11.22 containing RBM39. The association observed at 6q24.1 was new. The finding at ADAMTSL3 is consistent with the results from the analysis of the 180 "proband" SNPs. The associated SNP at 20q11.22 was less than 300kb to a "proband" SNP, rs143384 but this locus is not well known for its involvement in adult height. In a sex stratified analysis, one locus at 11q24.1 containing GRAMD1B was significant at < 10^{-6} in females only, while in males one locus at 12p11.1 containing SYT10 and ALG10 was significant at < 10^{-6}.

Replication

We selected one or two SNPs per locus for replication in 336 extremely tall individuals additionally collected from Netherlands and Sweden. We used the same set of 1,029 controls as we used in our GWAS analysis. We could replicate the association signals observed at 6q24.1 and 20q11.22 in all samples and the signal at 12p11.1 in males only ($P < 0.05$, Table 2a). Among these, the association at 20q11.22 was the most significantly replicated (rs2425073, $P = 6.09\times10^{-6}$, Table 2a). This was the only locus

reaching a genome-wide significance level in a combined analysis including all 820 extremely tall individuals as cases (allelic OR for G allele = 1.63, 95%CI: 1.38-1.92, $P = 8\times10^{-9}$, Table 2b).

Table 2a. Replication analysis of SNPs found in the discovery cohort at $P < 1\times10^{-6}$.

					Replication		
SNP	**Chr**	**Eff**	**fEff**	**OR**	**Low**	**Up**	**P**
All samples					N cases = 336		
rs1936208	6q24.1	C	0.45	1.22	1.01	1.46	3.62E-02
rs2554383	15q25.2	A	0.19	0.86	0.68	1.08	1.86E-01
rs2562776	15q25.2	G	0.18	0.83	0.65	1.05	1.15E-01
rs2425073	20q11.22	G	0.25	1.65	1.33	2.05	6.09E-06
Female					N cases = 238		
rs2852845	11q24.1	A	0.22	0.92	0.70	1.21	5.72E-01
Male					N cases = 98		
rs814669	12p11.1	T	0.44	1.44	1.06	1.97	2.12E-02

Table 2b. Combined analysis of SNPs found in the discovery cohort at $P < 1\times10^{-6}$.

					Combined		
SNP	**Chr**	**Eff**	**fEff**	**OR**	**Low**	**Up**	**P**
All samples					N cases = 820		
rs1936208	6q24.1	C	0.48	1.37	1.20	1.57	3.79E-06
rs2554383	15q25.2	A	0.16	0.69	0.58	0.82	2.10E-05
rs2562776	15q25.2	G	0.16	0.68	0.57	0.81	1.16E-05
rs2425073	20q11.22	G	0.25	1.63	1.38	1.92	8.00E-09
Female					N cases = 538		
rs2852845	11q24.1	A	0.17	0.66	0.53	0.82	1.45E-04
Male					N cases = 282		
rs814669	12p11.1	T	0.48	1.77	1.42	2.20	3.65E-07

Abbreviations: Chr, chromosome; Eff, the effective allele; fEff, the frequency of the effective allele; OR, odds ratio; Low, Up, lower and upper bound of the 95% confidence interval.

Discussion

Genome-wide association studies have successfully identified over 180 SNPs associated with human growth and adult height.[14-17] However, combined these common variants explain less than 10% of the heritability of height.[17] It has been suggested that rarer variants with larger effects may account for the missing heritability.[18] In search of these variants it has been proposed to sample at the extremes of a trait to maximize statistical power.[18-20] Here we present a genome-wide association study of adult height in a case-control study of Dutch extremely tall individuals compared with Dutch individuals of average height. We show several loci to be associated with extreme tall phenotype, including a genome-wide significant SNP at the relatively unknown gene RBM39 and SNPs located in the known growth gene ADAMTSL3, as identified in the OMIM database (http://www.ncbi.nlm.nih.gov/omim).

First we validated our sample by replicating the 180 "proband" SNPs robustly associated with height in previous GWAS.[14-17] We found 84 (46.7%) SNPs to be nominally associated with extreme tall phenotype. One of these associated SNPs (rs1351394) lies in the HMGA2 gene which has been associated with tall stature in the past.[30] In addition, the percentage associated with the phenotype is far beyond what one would expect under the null hypothesis of no association (5%). Therefore we believe our sample to be a valid set.

Next we performed a genome-wide association study and found one genome-wide significant SNP (rs2425073, $P = 8\times10^{-9}$) in the combined analysis of the discovery and replication cohorts. This SNP is in the gene for RNA binding motif protein 39 (RBM39, also known as CAPER), which is a transcriptional coactivator that stimulates transcription mediated by the progesterone and estrogen steroid hormone receptors.[31, 32] Thus far SNPs in the RBM39 gene have not been associated with adult height variation. We believe this locus could be of great interest as sex steroids are key hormones in human growth, especially during the pubertal growth spurt.[33] In addition, in the Nature paper one of the height SNPs (rs143384) near the RBM39 gene was associated with cis gene expression at this locus in the lymphocyte.[17] Finally, RBM39 has also been associated with tumor progression in breast cancer and cell viability in colorectal cancer.[34, 35] Additional research is necessary to determine the role of polymorphisms in the RBM39 gene in adult height variation.

Both in the validation analysis as in the GWA analysis several SNPs in the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif)-like protein 3 (ADAMTSL3) gene were significantly associated, albeit not genome-wide, with the

extreme height phenotype. The association between ADAMTSL3 SNPs and adult height variation has been extensively reported by previous studies.[14-16, 36-40] ADAMTSL3 is a secreted glycoprotein involved in the turnover of extracellular matrix components which is highly expressed in the liver, kidney, heart and skeletal muscle.[41] Interestingly ADAMTSL3 protein in concert with ADAMTS enzymes has been implicated to modulate fibrillin-1 function.[42] Mutations in the fibrillin-1 gene (FBN1) are known to cause Marfan syndrome, which has tall stature among its clinical features. However, a mutation in FBN1 that abolishes a binding site utilized by ADAMTSL3 causes Weill-Marchesani syndrome characterized by short stature.[42] Thus it has been suggested that ADAMTS enzymes with the help of ADAMTS-like proteins could be involved in the installation or remodeling of structural materials, such as collagens, in the fibrillin microenvironment, or they might participate in the activation of nearby latent growth factors.

The third locus identified and replicated was 6q24.1 which thus far has not been associated with height variation. This locus contains two pseudogenes ATP5F1P6 (ATP synthase, H+ transporting, mitochondrial Fo complex, subunit B1 pseudogene 6) and LOC100129554 (thyroid hormone receptor interactor 4 pseudogene). Further research is necessary to identify the function of these genes and the role of this locus in height variation.

In conclusion, we have performed a genome-wide association study using extreme tall phenotype as trait. We discovered a new genome-wide significant SNP in the gene for RBM39, which is involved in sex steroid mediated transcription. In addition, we found several SNPs in the gene for ADAMTSL3 to be associated with the extreme tall phenotype. ADAMTSL3 has been robustly associated with height variation and is implicated to modulate fibrillin-1 function. Finally, a new locus was identified at 6q24.1 which requires further research.

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Supplementary materials

Table S1. Association between 180 "proband" height-SNPs and extreme tall phenotype in a Dutch case-control sample.

						All				Male				Female			
SNP	**Chr**	**Mb**	**A1**	**A2**	**FreqA1**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**
rs425277	1	2.06	T	C	0.28	1.09	0.92	1.29	0.30	1.00	0.77	1.30	1.00	1.16	0.93	1.44	0.19
rs2284746	1	17.18	C	G	0.45	0.77	0.66	0.91	***1.5E-03***	0.78	0.61	1.00	**0.05**	0.78	0.64	0.96	**0.02**
rs1738475	1	23.41	G	C	0.37	0.93	0.80	1.09	0.40	0.90	0.70	1.15	0.39	0.97	0.79	1.19	0.74
rs4601530	1	24.92	T	C	0.25	0.94	0.79	1.12	0.48	1.01	0.77	1.33	0.92	0.90	0.71	1.13	0.35
rs7532866	1	26.61	G	A	0.33	0.92	0.78	1.08	0.30	0.92	0.71	1.18	0.51	0.93	0.75	1.16	0.52
rs2154319	1	41.52	C	T	0.23	1.04	0.86	1.25	0.68	0.96	0.70	1.30	0.77	1.09	0.86	1.37	0.49
rs17391694	1	78.40	T	C	0.12	1.42	1.14	1.78	*2.1E-03*	1.40	0.96	2.03	0.08	1.42	1.07	1.88	**0.01**
rs6699417	1	88.90	C	T	0.39	1.02	0.87	1.19	0.85	1.17	0.91	1.49	0.21	0.92	0.75	1.13	0.42
rs10874746	1	93.10	T	C	0.38	0.94	0.80	1.10	0.45	0.87	0.68	1.11	0.26	1.00	0.81	1.23	0.99
rs9428104	1	118.66	A	G	0.20	0.74	0.61	0.90	*2.7E-03*	0.71	0.52	0.97	**0.03**	0.76	0.59	0.99	**0.04**
rs11205277	1	148.16	G	A	0.45	1.28	1.10	1.49	*1.5E-03*	1.47	1.15	1.89	*2.3E-03*	1.17	0.97	1.43	0.11
rs17346452	1	170.32	C	T	0.29	1.06	0.90	1.26	0.47	1.24	0.95	1.62	0.11	0.96	0.77	1.19	0.70
rs1325598	1	175.06	A	G	0.42	0.94	0.80	1.10	0.43	0.84	0.65	1.08	0.16	1.00	0.81	1.22	0.99
rs1046934	1	182.29	C	A	0.37	1.19	1.01	1.39	**0.03**	1.08	0.85	1.38	0.53	1.29	1.04	1.59	**0.02**
rs10863936	1	210.30	G	A	0.49	1.13	0.97	1.32	0.11	1.06	0.83	1.36	0.63	1.17	0.96	1.42	0.11
rs6684205	1	216.68	G	A	0.30	1.08	0.92	1.27	0.36	0.88	0.68	1.15	0.36	1.22	0.99	1.51	0.06
rs11118346	1	217.81	T	C	0.46	0.91	0.79	1.07	0.25	0.96	0.75	1.22	0.74	0.87	0.72	1.06	0.17
rs10799445	1	225.98	C	A	0.22	0.90	0.75	1.09	0.28	1.20	0.91	1.59	0.20	0.73	0.57	0.93	**0.01**
rs4665736	2	25.04	C	T	0.46	0.83	0.71	0.98	**0.03**	0.79	0.61	1.01	0.06	0.87	0.71	1.07	0.18
rs6714546	2	33.21	A	G	0.27	0.80	0.67	0.95	**0.01**	0.90	0.68	1.19	0.46	0.73	0.58	0.92	*7.8E-03*
rs17511102	2	37.81	T	A	0.11	1.12	0.88	1.43	0.35	1.34	0.91	1.99	0.14	1.00	0.73	1.37	0.99

						All				Male				Female			
SNP	**Chr**	**Mb**	**A1**	**A2**	**FreqA1**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**
rs2341459	2	44.62	T	C	0.26	1.10	0.92	1.31	0.31	1.15	0.87	1.53	0.33	1.07	0.85	1.34	0.56
rs12474201	2	46.77	A	G	0.37	1.05	0.89	1.23	0.56	1.08	0.84	1.39	0.53	1.03	0.83	1.26	0.81
rs3791675	2	55.96	T	C	0.22	0.74	0.61	0.90	*2.5E-03*	0.63	0.46	0.88	*6.8E-03*	0.80	0.62	1.02	0.07
rs11684404	2	88.71	C	T	0.35	0.93	0.79	1.09	0.37	0.91	0.71	1.18	0.49	0.94	0.77	1.15	0.55
rs7567288	2	134.15	C	T	0.20	1.09	0.90	1.32	0.40	0.92	0.67	1.27	0.61	1.19	0.93	1.52	0.17
rs7567851	2	178.39	C	G	0.08	1.35	1.03	1.76	**0.03**	1.50	0.98	2.28	0.06	1.26	0.89	1.79	0.20
rs1351164	2	217.98	C	T	0.17	0.87	0.70	1.07	0.18	0.95	0.69	1.32	0.77	0.82	0.62	1.08	0.15
rs12470505	2	219.62	G	T	0.09	0.85	0.65	1.12	0.25	0.76	0.48	1.21	0.25	0.89	0.64	1.25	0.52
rs2629046	2	224.76	C	T	0.45	0.98	0.84	1.14	0.75	0.90	0.71	1.14	0.37	1.03	0.84	1.26	0.78
rs2580816	2	232.51	T	C	0.16	1.06	0.86	1.30	0.60	1.03	0.75	1.43	0.84	1.08	0.83	1.40	0.58
rs12694997	2	241.91	A	G	0.24	0.83	0.69	1.00	**0.05**	0.83	0.62	1.12	0.22	0.82	0.65	1.04	0.11
rs2597513	3	13.53	C	T	0.11	1.34	1.06	1.69	**0.02**	1.46	1.02	2.09	**0.04**	1.27	0.92	1.73	0.14
rs13088462	3	51.05	C	T	0.05	1.48	1.03	2.11	**0.03**	1.86	1.02	3.39	**0.04**	1.28	0.82	2.00	0.27
rs2336725	3	53.09	C	T	0.44	1.11	0.95	1.29	0.19	1.06	0.84	1.34	0.63	1.14	0.93	1.40	0.20
rs9835332	3	56.64	C	G	0.42	0.83	0.71	0.97	**0.02**	0.95	0.75	1.21	0.67	0.75	0.61	0.93	*6.8E-03*
rs17806888	3	67.50	C	T	0.11	1.12	0.88	1.41	0.36	1.10	0.75	1.60	0.64	1.13	0.83	1.53	0.44
rs9863706	3	72.52	T	C	0.22	0.87	0.73	1.05	0.15	1.10	0.83	1.46	0.50	0.75	0.58	0.95	**0.02**
rs6439167	3	130.53	T	C	0.21	0.78	0.65	0.95	**0.01**	0.79	0.58	1.08	0.14	0.78	0.61	0.99	**0.04**
rs9844666	3	137.46	A	G	0.24	0.98	0.82	1.18	0.84	1.24	0.93	1.66	0.14	0.84	0.67	1.06	0.14
rs724016	3	142.59	A	G	0.50	0.75	0.64	0.88	***3.9E-04***	0.73	0.57	0.94	**0.01**	0.76	0.62	0.94	**0.01**
rs572169	3	173.65	T	C	0.32	1.02	0.87	1.19	0.82	1.23	0.97	1.57	0.09	0.89	0.72	1.09	0.27
rs720390	3	187.03	A	G	0.39	1.18	1.01	1.38	**0.04**	1.39	1.08	1.79	*9.9E-03*	1.05	0.86	1.29	0.63
rs2247341	4	1.67	A	G	0.34	1.19	1.01	1.40	**0.03**	1.25	0.97	1.61	0.09	1.17	0.94	1.44	0.16
rs6449353	4	17.64	C	T	0.14	0.78	0.63	0.97	**0.03**	0.62	0.43	0.89	*9.8E-03*	0.91	0.69	1.20	0.51
rs17081935	4	57.52	T	C	0.20	1.09	0.90	1.31	0.38	0.92	0.67	1.26	0.61	1.18	0.93	1.50	0.17

						All				Male				Female			
SNP	**Chr**	**Mb**	**A1**	**A2**	**FreqA1**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**
rs7697556	4	73.73	T	C	0.46	1.20	1.03	1.41	**0.02**	1.07	0.82	1.38	0.63	1.29	1.05	1.57	**0.01**
rs788867	4	82.37	G	T	0.33	1.29	1.10	1.52	*2.1E-03*	1.22	0.95	1.57	0.12	1.35	1.09	1.67	*5.9E-03*
rs10010325	4	106.33	A	C	0.49	1.06	0.91	1.24	0.42	1.04	0.82	1.34	*0.73*	1.08	0.89	1.31	0.45
rs7689420	4	145.79	T	C	0.15	0.60	0.47	0.76	***2.1E-05***	0.60	0.41	0.87	*7.8E-03*	0.60	0.44	0.82	*1.1E-03*
rs955748	4	184.45	A	G	0.24	0.96	0.81	1.15	0.69	0.86	0.64	1.16	0.33	1.03	0.82	1.30	0.79
rs1173727	5	32.87	T	C	0.42	1.22	1.05	1.43	**0.01**	1.44	1.13	1.84	*3.7E-03*	1.09	0.89	1.34	0.40
rs11958779	5	55.04	G	A	0.31	1.31	1.11	1.55	*1.1E-03*	1.59	1.23	2.06	***4.4E-04***	1.14	0.93	1.41	0.22
rs10037512	5	88.39	C	T	0.43	0.85	0.72	0.99	**0.04**	0.92	0.71	1.18	0.49	0.82	0.66	1.01	0.07
rs13177718	5	108.14	T	C	0.07	1.02	0.77	1.36	0.87	1.26	0.84	1.90	0.26	0.88	0.59	1.30	0.51
rs1582931	5	122.69	A	G	0.45	1.14	0.96	1.35	0.14	1.46	1.10	1.93	*8.1E-03*	0.98	0.79	1.23	0.88
rs274546	5	131.73	A	G	0.35	0.82	0.70	0.97	**0.02**	0.86	0.67	1.10	0.23	0.81	0.65	1.00	**0.05**
rs526896	5	134.38	G	T	0.26	0.84	0.69	1.02	0.08	0.97	0.71	1.31	0.82	0.76	0.59	0.98	**0.03**
rs4282339	5	168.19	A	G	0.20	0.98	0.81	1.19	0.87	0.88	0.65	1.18	0.40	1.09	0.85	1.39	0.51
rs12153391	5	171.14	A	C	0.24	0.89	0.73	1.08	0.22	0.84	0.62	1.14	0.26	0.93	0.73	1.19	0.57
rs889014	5	172.92	T	C	0.33	0.89	0.75	1.05	0.17	1.10	0.85	1.42	0.46	0.77	0.61	0.95	**0.02**
rs422421	5	176.45	T	C	0.21	0.81	0.67	0.98	**0.03**	0.66	0.49	0.90	*8.3E-03*	0.93	0.72	1.19	0.55
rs6879260	5	179.66	T	C	0.36	0.90	0.77	1.06	0.22	0.97	0.75	1.25	0.82	0.86	0.69	1.06	0.16
rs3812163	6	7.67	T	A	0.46	1.34	1.14	1.57	***3.0E-04***	1.15	0.90	1.48	0.26	1.50	1.22	1.85	***1.5E-04***
rs1047014	6	19.95	C	T	0.26	1.27	1.07	1.51	*6.8E-03*	1.39	1.06	1.83	**0.02**	1.21	0.96	1.51	0.10
rs806794	6	26.31	G	A	0.25	0.85	0.71	1.02	0.07	0.92	0.70	1.22	0.56	0.80	0.63	1.02	0.07
rs3129109	6	29.19	T	C	0.36	0.79	0.67	0.93	*5.0E-03*	0.83	0.64	1.08	0.17	0.76	0.62	0.95	**0.01**
rs2256183	6	31.49	A	G	0.48	1.30	1.12	1.52	***6.6E-04***	1.52	1.20	1.94	***6.4E-04***	1.16	0.96	1.42	0.13
rs6457620	6	32.77	C	G	0.47	0.92	0.79	1.07	0.29	0.82	0.65	1.04	0.10	1.00	0.82	1.22	0.98
rs2780226	6	34.31	C	T	0.09	1.39	1.07	1.81	**0.01**	1.58	1.04	2.41	**0.03**	1.28	0.91	1.79	0.15
rs6457821	6	35.51	A	C	0.02	0.66	0.38	1.15	0.14	0.51	0.19	1.36	0.18	0.75	0.38	1.46	0.40

						All				Male				Female			
SNP	**Chr**	**Mb**	**A1**	**A2**	**FreqA1**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**
rs9472414	6	45.05	A	T	0.19	0.92	0.75	1.13	0.42	1.12	0.82	1.54	0.48	0.81	0.62	1.05	0.11
rs9360921	6	76.32	G	T	0.11	1.21	0.96	1.52	0.12	0.88	0.59	1.31	0.53	1.44	1.07	1.93	**0.02**
rs310405	6	81.86	G	A	0.46	0.87	0.74	1.01	0.06	0.92	0.72	1.17	0.49	0.83	0.68	1.02	0.07
rs7759938	6	105.49	C	T	0.31	1.23	1.05	1.45	**0.01**	1.21	0.93	1.58	0.15	1.23	1.00	1.52	**0.05**
rs1046943	6	109.89	G	A	0.41	0.84	0.71	0.98	**0.02**	0.71	0.55	0.91	*7.5E-03*	0.92	0.75	1.12	0.39
rs961764	6	117.63	C	G	0.40	0.98	0.84	1.15	0.82	0.79	0.61	1.02	0.07	1.13	0.92	1.39	0.24
rs1490384	6	126.89	C	T	0.49	0.89	0.76	1.04	0.15	0.79	0.61	1.01	0.06	0.97	0.79	1.19	0.78
rs6569648	6	130.39	C	T	0.23	1.06	0.89	1.27	0.50	0.92	0.69	1.21	0.53	1.20	0.94	1.52	0.14
rs7763064	6	142.84	A	G	0.27	0.89	0.75	1.06	0.20	0.87	0.65	1.16	0.35	0.89	0.71	1.11	0.30
rs543650	6	152.15	T	G	0.40	0.84	0.72	0.98	**0.03**	0.90	0.70	1.15	0.40	0.79	0.64	0.97	**0.03**
rs9456307	6	158.85	A	T	0.05	0.56	0.38	0.84	*4.7E-03*	0.45	0.22	0.90	**0.02**	0.63	0.39	1.02	0.06
rs798489	7	2.77	T	C	0.26	0.80	0.67	0.96	**0.01**	0.81	0.61	1.08	0.15	0.79	0.63	1.00	**0.05**
rs4470914	7	19.58	T	C	0.18	1.15	0.94	1.39	0.17	1.10	0.81	1.49	0.54	1.21	0.93	1.56	0.15
rs12534093	7	23.47	A	T	0.22	0.91	0.74	1.11	0.34	0.78	0.56	1.09	0.14	0.99	0.77	1.27	0.93
rs1708299	7	28.16	A	G	0.32	1.26	1.07	1.49	*5.0E-03*	1.30	1.01	1.67	**0.04**	1.25	1.01	1.55	**0.04**
rs6959212	7	38.09	T	C	0.30	0.95	0.81	1.13	0.59	0.93	0.71	1.21	0.58	0.97	0.78	1.21	0.79
rs42235	7	92.09	T	C	0.31	1.11	0.94	1.32	0.22	1.09	0.83	1.44	0.51	1.14	0.91	1.42	0.26
rs822552	7	148.28	G	C	0.22	1.10	0.88	1.37	0.40	1.09	0.77	1.53	0.64	1.13	0.84	1.51	0.42
rs2110001	7	150.15	G	C	0.29	1.21	1.01	1.45	**0.04**	1.20	0.90	1.62	0.22	1.22	0.96	1.54	0.10
rs1013209	8	24.17	T	C	0.23	1.00	0.83	1.19	0.96	1.08	0.81	1.45	0.58	0.93	0.74	1.17	0.54
rs7460090	8	57.36	C	T	0.10	0.70	0.53	0.92	**0.01**	0.64	0.41	1.00	**0.05**	0.73	0.52	1.04	0.08
rs6473015	8	78.34	C	A	0.30	1.14	0.96	1.35	0.12	1.21	0.93	1.57	0.16	1.10	0.88	1.36	0.42
rs6470764	8	130.79	T	C	0.19	0.67	0.54	0.82	***1.3E-04***	0.57	0.41	0.80	*1.0E-03*	0.75	0.57	0.98	**0.03**
rs12680655	8	135.71	G	C	0.37	0.94	0.80	1.10	0.42	0.98	0.76	1.27	0.89	0.91	0.74	1.12	0.38
rs7864648	9	16.36	T	G	0.31	1.05	0.87	1.26	0.64	0.93	0.69	1.26	0.63	1.12	0.88	1.42	0.35

						All				Male				Female			
SNP	**Chr**	**Mb**	**A1**	**A2**	**FreqA1**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**
rs11144688	9	77.73	A	G	0.00	0.41	**0.05**	3.53	0.42	0.00	0.00	Inf	0.98	0.45	**0.05**	4.09	0.48
rs7853377	9	85.74	G	A	0.24	1.00	0.84	1.20	0.98	1.01	0.76	1.36	0.92	0.98	0.78	1.24	0.87
rs8181166	9	88.31	G	C	0.49	0.89	0.76	1.04	0.15	0.87	0.68	1.11	0.26	0.90	0.74	1.11	0.33
rs2778031	9	90.03	T	C	0.25	1.05	0.88	1.26	0.56	1.16	0.87	1.54	0.30	0.99	0.79	1.25	0.96
rs9969804	9	94.47	A	C	0.46	1.16	0.99	1.36	0.06	0.99	0.77	1.26	0.91	1.32	1.07	1.62	*8.8E-03*
rs1257763	9	95.93	A	G	0.02	1.13	0.66	1.94	0.65	1.41	0.66	3.00	0.38	0.96	0.44	2.09	0.92
rs473902	9	97.30	G	T	0.01	1.06	0.47	2.38	0.89	1.28	0.32	5.20	0.73	0.92	0.34	2.49	0.87
rs7027110	9	108.64	A	G	0.24	1.14	0.96	1.36	0.13	0.81	0.61	1.09	0.17	1.37	1.10	1.71	*4.5E-03*
rs1468758	9	112.85	T	C	0.23	0.85	0.70	1.02	0.09	0.92	0.68	1.24	0.58	0.80	0.63	1.02	0.07
rs751543	9	118.16	C	T	0.25	0.92	0.76	1.13	0.44	0.85	0.61	1.18	0.34	0.97	0.75	1.26	0.85
rs7466269	9	132.45	G	A	0.35	0.76	0.64	0.89	***9.5E-04***	0.71	0.55	0.93	**0.01**	0.79	0.64	0.98	**0.03**
rs7849585	9	138.25	T	G	0.32	1.10	0.93	1.29	0.28	0.97	0.74	1.28	0.83	1.18	0.95	1.45	0.13
rs7909670	10	12.96	T	C	0.42	0.96	0.82	1.12	0.58	0.87	0.67	1.12	0.28	1.01	0.83	1.23	0.94
rs2145998	10	80.79	A	T	0.47	0.82	0.71	0.96	**0.01**	0.76	0.60	0.97	**0.03**	0.87	0.71	1.06	0.17
rs11599750	10	101.80	T	C	0.37	0.81	0.69	0.96	**0.01**	0.72	0.56	0.94	**0.02**	0.88	0.72	1.08	0.23
rs2237886	11	2.77	T	C	0.11	1.08	0.85	1.38	0.53	1.00	0.67	1.47	0.99	1.13	0.83	1.54	0.44
rs7926971	11	12.65	G	A	0.48	1.05	0.90	1.23	0.52	0.98	0.77	1.26	0.90	1.08	0.88	1.33	0.44
rs1330	11	17.27	T	C	0.36	0.96	0.82	1.13	0.64	1.04	0.81	1.34	0.73	0.91	0.74	1.13	0.41
rs10838801	11	48.05	G	A	0.33	1.19	1.01	1.40	**0.03**	1.01	0.78	1.32	0.92	1.31	1.07	1.61	*9.7E-03*
rs1814175	11	49.52	T	C	0.37	1.13	0.95	1.34	0.16	0.96	0.72	1.26	0.75	1.26	1.01	1.57	**0.04**
rs5017948	11	51.27	A	T	0.20	1.20	0.99	1.45	0.06	0.88	0.64	1.21	0.43	1.47	1.15	1.89	*2.2E-03*
rs3782089	11	65.09	T	C	0.07	0.87	0.64	1.19	0.38	0.88	0.53	1.47	0.63	0.85	0.57	1.26	0.42
rs7112925	11	66.58	T	C	0.34	0.77	0.65	0.92	*3.5E-03*	0.74	0.56	0.97	**0.03**	0.80	0.64	1.00	**0.05**
rs634552	11	74.96	T	G	0.13	1.14	0.92	1.42	0.22	1.20	0.87	1.68	0.27	1.12	0.84	1.50	0.43
rs494459	11	118.08	T	C	0.43	1.02	0.88	1.19	0.78	1.06	0.84	1.34	0.64	0.98	0.80	1.20	0.84

						All				Male				Female			
SNP	**Chr**	**Mb**	**A1**	**A2**	**FreqA1**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**
rs654723	11	128.09	C	A	0.34	0.88	0.74	1.04	0.13	0.79	0.61	1.04	0.09	0.94	0.75	1.17	0.55
rs2856321	12	11.75	G	A	0.37	1.19	1.02	1.40	**0.03**	1.11	0.87	1.42	0.40	1.25	1.02	1.54	**0.03**
rs10770705	12	20.75	A	C	0.35	1.23	1.05	1.44	*9.8E-03*	1.28	1.00	1.64	**0.05**	1.20	0.98	1.48	0.08
rs2638953	12	28.43	G	C	0.32	0.91	0.77	1.07	0.26	1.07	0.84	1.38	0.58	0.82	0.66	1.02	0.07
rs2066807	12	55.03	G	C	0.07	1.13	0.84	1.52	0.43	1.07	0.66	1.74	0.77	1.16	0.79	1.71	0.44
rs1351394	12	64.64	C	T	0.49	0.83	0.72	0.97	**0.02**	0.86	0.68	1.08	0.19	0.83	0.68	1.02	0.07
rs10748128	12	68.11	T	G	0.38	1.15	0.98	1.34	0.09	1.19	0.93	1.54	0.17	1.12	0.91	1.37	0.29
rs11107116	12	92.50	T	G	0.23	1.26	1.06	1.50	**0.01**	1.14	0.87	1.50	0.35	1.35	1.07	1.70	**0.01**
rs7971536	12	100.90	A	T	0.48	0.97	0.82	1.15	0.74	1.06	0.81	1.38	0.66	0.91	0.73	1.14	0.42
rs11830103	12	122.39	G	A	0.22	1.36	1.13	1.63	*1.2E-03*	1.35	1.01	1.79	**0.04**	1.35	1.06	1.72	**0.01**
rs7332115	13	32.05	G	T	0.37	1.03	0.88	1.20	0.73	1.17	0.91	1.51	0.21	0.93	0.76	1.15	0.52
rs3118905	13	50.00	A	G	0.24	0.96	0.80	1.15	0.64	0.71	0.53	0.97	**0.03**	1.13	0.90	1.42	0.30
rs7319045	13	90.82	A	G	0.39	1.11	0.95	1.30	0.19	1.18	0.92	1.50	0.19	1.06	0.86	1.30	0.58
rs1950500	14	23.90	T	C	0.33	1.07	0.92	1.25	0.39	1.01	0.79	1.29	0.95	1.12	0.91	1.37	0.28
rs2093210	14	60.03	C	T	0.43	1.19	1.02	1.40	**0.03**	1.15	0.90	1.47	0.27	1.22	0.99	1.50	0.06
rs1570106	14	67.88	T	C	0.19	0.97	0.80	1.18	0.78	0.94	0.68	1.29	0.69	0.98	0.77	1.26	0.88
rs862034	14	74.06	A	G	0.32	0.82	0.69	0.97	**0.02**	0.73	0.56	0.96	**0.03**	0.88	0.70	1.10	0.27
rs7155279	14	91.56	T	G	0.37	0.82	0.70	0.97	**0.02**	0.70	0.54	0.91	*6.8E-03*	0.94	0.76	1.16	0.57
rs16964211	15	49.32	A	G	0.05	0.71	0.49	1.02	0.06	0.51	0.26	0.98	**0.04**	0.82	0.53	1.29	0.39
rs7178424	15	60.17	T	C	0.43	0.85	0.73	0.99	**0.03**	0.91	0.71	1.17	0.46	0.81	0.66	0.98	**0.03**
rs10152591	15	67.84	C	A	0.10	0.86	0.66	1.13	0.28	0.81	0.52	1.26	0.34	0.89	0.63	1.26	0.51
rs12902421	15	69.95	C	T	0.03	1.20	0.76	1.91	0.43	1.39	0.67	2.87	0.37	1.09	0.60	1.97	0.79
rs5742915	15	72.12	C	T	0.47	1.08	0.92	1.28	0.35	1.37	1.05	1.79	**0.02**	0.93	0.75	1.15	0.49
rs11259936	15	82.37	A	C	0.47	0.68	0.58	0.80	***2.3E-06***	0.78	0.61	1.00	**0.05**	0.63	0.51	0.77	***7.4E-06***
rs16942341	15	87.19	T	C	0.02	0.63	0.33	1.18	0.15	0.59	0.22	1.58	0.29	0.67	0.29	1.53	0.34

						All				Male				Female			
SNP	**Chr**	**Mb**	**A1**	**A2**	**FreqA1**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**
rs2871865	15	97.01	G	C	0.10	0.84	0.64	1.10	0.20	0.86	0.56	1.30	0.47	0.83	0.58	1.18	0.29
rs4965598	15	98.58	C	T	0.34	1.14	0.97	1.33	0.12	1.06	0.83	1.37	0.63	1.18	0.97	1.45	0.11
rs11648796	16	0.73	G	A	0.23	1.22	1.01	1.47	**0.04**	1.50	1.12	2.00	*6.2E-03*	1.06	0.83	1.35	0.66
rs26868	16	2.19	A	T	0.44	1.06	0.90	1.24	0.47	1.17	0.91	1.51	0.23	1.00	0.82	1.23	0.99
rs1659127	16	14.30	A	G	0.33	1.16	0.98	1.38	0.09	1.01	0.77	1.32	0.93	1.28	1.02	1.61	**0.03**
rs8052560	16	87.30	C	A	0.13	1.03	0.78	1.35	0.85	1.23	0.82	1.84	0.32	0.90	0.62	1.30	0.57
rs4640244	17	21.22	G	A	0.37	0.97	0.83	1.14	0.75	1.06	0.83	1.36	0.66	0.92	0.75	1.13	0.44
rs3110496	17	24.94	A	G	0.29	0.99	0.84	1.17	0.92	0.97	0.74	1.28	0.84	0.99	0.80	1.23	0.95
rs3764419	17	26.19	A	C	0.35	0.84	0.71	0.98	**0.03**	0.86	0.67	1.10	0.23	0.84	0.67	1.04	0.11
rs17780086	17	27.37	A	G	0.14	0.89	0.71	1.11	0.31	1.20	0.85	1.68	0.30	0.71	0.53	0.96	**0.03**
rs1043515	17	34.18	A	G	0.47	1.15	0.98	1.34	0.09	1.03	0.80	1.32	0.84	1.21	0.99	1.48	0.06
rs4986172	17	40.57	T	C	0.31	0.79	0.67	0.94	*8.5E-03*	0.88	0.68	1.15	0.34	0.75	0.59	0.94	**0.01**
rs2072153	17	44.75	C	G	0.30	1.16	0.99	1.36	0.07	1.46	1.14	1.87	*2.9E-03*	0.98	0.80	1.21	0.87
rs4605213	17	46.60	C	G	0.33	1.06	0.90	1.26	0.49	1.16	0.88	1.51	0.29	1.01	0.81	1.26	0.92
rs227724	17	52.13	T	A	0.34	1.08	0.92	1.28	0.36	1.17	0.90	1.53	0.24	1.02	0.82	1.26	0.85
rs2079795	17	56.85	T	C	0.36	1.08	0.92	1.27	0.37	1.17	0.91	1.51	0.22	1.02	0.82	1.25	0.88
rs2665838	17	59.32	G	C	0.30	1.37	1.15	1.63	***3.7E-04***	1.28	0.96	1.71	0.09	1.41	1.14	1.76	*2.0E-03*
rs11867479	17	65.60	T	C	0.37	1.22	1.04	1.43	**0.01**	1.59	1.25	2.03	***2.0E-04***	1.01	0.82	1.24	0.91
rs4800452	18	18.98	C	T	0.18	0.75	0.61	0.92	*5.3E-03*	0.77	0.55	1.07	0.12	0.73	0.57	0.95	**0.02**
rs9967417	18	45.21	G	C	0.44	1.13	0.97	1.32	0.13	1.05	0.82	1.35	0.69	1.17	0.96	1.43	0.11
rs17782313	18	56.00	C	T	0.25	1.22	1.03	1.45	**0.02**	1.31	0.99	1.72	0.06	1.16	0.93	1.44	0.19
rs12982744	19	2.13	G	C	0.39	1.29	1.11	1.50	*1.2E-03*	1.08	0.84	1.38	0.54	1.43	1.17	1.74	***4.0E-04***
rs7507204	19	3.38	C	G	0.24	1.19	0.99	1.43	0.07	1.19	0.89	1.58	0.24	1.20	0.94	1.53	0.15
rs891088	19	7.14	G	A	0.26	1.03	0.87	1.23	0.73	0.95	0.72	1.26	0.74	1.08	0.87	1.35	0.47
rs4072910	19	8.55	C	G	0.34	0.75	0.60	0.92	*7.3E-03*	0.78	0.55	1.10	0.16	0.72	0.55	0.95	**0.02**

						All				Male				Female			
SNP	**Chr**	**Mb**	**A1**	**A2**	**FreqA1**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**	**OR**	**Low**	**Up**	**P**
rs2279008	19	17.14	C	T	0.24	0.82	0.68	0.99	**0.03**	0.93	0.70	1.23	0.60	0.76	0.59	0.97	**0.03**
rs17318596	19	46.63	A	G	0.38	1.18	1.01	1.37	**0.04**	1.04	0.81	1.32	0.78	1.28	1.04	1.57	**0.02**
rs1741344	20	4.05	C	T	0.38	1.04	0.89	1.22	0.64	1.13	0.88	1.46	0.33	0.98	0.80	1.20	0.85
rs2145272	20	6.57	G	A	0.38	1.27	1.08	1.48	*3.0E-03*	1.56	1.22	2.00	***4.1E-04***	1.11	0.91	1.36	0.31
rs7274811	20	31.80	T	G	0.21	0.88	0.73	1.06	0.18	0.84	0.63	1.13	0.25	0.91	0.71	1.15	0.43
rs143384	20	33.49	G	A	0.44	1.49	1.25	1.78	***9.6E-06***	1.41	1.07	1.86	**0.02**	1.54	1.23	1.94	***2.1E-04***
rs237743	20	47.34	A	G	0.23	1.19	1.00	1.42	**0.05**	1.29	0.98	1.68	0.07	1.16	0.91	1.47	0.23
rs2834442	21	34.61	T	A	0.36	1.00	0.85	1.17	0.97	1.11	0.87	1.43	0.40	0.94	0.76	1.15	0.54
rs4821083	22	31.39	C	T	0.14	0.85	0.68	1.07	0.16	1.02	0.73	1.44	0.89	0.74	0.55	1.01	**0.05**

Abbreviations: Chr, chromosome; Mb, million basepair; A1and A2, allele 1 and 2; FreqA1, frequency of A1; OR, allelic odds ratio for A1; Low and Up, 95% confidence intervals of OR. Legend: *Bold*: P < 0.05; *Italic*: P < 0.01; *Bold Italic*: P < 0.001.

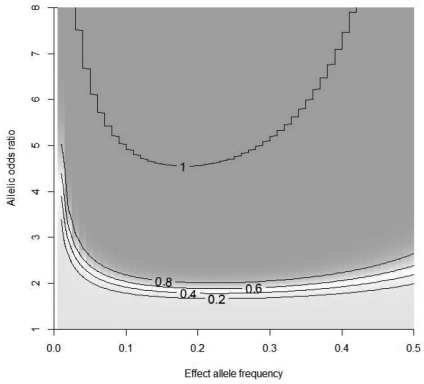
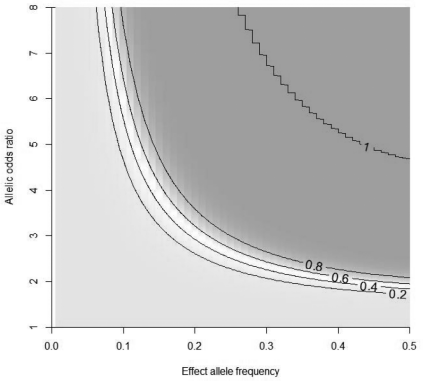


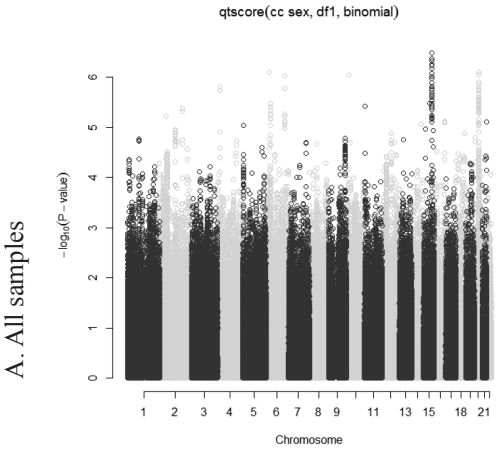
Figure S1. Statistical power to detect effective alleles at the genome-wide significance threshold ($P < 5\times10^{-8}$) using on 484 cases and 1029 controls.

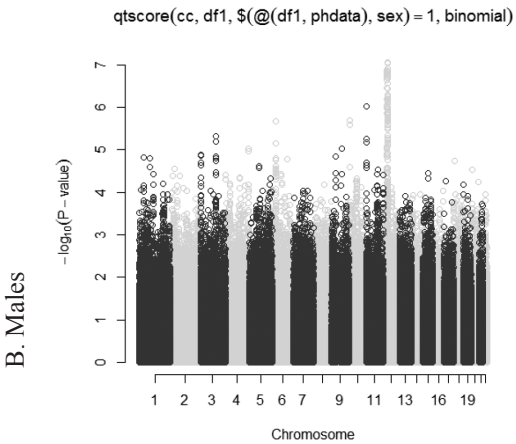
A. Dominant allele

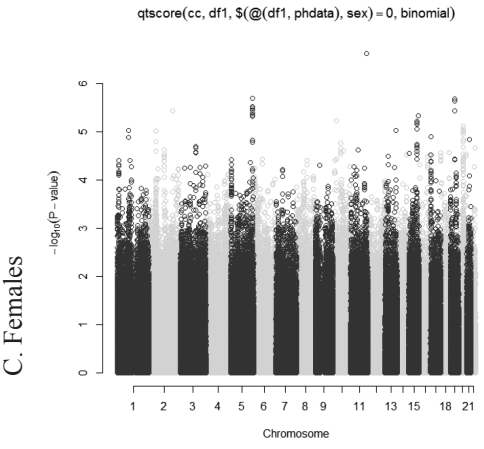


B. Recessive allele

Figure S2. Manhattan plot from single SNP GWA analysis.







Chapter 8

General discussion

Part 1 - High-dose sex steroid treatment of constitutional tall stature

Often new therapies emerge when extremes of nature give us insight in the pathophysiologic mechanism leading to a better understanding of normal physiology. Extensive scientific research is done to demonstrate effectiveness and short-term safety. While patients and physicians certainly consider the equally important question of long-term safety, truth is that studies aiming to answer this question are often few and far between. In the case of human growth our understanding of the effects of the sex steroids on pubertal growth spurt and epiphyseal closure comes from several pathologic conditions. Girls with precocious puberty with clearly elevated estrogen levels show increased height velocity and premature epiphyseal closure.[1-3] In addition, a man has been described with estrogen resistance. He had a normal prepubertal growth and normal timing of onset of secondary sex characteristics. Despite full masculinization his growth persisted into adulthood because of lack of epiphyseal closure.[4] Later in vitro studies showed that the tibial growth plate of ovariectomized rabbits completely fuses after 8 weeks of estrogen while it stayed intact when vehicle was given.[5] The knowledge that sex steroids are needed for epiphyseal closure and cessation of growth led to the development of high-dose sex steroid treatment to reduce adult height of tall boys and girls. While multiple studies evaluated effectiveness and short-term side effects, long-term side effects remained speculative.[6-70] While experience with low-dose estrogen use for contraception and high-dose anabolic steroid use in athletes had not revealed any long-term side effects the possibility of adverse reproductive effects of high-dose sex steroid treatment for tall stature in boys and girls has been acknowledged for many years.[21, 63, 71-73] In fact, in the Netherlands the publicly available drug information specifically states that the long-term effects on reproductive function are unknown (www.fk.cvz.nl).

High-dose estrogen treatment

In 1995 the first study was published assessing long-term effects of high-dose estrogen treatment in adolescence to reduce adult height of tall girls. With a mean follow-up period of 10 years and a average age of 25 years most of the girls had not yet attempted to conceive and no definite conclusions were drawn.[74-76] Until 2004 high-dose estrogen treatment in general was considered to be safe. That year an Australian study reported reduced fertility in treated women with an average age of 35 years after a mean follow-up period of 20 years.[77] The investigators compared 190 treated tall women to 180 untreated tall women and found treated women to have an increased time to pregnancy and to have

an increased risk of infertility. They reported that treated women were 40% less likely to conceive in any given menstrual cycle of unprotected intercourse. While well powered, this study had only used a standardized questionnaire by means of telephone interviewing to assess fertility and thus were unable to elucidate any of the possible mechanisms involved.

Fertility

This thesis confirms in two independent cohorts that tall girls treated with high-dose estrogen in adolescence are at increased risk of infertility in later life. In our first cohort from Erasmus Medical Center in Rotterdam we studied fertility outcome of 413 tall women aged 23-48 years, of whom 239 women had been treated with 200 µg ethinyl estradiol (EE) in adolescence. We show that time to first pregnancy is significantly increased in treated women. Fifty-six percent of treated women conceived their first pregnancy within 12 months of unprotected intercourse compared with 79% of untreated women and 80-85% of the general population as reported in the literature.[78] In addition, we report that treated women have a fourfold decreased odds of achieving a pregnancy and a threefold increased odds of needing infertility treatments. Most importantly, we show that treated women have a threefold decreased odds of achieving at least one live birth resulting in almost one third of the treated women suffering from involuntary childlessness at the time of study. We replicated these results in our second cohort from University Medical Center Groningen, where we studied fertility outcome of 125 tall women aged 20-42 years, of whom 52 women had been treated with 100 µg EE and 43 women with 200 µg EE. We confirm that time to first pregnancy is increased in treated women and that these women more often need infertility treatments. Fifty-nine percent of women treated with 200 µg EE and 69% of women treated with 100 µg EE conceived their first pregnancy within 12 months of unprotected intercourse compared with 80% of untreated women. Finally, also in this cohort women treated with 200 µg EE had decreased odds of achieving a live birth, while this could not be demonstrated for women treated with 100 µg EE.

While our results are in line with the 2004 Australian paper, several differences need to be discussed. Whereas in our population only EE was used, either 100 or 200 µg, about half of the Australian women had used diethylstilboestrol (DES) 3 mg daily and the other half 150 µg EE.[77] The reduction in fecundability was similar in DES and EE treated women. The results of the time to first pregnancy analyses are comparable between all studies, about 55-65% of treated women compared to 80% of untreated

women. Remarkably 20% of the Australian women, both treated and untreated, had conceived their first pregnancy while they were reportedly trying not to get pregnant. These women were excluded from further analyses. In our studies only a handful of women reported unplanned pregnancies and we included them in the analyses while recording their time to first pregnancy as 1 month. We believe that these women need to be included because they have proven albeit unplanned fertility, excluding them may cause overestimation of the treatment effect. Had the Australian study used the same approach their time to first pregnancy analyses would have been more optimistic with 74% of untreated women achieving pregnancy in the first year, compared with 85% of untreated women. The results of the analyses of outcome of fertility across all studies show increased risk of infertility for treated women, who more often seek medical attention for infertility and more often need infertility treatments. We have higher risk estimates than the Australian study, possibly due to a difference in the cohort analyzed. The Australian study included all participating women in this analysis irrespective of whether they attempted to conceive. We decided to study fertility outcome as well as time to pregnancy only in women had attempted to conceive. We believe this to be the true group of women at risk of experiencing and thus reporting infertility. Although theoretically any woman with sexual intercourse is 'at risk' of conceiving, in reality contraceptive methods are very accurate and thus the conscious decision to not get pregnant is an effect modifier in our study. We, therefore, stratified for the effect modifier by excluding women who had not attempted to conceive. This way we believe our results reflect the true odds tall women treated with high-dose estrogen face when choosing to attempt to conceive. We believe this difference in analysis may also explain the marked difference in the risk for involuntary childlessness.

The association between high-dose estrogen treatment and infertility in later life does not prove causation. Alternative hypotheses need to be considered, while methodological considerations will be discussed later;

1) It is possible that treated women have a mentality to seek medical attention sooner than others and are more likely to choose treatment if available. Thus mentality could confound our association between high-dose estrogen treatment and receiving infertility treatments in later life. However, we believe that our conclusions are not affected by such confounding because we also assessed fertility based on time to first pregnancy which is not prone to such confounding.

2) Infertility could also be caused by the partners of these women. Women commonly choose taller partners and tall women are generally believed to choose tall men

as their partners.[79-81] Tall men in turn are at increased risk of developing varicocele, which may cause reduced fertility.[76] Treated women were taller, despite treatment, than untreated women and thus may choose taller potentially less fertile men as their partners which could confound the association between high-dose estrogen treatment and infertility. We therefore asked our participant about their partners and possible fertility issues. In both our cohorts we consistently found a low reported prevalence of a male contributing factor (~5-15%) in women and their partners who had visited an infertility clinic. In addition, no significant differences between treated and untreated women were observed. Finally, mean height of the male partners was found to be around 188 cm in both treated and untreated women, which is less than one standard deviation above the mean for Dutch standards. Thus, although tall women in general choose taller partners, these men cannot be considered extremely tall nor potentially less fertile.

3) Treated women differ from untreated women with regard to their predicted and adult height. Women who as girls had chosen to be treated for their tall stature were in general taller at first presentation and had higher predicted heights than girls who decided not to be treated. In addition, despite treatment adult height of treated women is higher than that of untreated women. Therefore, tall stature itself could be a confounder. We studied several clinical parameters representing ovarian function at first presentation such as Tanner stage and age at menarche and found no differences between treated and untreated women. In the available literature height is negatively correlated with reproductive success in Western society, however this is attributed to sexual selection and the chance of finding a mate rather than the fertility of these women which has not been studied in detail.[82] The height difference between untreated and treated women equals 1 percentile (98th vs. 99th percentile) according to Dutch standards.[83] From a population perspective it seems unlikely that this could explain 15-25% reduction in achieving a first pregnancy in the first year, but we cannot fully exclude height as a possible confounder.

4) The possibility of an underlying condition associated with both tall stature and reduced fertility, such as partial hypogonadotropic hypogonadism, needs to be considered. However, hypogonadotropic hypogonadism is a secondary growth disorder and its growth pattern is distinctively different from constitutional tall stature. In hypogonadotropic hypogonadism growth during childhood is unremarkable and tall stature does not become evident until the teenage years when growth continues because of lack of epiphyseal closure.[84] Bone age is delayed because of low circulating sex steroid levels. Constitutional tall stature is characterized by accelerated growth velocity in early childhood and tall stature becomes apparent at the age of 3 to 4 years.[48] Bone age is generally

slightly advanced. We believe we can exclude the possibility of hypogonadotropic hypogonadism in these women, even though serum gonadotropin and sex steroid concentrations were not measured at initial presentation, as we excluded women with secondary growth disorders and found no hypogonadotropic anovulation at follow-up.

All alternative hypotheses considered, an important observation supporting causality between high-dose estrogen treatment and infertility is the dose-response relationship shown in one of our studies. Treated women had an increased time to pregnancy and more often sought medical attention for infertility. Of these, women treated with 200 µg of EE significantly more often experienced fertility problems than women treated with 100 µg. These results suggest an important role of estrogen dose on fertility outcome and a possible lead towards explaining the mechanism behind the loss of fertility in tall women treated with high-dose estrogen in adolescence.

Ovarian function

Having shown the dose-dependent effects of high-dose estrogen treatment on long-term fertility outcome of tall girls treated in adolescence, we set out to improve our understanding of the mechanisms involved. Of the women who had seen a doctor because they were having trouble becoming pregnant in about 60% a diagnosis had been made across all three studies. When studied in detail none of the diagnoses, such as PCOS or endometriosis, were seen more often in treated women compared with untreated women. As we observed a significant reduction in the number of live births in treated women we studied whether there was a possible increase in miscarriages. However, an increased risk of miscarriages in treated women was not found. Finally, we studied ovarian function using the WHO classification based on serum gonadotropin levels.[85, 86] No women presented with hypogonadotropic anovulation (WHO-1). Normogonadotropic anovulation was diagnosed in 6% of both treated and untreated tall women, this frequency is comparable with the incidence reported in the general population.[87, 88] We show an increased frequency of women with a hypergonadotropic profile. These women had, next to abnormally high serum FSH levels, decreased serum levels of AMH and Inhibin B as well as lower antral follicle counts compared with normogonadotropic tall women. This hormonal profile fits with the diagnosis of imminent ovarian failure.[89, 90] Imminent ovarian failure precedes the onset of cycle irregularity and hence the menopausal transition by 3–10 yr and may be considered an early sign of advanced ovarian aging in young women.[90, 91] To account for normal changes in ovarian function in the late reproductive stages, treated and untreated women were divided into two age categories.[92] Taking these

age categories into account, the odds of imminent ovarian failure in treated women was almost threefold higher than in untreated women. Thus treated women are at increased of accelerated ovarian aging with concomitant follicle pool depletion. This may lead to earlier menopause in these women. The validity of our results need to be discussed, while methodological issues will be examined later. As we are the first to study ovarian function in later life of girls treated with high-dose estrogen in adolescence no comparison with other studies can be made, but several issues need to be reviewed;

1) Our main goal was to study ovulatory function. Unfortunately, there is no established method to ascertain the completion of a normal ovulatory cycle in a woman. Therefore several diagnostic approaches had to be combined. We measured serum hormone values in the early follicular phase and classified ovulatory function according to WHO-criteria based on gonadotropin levels. In addition, we used serum androgen levels and transvaginal ultrasound scanning of the ovaries to diagnose PCOS, while serum prolactin and TSH were measured to exclude hyperprolactinemia and primary hypothyroidism. However, other common tests in the evaluation of infertility such as tubal patency assessment, measurement of midluteal serum progesterone levels or hysteroscopy were not performed. While we hypothesize that the association between high-dose estrogen treatment and infertility is based on disruption of folliculogenesis, we cannot exclude the possibility of other pathologies not currently studied.

2) Half of the women included in the early follicular phase assessment were using oral contraceptives at the time of study. We included these women in the analyses based on the knowledge that events during the pill-free interval resemble those observed during the early follicular phase.[93-95] However, the rise in FSH has been shown to be steeper than in the normal menstrual cycle and a higher cut-off point was found to be the upper limit of the range in FSH on day 7 of the pill-free interval in healthy OCP using female volunteers.[93] In other words, although comparable, ovarian activity is not exactly the same in the pill-free interval as it is in the normal menstrual cycle. In fact the inhibitory effect of contraceptives is meant to be sufficient to arrest and repress the amount of activity present at the end of the 7-day pill-free interval.

3) We have not reported on menstrual cycle characteristics of these women for several reasons. Firstly, women using contraceptives could not give reliable information regarding their current menstrual cycle. Secondly, menstrual cycle history was obtained but our questionnaire in hindsight was not detailed enough to separate irregular cycles right after menarche or contraceptive use from true oligomenorrhea. Finally, anovulatory infertility can be associated with oligo/amenorrhea but also with apparently normal

menstrual cycles.

4) Notably the incidence of imminent ovarian failure in untreated women is increased when compared with the general population.[90] The observed difference is likely due to selection because only a small number of eligible untreated women participated in the ovarian function analysis. Future research is necessary to determine whether tall women intrinsically have an increased risk of infertility and/or ovarian failure. While taking these considerations into account we wish to emphasize that imminent ovarian failure was not diagnosed based on a hypergonadotropic profile alone but included hallmark parameters such as reduced serum AMH and Inhibin B levels and decreased antral follicle count. Serum AMH is currently the best marker for primordial follicle pool size because in the ovary it is expressed in granulosa cells of follicles that have undergone recruitment but have not yet been selected for dominance.[95-97] In addition, AMH plays an important role in regulating folliculogenesis as it is involved in determining the individual FSH threshold of early antral follicles.[98, 99] Antral follicle count decreases during reproductive aging and it is used to estimate ovarian reserve assuming that the number of visible antral follicles reflects the size of the primordial follicle pool.[100, 101] Inhibin B is produced by the developing preantral and early antral follicles, and its circulating concentrations are maximal during the early to midfollicular phase.[102] Early follicular inhibin B levels decrease during reproductive aging leading to increasing FSH concentrations.[103]

While our research has taken us a step closer to understanding the association between high-dose estrogen treatment and infertility, the mechanism behind the observed accelerated follicle loss remains unknown. We hypothesize that the effects of high-dose estrogen could be directly at the follicle level or indirectly through other intra-ovarian regulatory hormones such as IGF-1 or AMH. Studies on physiological levels of estrogen have shown that human granulosa cells are a site of estrogen reception, while it is still uncertain whether the human oocyte is also estrogen responsive.[104] Among the local intrafollicular actions of estrogen is its responsibility for facilitating the differentiation of granulosa cells, including the induction of receptor systems for FSH and LH and it can influence post-receptor mechanisms.[105] Studies on estrogen depleted ovaries have shown that folliculogenesis halts in the antral stage causing infertility due to the inability to ovulate.[105] Animal studies have shown that IGF-1 is required for reproduction. It has been suggested that IGF-1 promotes fertility by limiting the recruitment of primordial follicles in the growing pool thus conserving the resting pool.[106] Interestingly, it has been shown that serum IGF-1 levels are greatly reduced during high-dose estrogen treatment.[30] One study, in fact, has shown that serum IGF-1 levels are lower in girls receiving

200 µg EE compared with 100 µg EE after 12 months of high-dose estrogen treatment.[38]

High-dose androgen treatment

Several studies have been published of long-term effects of high-dose androgen treatment in adolescence to reduce adult height of tall boys. Follow-up thus far had been up to ten years after cessation of treatment. Only one study assessed fatherhood in previously treated men and found no differences between treated and untreated men. However, most men at the time of study had no wish for children yet. Instead all studies mostly focused on testicular function. One study demonstrated normalization of gonadotropin levels in tall boys after discontinuation of treatment with up to 48 months of follow-up, although transient hypergonadotropic LH- and FSH- secretory patterns were observed.[12] Another study found marginally higher serum follicle stimulating hormone levels and lower serum luteinizing hormone levels in androgen treated tall boys compared with untreated tall boys at an average follow-up of ten years.[76] Finally, one study found lower testosterone levels in treated men compared with controls of average height.[26] Several studies have reported a reduction in testicular volume in tall adolescent boys treated with high-dose androgen. This process is likely to be reversible since testicular volume normalizes after discontinuation of therapy.[55, 58] In addition, no differences in sperm quantity or quality has been found at follow-up.[26, 55, 58]

Fertility

This thesis presents the first study to evaluate long-term fertility outcome in tall boys treated with high-dose androgen in adolescence with a mean period of follow-up of more than 20 years. We show that fatherhood is not affected by this treatment. Treated men had a normal time to first pregnancy, they had a low rate of infertility and most men had achieved to father at least one child. While there are no other studies to compare our results with we need to consider the possibility of underestimating the true effect of treatment. About half the men studied had attempted to achieve fatherhood and were included in the fertility analyses. We did not compare these men to those who had not yet started to conceive, nor did we study the age at which the men chose to father a child. We have no reason to assume a difference in mentality towards family planning but we cannot exclude such possibility either. For example men who fathered a child could have done so at a relatively young age while the other men may be waiting with their attempt at fatherhood until later in life which could result in fertility problems not observed in our study. It is also possible that men with reproductive problems are less likely to

participate. These men may be afraid to hear the results of our tests, or they could have already had their fertility tested before being confronted by our invitation to participate. Finally, only one third of the eligible men participated in the study. Our results therefore need to be interpreted with caution.

Testicular function

Next to fertility we studied whether high-dose androgen treatment has long-term effects on testicular function. We show that testicular volume and morphology as well as semen quantity and quality are normal in treated men and do not differ from untreated men. This suggests that no severe long-term complications on spermatogenesis are apparent after 21 years of follow-up, which is in agreement with findings of previous studies performed directly after cessation of treatment or after a few years of follow-up.[55, 58, 75] One study showed seminal parameters to be slightly lower, albeit mostly not significantly, in treated men compared with healthy controls of average height.[26] The authors discuss that using untreated tall men as controls may introduce bias as these men may have remained untreated for a reason. However, prevalence of varicocele is associated with increased height and in this study indeed tall treated men had varicocele more often than the controls. Therefore, no conclusions could be drawn about whether seminal parameters were lower because of high-dose androgen treatment or because of the prevalence of varicocele. We know from clinical experience that untreated men declined treatment because of satisfaction with the predicted height or fear of possible side effects, not because of doctors warnings based on underlying disease. Therefore, we chose to compare tall treated men with tall untreated men. We found a similar prevalence (20%) of varicocele in treated and untreated tall men and we found no differences in seminal parameters. We conclude that high-dose androgen treatment does not influence spermatogenesis in the long term. The prevalence of varicocele was lower than previously reported (40%).[76] This discrepancy is likely caused by ultrasound imaging we used, which is more sensitive than the Doppler stethoscope. In fact, one other study using ultrasonography reported a prevalence similar to ours (26%).[26]

Finally, we studied serum hormone values of the hypothalamic-pituitary-gonadal axis. We found normal values for the hormones involved in Sertoli cell function. This is in contrast with earlier findings of marginally increased FSH levels in treated men.[76] This association may have been lost due to the age-related increase of FSH as seen in our longitudinal follow-up and in a previous study.[107] However, Leydig cell function was significantly affected by high-dose androgen treatment. In treated men both

serum testosterone and non-SHBG-bound testosterone levels were significantly reduced compared with untreated men, while serum LH levels were normal. Significantly lower testosterone levels have been found before, however values then were well within the normal range (19.9 vs. 23.9 nmol/L) and not much was made of this difference.[26] In our study testosterone levels in treated men, albeit still within the normal range, were again significantly lower (13.3 vs 15.2 nmol/L) and approach clinically relevant cut-off values. Published normal ranges of serum testosterone in healthy men are typically above 10.4 nmol/L. Levels below 8.7 nmol/L are considered unequivocal hypogonadism, while values below 12.0 nmol/L have been associated with mild sexual dysfunctioning.[108-110] We hypothesize that the decreased testosterone levels may be caused by reduced Leydig cell growth during puberty and suboptimal functioning of the Leydig cells in later life. In humans, there are three waves of Leydig cell growth: the third is during puberty when it is strictly under the control of luteinizing hormone.[111] High-dose androgen treatment in tall boys is given during puberty and suppresses the hypothalamic-pituitary-gonadal axis.[12, 58] This results in low levels of LH during the third wave of Leydig cell growth. It is therefore not unlikely that Leydig cell growth and function would be impaired in these men. During treatment testicular volumes remain prepubertal or decreased, based on our and previous results this effect of treatment appears to be fully reversible. However, some measurable effect of treatment remains as we found an association between age at which treatment was started and testicular volume in later life. Men treated at a younger age as boys had lower testicular volumes but also lower inhibin B and FSH levels, while semen parameters and fatherhood were not affected. It is likely that spermatogenesis has such a reserve capacity that small differences in testicular function do not influence fertility. But we cannot exclude the possibility that our study may be underpowered to detect infertility in men treated at a young age nor the possibility that infertility may only be evident in men who wish to father a child at a more advanced age. However, the mean age of the population currently studied is 35 years old, which corresponds to the average age at which Dutch men father their children.[112] We therefore believe our results to be a good representation of the chances of fatherhood in this cohort of tall men.

Methodological considerations

In any observational cohort study it is important to carefully consider possible biases when interpreting the results. In our studies we need to consider the possibility of selection bias as we were unable to follow-up all eligible men and women. From our first co-

hort of women from Erasmus Medical Center 71% of the eligible women participated in our study. The response rate in the treated group was higher (71%) than in the untreated (62%), and treated women with reproductive problems may have been more interested in participating. A sensitivity analysis was done to see if such a selective response could explain whether the increased infertility we recorded was associated with treatment. First, we assumed that treated non-participants had no infertility and a time to first pregnancy of 6 months. When this hypothetical group of non-participants was added to the analysis, the time to first pregnancy remained significantly longer in treated women compared to untreated women (logrank $P = 0.03$). The scenario in which we included a hypothetical group of all non-participants, both treated and untreated, that was fertile the significant treatment effect remained (logrank $P < 0.001$). Selective non-response of women with low fertility in the untreated group would also suggest increased infertility in the treated women. When we assumed that untreated non-respondents had the same prevalence of infertility as the treated respondents (TTP 56% after 1 year and 70% after 2 years), a significant treatment effect still remained (logrank $P = 0.001$). No sensitivity analysis was performed in the second cohort of women (57% participation) nor in the cohort of men (31% participation). We therefore cannot fully exclude the possibility of selection bias in these studies. The time to pregnancy data is self reported and may be confounded by recall bias. However, we believe that our conclusions are not affected by such bias because we also assessed fertility based on data such as having received infertility treatments which is not prone to recall bias and showed similar results. In our second cohort of women from University Medical Center Groningen women treated with 200 µg EE had started treatment in earlier years and as a result had a longer duration of follow-up and were older at the time of study. We, therefore, cannot fully exclude the possibility of bias due to differential follow-up. While this is corrected for by the use of the time to event analysis in the calculation of time to first pregnancy, it is possible that with similar follow-up time women treated with 100 µg EE would have had overall fertility outcome similar to women treated with 200 µg EE. We therefore performed a sensitivity analysis to see if differential follow-up could explain the observed difference in fertility outcome between women treated with 100 µg EE and women treated with 200 µg EE. We assumed that participating women, both treated with 100 and 200 µg EE, who had not attempted to conceive had overall fertility outcome comparable to women treated with 200 µg EE. When this hypothetical group of treated women who had not attempted to conceive was added to the analysis, the significant trends of increasing fertility problems with increasing estrogen dose remained (trend $P<0.05$).

Conclusions

In conclusion, we evaluated fertility and gonadal function in later life of two independent cohorts of tall women and one cohort of tall men who did or did not receive high-dose sex steroid treatment in adolescence. We found that estrogen treated women experienced more difficulties conceiving and more often received medical treatment for infertility compared with untreated women. Treated women had a decreased chance of achieving at least one live birth. We report a dose-response relationship between fertility in later life and the estrogen dose used, as women treated with 200 µg of EE significantly more often experienced fertility problems than women treated with 100 µg. Our results suggest an important role of estrogen dose on fertility outcome. Finally, we showed that treated women were at increased risk of being diagnosed with imminent ovarian failure. They exhibit signs of accelerated ovarian aging with concomitant follicle pool depletion, which may be the basis of the observed infertility. However, the mechanism behind this accelerated follicle loss by high-dose estrogen treatment remains unknown. In men we found no long-term impact of this treatment on fatherhood or semen quality. Testosterone production, however, is reduced in androgen treated men.

Recommendations

For future research

Since it is now clearly established that high-dose estrogen treatment in associated with infertility in a dose-dependent manner we recommend future research to focus on the possible mechanisms involved. We have shown that the observed infertility could be caused by accelerated ovarian aging with concomitant follicle pool depletion but additional research is necessary to unravel the mechanisms leading to this follicle loss. Future studies, possibly animal models, should focus on ovarian function during and shortly after treatment to detect any early changes that may explain the effect of treatment. In addition, confirmation of the observed accelerated follicle loss by another long-term follow-up study would be desirable. Another possible way of confirming our results and hypotheses would be to study age at menopause in these women. Unfortunately, such study is not possible until 15 years from now when the women will on average have achieved menopausal age. Finally, research could focus on why not all treated women

experienced infertility. Is it possible that some women are more susceptible than other women to this long-term side effect of high-dose estrogen treatment.

Although high-dose androgen treatment does not seem to affect semen quality and fatherhood, we wish to emphasize that our results need to be interpreted with care and that confirmation in an independent cohort is necessary. Since testosterone levels will continue to decline as these men age, we believe our results may be of clinical relevance to androgen treated tall men as they grow older. The possible clinical relevance is illustrated by the results of a recent cohort study of men over 65 years old, which found low levels of non-SHBG-bound testosterone to be associated with worse baseline frailty status.[113] In addition, LH levels may only rise as soon as clinically relevant low serum testosterone levels are observed. Therefore, future studies are required to fully elucidate the effects of treatment on LH and testosterone levels.

Another possible long-term side-effect not well studied to date is the risk of malignancy. Although there have been no reported cases of malignancies in girls treated for tall stature, the risk of cancer in young women receiving estrogens remains uncertain. For example, an associations has been reported of oral contraceptive use at a young age and the duration of use with increased risk of breast cancer.[73, 114] Several other studies have reported a higher risk of epithelial ovarian malignancies in women who ever received hormone replacement therapy than in women who never received such treatment. The risk increased with long term use.[115] In men testosterone treatment is associated with enhancing a pre-existing malignancy of the prostate.[116]

For clinical practice

We expect that for many girls the benefits of reducing adult height with a few centimeters do not outweigh the risks of infertility, reduced fecundity and possibly early menopause. Based on our results we advocate to no longer continue the treatment of tall girls with high-dose estrogens for the purpose of reducing their adult height. We recommend to counsel women who were treated in the past about the risks of infertility and unwanted childlessness. We would advise to women who desire to have children to not delay conceiving unnecessarily if possible. When treated women are unable to conceive within the first year of attempting pregnancy we recommend consultation with a gynecologist for further investigation. Diagnostic testing should include measuring AMH to determine ovarian reserve. Furthermore, we advise to consider screening for decreased ovarian reserve by measuring AMH in women who seek answers about their fertility but are not yet able or ready to fulfill their desire to have children. If normal ovarian reserve is found

this screening could be repeated after two to three years to catch any rapid decline since. The treatment of tall boys with high-dose androgens to reduce adult height does not affect fatherhood or semen quality. Continuation of treatment when requested thus seems safe. However, we did observe reduced testosterone levels in treated men which may cause clinically relevant symptoms in the future. We therefore recommend measuring testosterone levels in treated men as they age.

Part 2 - Genetic determinants of constitutional tall stature

Initially the study of genetics of height mainly focused on short stature, with many possible candidate genes identified.[117-175] Notably, however, few genes were reproducibly associated with height variation. In recent years, focus has shifted to genome wide association studies in large populations of average height. While multiple genetic loci have been robustly associated with height variation, effects sizes were small and cumulatively explained approximately five to ten percent of the population height variation.[176-185] Additional strategies are therefore necessary. Sampling at the extremes of a quantitative trait while using common controls has been shown to maximize statistical power as much of the information is provided by individuals in the tails of the distribution.[181] While genetic determinants of height have been extensively studied in the very short, very few authors have attempted to study these genes in the extremely tall. In view of the availability of a large population of tall individuals participating in our study on long-term effects of high-dose sex steroid treatment we initiated several studies on the genetic determinants of tall stature.

Candidate gene analysis

Using a case-control design we have been able to show several single nucleotide polymorphisms (SNPs) to be associated with extremely tall stature. This included a SNP in the high mobility group-A2 gene (HMGA2), which is involved in the cell cycle, and several SNPs in the growth hormone (GH)/insulin-like growth factor-1(IGF-1) axis. Before reviewing our result the validity of our design needs to be discussed.

In genetic association studies it is important to have a clear case definition, to determine that the disease is heritable and to select proper controls.[186, 187] Height is an easily and accurately measurable phenotype to use as case definition, but does vary with age and gender within the same population. To properly define cases we have chosen to define extremely tall stature as having a height standard deviation score (SDS) over +2 according to Dutch standards.[83] In addition, height is highly heritable (h2 ~ 0.80–0.90).[188, 189] Finally, we have chosen to compare one tail of the distribution i.e. extremely tall stature to the rest of the normal distribution i.e. height SDS less than +2. It may be argued to use individuals with extremely short stature i.e. height SDS less than -2 as controls to maximize power because there could be genetic clustering at the extremes which could enhance the frequency difference.[190] However, tall and short stature are not necessarily genetic counterparts and comparing both extremes of the distribution could enhance but

may well reduce frequency difference. In addition, others have compared both tails of the distribution and found no association for genes of the GH/IGF-1 axis.[129] We have therefore chosen to compare one extreme of the distribution to the rest of the normal distribution.

To avoid spurious associations due to population stratification, cases and controls should be matched by ethnic origin. In our studies this was accomplished by including only Caucasian individuals of Dutch ancestry, defined as being born to Dutch parents who themselves were born in the Netherlands. We did not need to match for age and gender as our outcome variable is corrected for these two confounders. Another important factor in the analysis of case-control genetic association studies is for the control group to follow the Hardy–Weinberg equilibrium (HWE).[191] Conformity with HWE suggests that several conditions are met including absence of recent mutations and genetic drift and conformance with mendelian segregation and random mating. When HWE fails population stratification or selection bias are possible, but probably genotyping error occurred. In our controls all genotype distributions were in HWE.

Finally, to determine the validity of our controls we compared our results to previous publications. The allele frequency observed in our controls was comparable with the frequency found in other studies of Caucasian subjects. For example, the HMGA2 SNP C-allele frequency we observed in controls (0.50) was similar to previously reported allele frequencies (0.48-0.54), as was the direction of the allele effect.[183] Also in line with previous studies we found IGFBP-3 serum levels in controls to be highest in carriers of the AA genotype and decline in a stepwise manner per C-allele.[121] Several of our cases had received high-dose sex steroids to reduce adult height. This may cause underestimation of effect sizes in cases. However, we have chosen to not correct for this possible treatment effect because of the uncertainty in height prediction models making any estimate of such an effect inaccurate. We thus use current height only, moreover because in our case-control study design it does not affect our primary objective of comparing cases to controls. To achieve a higher power we included multiple cases from families while adjusting for their familial correlation. This is known as enrichment sampling and increases power as these cases may be more homogeneous in genetic etiology.[192] To account for family structure we used a pairwise kinship matrix specifying the degree of relatedness between each pair of individuals in the analyses.[193]

Concerning the results of our candidate gene association analyses several points need discussion. We found polymorphic variation in several genes of the GH/IGF-1 axis to be associated with tall stature. While this is not unexpected from a biological point

of view as the GH/IGF-1 axis is a key regulator of somatic growth in humans, genetic association studies have failed thus far to robustly associate genes of the GH/IGF-1 axis with height variation.[129] In addition, genome wide association analyses have found 180 replicated loci to influence adult height of which only a few are of the GH/IGF-1 axis.[179]

The significant association observed in our study could be a result of our approach to study the extreme of the distribution and thereby maximizing our power to detect differences. In fact, as the Dutch are the tallest people in the world we have studied the worldwide furthest possible tail of the height distribution. Several of the variants we studied have potential functional significance, such as the IGFBP-3 SNP which has been shown to affect circulating IGFBP-3 levels.

It has been argued that using functional variants increases the chance of detecting trait associated genes, because a priori they are most likely to be of functional significance and to influence directly the traits under study. In fact, these are the variants to which random SNP searches are likely to lead. In addition, even if not the causative variant in a gene, such SNPs are as likely to be in linkage disequilibrium with the causative allele as randomly placed SNPs are.[194] Finally, we wish to argue that our cohort of tall individuals might be more homogenous than common population based studies. While height is a normally distributed variable following a Gaussian distribution, the tails of the distribution do not only consist of individuals tall or short by constitution but also include individuals with primary or secondary growth disorders. Most population based studies do not mention excluding individuals with these growth disorders and they are probably not capable of identifying such individuals in their cohort as it was initially collected for a completely different phenotype and height just happened to be measured. We have set out to collect a sample of constitutionally tall men and women and we have excluded individuals with primary or secondary growth disorders leading to a possibly more homogenous sample than most other studies.

Next to SNPs in genes of GH/IGF-1 axis we have found the HMGA2 SNP to be associated with being extremely tall. This shows that common variants robustly associated with height in the general population also associate with height at an extreme tail of the height distribution. Thus, our results support the hypothesis that with complex traits the extremes of the distribution represent the sum of all low penetrance common variants rather than the hypothesis that they are caused by a few moderately penetrant rare variants. Such polygenic model for the genetic architecture of quantitative traits may imply that only studying the extremes of the distribution could be an efficient and successful method.

None of the candidate gene SNPs associated with gonadal steroids and growth factors of the skeletal axis were associated with tall stature while they had been associated with height variation in the past. Non-replication is a common problem in candidate gene studies due to either false-positive or false-negative results.[195] Although certainly possible, false-negative results due to errors in our study design seems unlikely as we clearly replicated the HMGA2 SNP association in both cases and controls. In addition, the chance of false-positive results in the previously referenced studies seems likely as quite often only a single study had shown an association. The improved understanding of the limitations of the hypothesis driven approach of candidate gene studies has led to an increased demand for replication of results and the need of the gene finding approach applied in genome wide association studies. The HMGA2 SNP was initially identified through a gene finding approach, our replication of this SNP in a cohort of extremely tall stature clearly shows the value of this new method. We believe that combining reports from GWAS and candidate gene studies will lead to identifying new SNPs and genes involved in height variation.

Genome wide association analysis

Finally, we performed a genome-wide association study of adult height in a case-control study of Dutch extremely tall individuals compared with Dutch individuals of average height. We show several loci to be associated with extreme tall phenotype, including a genome-wide significant SNP at the relatively unknown gene RBM39, SNPs located in the known growth gene ADAMTSL3 and SNPs at 6q24.1 containing two pseudogenes which has not been associated with height variation before. We wish to discuss the design of this study before reviewing the results.

Compared to most GWAS of adult height using thousands of individuals, our discovery cohort of 500 is relatively small. However, our discovery cohort consisted of 500 individuals with heights over the 97th percentile which is equivalent to a general population based sample of 16.000 individuals. Therefore, as shown by our power analysis we have good power to detect common SNPs with moderate effects or rare SNPs with large effects. However, as in most GWAS, we have limited power to detect SNPs with small effects. To minimize the misclassification of cases or controls we excluded 964 controls falling in the upper middle range of the height distribution as oversampling of controls does not provide a noticeable gain in statistical power. We validated our sample by replicating the 180 SNPs robustly associated with height in previous GWAS.[176-179] We found 84 (46.7%) SNPs to be nominally associated with extreme tall phenotype, which

is far beyond what one would expect under the null hypothesis of no association (5%). Therefore we believe our sample to be a valid set. In addition, one of these associated SNPs (rs1351394) lies in the HMGA2 gene which has been associated with tall stature in previous studies.[183, 184]

We wish to discuss several results of our GWAS. First, we found no genome-wide significant hits in our discovery cohort. Our study, therefore, does not support the hypothesis that rare variants with large effects may explain the missing heritability of height.[181] This is unexpected as we used the study design proposed in the literature to maximize statistical power and we show in our power calculations to have good power to detect these variants under an additive model.[181, 190, 196] A possible explanation could be that rare variants with small to moderate effects explain the missing heritability. We did not have sufficient power to detect these variants. In addition, we had low power to detect variants under a recessive model, especially those with frequencies lower than ten percent. Larger studies or consortia using our design are needed to find these rare variants under additive or recessive models.

Next we identified three loci with at least one SNP showing P values $< 10^{-6}$. These loci were 6q24.1, 15q25.2 containing ADAMTSL3, and 20q11.22 containing RBM39. In the combined analysis of the discovery and replication cohorts the SNP (rs2425073) in the gene for RNA binding motif protein 39 (RBM39) reached genome-wide significance. RBM39 is a transcriptional coactivator that stimulates transcription mediated by the progesterone and estrogen steroid hormone receptors.[197, 198] While RBM39 has not been associated with height variation before it could be a plausible candidate as sex steroids are key hormones in human growth. Additional research is necessary to determine the role of polymorphisms in the RBM39 gene in adult height variation. In addition, we believe this locus could be of interest when studying the mechanism behind the observed infertility and accelerated follicle loss in tall women treated with high-dose estrogen. While we have demonstrated that estrogen dose is associated with infertility, it remains to be determined whether tall women in general are more susceptible to these adverse effects of high-dose estrogen treatment. Genetic variants associated with height in genes involved in sex steroid function could explain such susceptibility. The second locus identified contains the gene “a disintegrin and metalloproteinase with thrombospondin motif-like protein 3” (ADAMTSL3), which we found in both the validation analysis and the GWA analysis to be significantly associated, albeit not genome-wide, with the extreme height phenotype. ADAMTSL3 is a secreted glycoprotein involved in the turnover of extracellular matrix components which is robustly associated with adult

height variation.[176-178, 199-203] Interestingly ADAMTSL3 protein in concert with ADAMTS enzymes has been implicated to modulate fibrillin-1 function.[204] Mutations in the fibrillin-1 gene (FBN1) are known to cause Marfan syndrome, which has tall stature among its clinical features. However, a mutation in FBN1 that abolishes a binding site utilized by ADAMTSL3 causes Weill-Marchesani syndrome characterized by short stature. Thus it has been suggested that ADAMTS enzymes with the help of ADAMTS-like proteins could be involved in the installation or remodeling of structural materials, such as collagens, in the fibrillin microenvironment, or they might participate in the activation of nearby latent growth factors. Finally, the third locus identified and replicated was 6q24.1 which thus far has not been associated with height variation. This locus contains two pseudogenes ATP5F1P6 (ATP synthase, H+ transporting, mitochondrial Fo complex, subunit B1 pseudogene 6) and LOC100129554 (thyroid hormone receptor interactor 4 pseudogene). Further research is necessary to identify the function of these genes and the role of this locus in height variation.

Conclusions

In conclusion, first we have performed a candidate gene analysis and we have shown several common polymorphisms in the IGF-1, IGFBP-3 and HMGA2 genes and haplotype at the GH1 gene to be associated with extremely tall stature in a Dutch cohort. Our study shows that these genes are not only associated with height variation in the general population but also play an important role at one of the extremes of the height distribution. Next we have performed a genome-wide association study using extreme tall phenotype as trait. We discovered a new genome-wide significant SNP in the gene for RBM39, which is involved in sex steroid mediated transcription. In addition, we found several SNPs in the gene for ADAMTSL3 to be associated with the extreme tall phenotype. ADAMTSL3 has been robustly associated with height variation and is implicated to modulate fibrillin-1 function. Finally, a new locus was identified at 6q24.1 which requires further research.

Recommendations

For future research

Based on our results we recommend sampling at the extremes of the distribution when studying the genetics of complex traits. Further research is needed to fully understand the role of common polymorphisms in the genetic architecture of quantative traits and to explain the missing heritability of adult height. The role of possible rare variants with moderate to large effect sizes remains elusive. We were unable to detect such variants at genome-wide significance using our sample size and study design. It would be of great value to collect larger cohorts of extremely tall or short individuals. We wish to emphasize the need for excluding individuals with primary or secondary growth disorders, as they also cluster at the extremes. We recommend continuing the gene finding approach by means of genome wide association analysis in large cohorts or consortia. We believe that the candidate gene approach still has merit to confirm robustly associated polymorphisms. In addition, the results of the candidate gene analyses presented in this thesis require replication. Finally, genes containing common variants with small effects may also harbor rare variants with moderate effects. Thus, rare variants and their role in height variation may be identified by sequencing genomic regions identified by common variants, especially if extreme phenotypes are used, as these seem to more often represent effects of loss-of-function alleles.[190]

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Chapter 9

Summary

Summary

This doctoral thesis describes in a large group of constitutional tall men and women the long-term effects of high-dose sex steroid treatment to reduce adult height and the genetic determinants of tall stature. The chapters in the first part of this thesis focus on long-term fertility outcome of tall boys and girls treated with high-dose sex steroids in adolescence. In addition, a dose-response relationship of estrogen dose and fertility is studied. The chapters in the second part concentrate on the association of tall stature with several common polymorphisms in the GH/IGF-1 axis, as well as with the recently discovered HMGA2 gene SNP. Finally a genome wide association study is presented.

Chapter 1

This chapter gives a general introduction to the topics studied in this thesis. It introduces constitutional tall stature and the different aspect of high-dose sex steroid treatment. It also presents the current insight in genetic determinants of growth. Finally, the aims of our studies are presented and an outline of this thesis is given.

Part 1 - High-dose sex steroid treatment of constitutional tall stature

Chapter 2

High-dose estrogen treatment to reduce adult height of tall girls may interfere with fertility. We evaluated fertility and ovarian function in tall women who did or did not receive such treatment. Fertility outcome of 239 estrogen treated tall women is presented in chapter 2 and compared with 174 tall women who had not received high-dose estrogen treatment in adolescence. As girls these women had visited the Erasmus Medical Center for their tall stature. Our results confirm earlier findings that treated women experience more difficulties getting pregnant and more often receive infertility treatments. Unexpected in light of earlier findings was our observation of an increased risk of suffering from involuntary childlessness in treated women. At the time of study one third of treated women had not been able to achieve a live birth while attempting to conceive for a median of 40 months. When studying serum hormone levels and antral follicle counts of 119 estrogen treated tall women and 55 untreated tall women we found a considerable number of treated women to have increased serum FSH levels along with decreased AMH levels and low antral follicle counts. Therefore, we conclude that treated women seem to suffer from accelerated follicle loss with concomitant follicle pool depletion,

which may be the basis of the observed subfertility. However, the mechanism behind this accelerated follicle loss by high-dose estrogen treatment remains unknown and requires future research.

Chapter 3

Recent studies have shown that tall women treated with high-dose estrogen in adolescence are at increased risk of infertility in later life and that their fecundity is reduced. No such association has been reported on low dose estrogen treatment used for contraception. This raises the questions whether there is a dose-response relationship. To study the effect of estrogen dose we compared in chapter 3 the fertility outcome of 30 untreated tall women to 52 tall women treated with 100 µg ethinyl estradiol (EE) and 43 tall women treated 200 µg EE in adolescence. These women had as girls visited the University Medical Center Groningen for their tall stature. This study once again confirms that high-dose estrogen treated women have an increased time to conception and experience more fertility problems compared to untreated women. New is that this study shows that the observed reduced fertility is dependent on estrogen dose. We found a significant trend of increasing time to conception with increase in estrogen dose. As a consequence the number of women seeking medical attention for fertility problems is also higher with increasing estrogen dose; i.e. women treated with 200 µg EE experienced more fertility problems than women treated with 100 µg EE, who in turn experienced more fertility problems than untreated women. Finally, women treated with 200 µg EE had a significantly reduced chance of achieving at least one live birth. In this study we did not observe a reduced fecundity in women treated with 100 µg EE, possibly due to a relatively small sample size. Based on these results estrogen likely plays a dose-dependent role in the previously found accelerated ovarian ageing with concomitant follicle pool depletion.

Chapter 4

High-dose sex steroid treatment to reduce adult height of tall boys and girls has been shown to interfere with fertility in women. No such data are available in men. We evaluated fertility and testicular function in tall men who did or did not receive high-dose androgen treatment in adolescence. In chapter 4 we present fatherhood of 60 androgen treated tall men compared with 56 tall men who had not received treatment. As boys these men had visited the Erasmus Medical Center for their tall stature. Our results show that there is no long-term impact on fatherhood in treated men. Time to first pregnancy

did not differ between treated and untreated men and the number of men that had experienced infertility was similar in both groups. Most importantly, over 85% of treated and untreated men who had attempted to achieve fatherhood had achieved at least one live birth. These results contrast with recent findings of increased risk for infertility in later life in high-dose estrogen treated tall girls. Testicular ultrasound examination showed no major abnormalities in treated men and semen parameters were comparable in androgen treated and untreated tall men. This suggests that there is no long-term complications of high-dose androgen treatment on spermatogenesis. However, we found testosterone production to be reduced in androgen treated men. Testosterone levels in the androgen treated men, albeit still within the normal range, were significantly lower compared with untreated men and approach clinically relevant cut-off values. Since testosterone levels will continue to decline as these men age, we believe our results may be of clinical relevance to androgen treated tall men as they grow older.

Part 2 - Genetic determinants of constitutional tall stature

Chapter 5

Human growth and adult height are considered highly heritable polygenic traits that reflect the input of multiple genes interacting with environmental factors such as nutrition. The growth hormone (GH)/insulin-like growth factor-1(IGF-1) axis is the key regulator of somatic growth in humans and its genes are plausible candidates to study the genetics of height variation. In chapter 5 we present a case-control study of 166 tall cases with height >2 SDS and 206 controls with normally distributed height <2 SDS. We assessed the role of polymorphic variation in the GH/IGF-1 axis in the regulation of extreme tall stature. Seven common polymorphisms in the GH1, GH receptor (GHR), IGF1 and IGFBP3 genes were analyzed. The IGFBP-3 -202 C-allele occurred more frequently in cases than in controls. Remarkably, the C-allele was also associated with increased height within the cases. Carriers of one or two copies of the C-allele were on average almost 0.5 SDS taller. Polymorphic variation explained 5.8% of circulating IGFBP-3 levels and were highest in carriers of the AA genotype. For the IGF-1 CA-repeat we observed a higher frequency of homozygous 192-bp carriers among tall males compared to control males. No association with serum IGF-1 levels was observed. Finally we observed a significant association between tall stature and a haplotype at the GH1 gene. Therefore, we conclude that polymorphic variation in the GH1, IGF1 and IGFBP3 genes is associated with extreme tall stature.

Chapter 6

A high mobility group-A2 (HMGA2) gene SNP has been robustly associated with height in the general population. Few have attempted to study this gene in extreme tall stature. In chapter 6 we studied common genetic variation in the HMGA2 gene in a case-control study of 116 tall cases with height >2 SDS and 103 controls with normally distributed height <2 SDS. We found that the HMGA2 C-allele was significantly more frequent in cases than in controls under an additive model. Using a logistic regression model we calculated that carrying the C-allele significantly increased the odds of being a case. In addition, we replicate what has been found previously as in our normal height controls the ones carrying the C-allele were significantly taller than those carrying only T-alleles. Our replication of the association of the HMGA2 C-allele with increased odds of being tall suggests that this common SNP, that is robustly associated with height variation in the general population, may also play an important role in the regulation of extreme tall stature.

Chapter 7

Genome-wide association studies have identified over 180 loci robustly associated with height variation. These studies explain however only 10% of the phenotypic variation. Sampling at the extremes of a quantitative trait while using common controls has been shown to maximize statistical power. In chapter 7 we studied genome-wide polymorphic variation in 820 Dutch extremely tall individuals compared with 1029 individuals of average height. We discovered a new genome-wide significant SNP in the gene for RBM39, which is involved in sex steroid mediated transcription. In addition, we found several SNPs in the gene for ADAMTSL3 to be associated with the extreme tall phenotype. ADAMTSL3 has been robustly associated with height variation and is implicated to modulate fibrillin-1 function. Finally, a new locus was identified at 6q24.1 which requires further research.

Chapter 8

This chapter discusses our results in the context of the current literature. We emphasize the strengths and weaknesses of our studies, the clinical implications of our results and our recommendations for future research.

Chapter 10

Samenvatting

Samenvatting

Dit proefschrift beschrijft de lange termijn effecten van hoge doses geslachtshormoonbehandeling ter groeiremming tijdens de puberteit in een grote groep van constitutioneel lange mannen en vrouwen. Tevens bespreekt het de genetische determinanten van lange gestalte. De hoofdstukken in het eerste deel van dit proefschrift zijn gewijd aan de vruchtbaarheid op de lange termijn van lange jongens en meisjes behandeld met hoge doses geslachtshormonen tijdens de puberteit. De hoofdstukken in het tweede deel bestuderen de associatie tussen lange gestalte en genetische variaties door middel van kandidaatgen analyse en genoombrede associatiestudie.

Hoofdstuk 1

Dit hoofdstuk geeft een algemene inleiding over constitutioneel lange gestalte en de verschillende aspecten van de behandeling met hoge doses geslachtshormonen. Daarnaast worden de huidige inzichten in de genetische determinanten van groei geïntroduceerd. We beschrijven de doelstellingen van de uitgevoerde studies en de opzet van het proefschrift.

Deel 1 - Lange termijn effecten van hoge doses geslachtshormoonbehandeling.

Hoofdstuk 2

De behandeling met hoge doses oestrogeen ter groeiremming van lange meisjes tijdens de puberteit veroorzaakt mogelijk verminderde vruchtbaarheid op latere leeftijd. Om die reden hebben wij in hoofdstuk 2 de vruchtbaarheid en ovariële functie bestudeerd van 239 lange vrouwen die in het verleden deze behandeling hadden ondergaan en de uitkomsten vergeleken met 174 lange vrouwen die in het verleden geen behandeling hadden ondergaan. Alle onderzochte vrouwen hadden als meisje het Erasmus Medisch Centrum bezocht voor evaluatie van lange gestalte. Onze resultaten bevestigen dat behandelde vrouwen meer vruchtbaarheidsstoornissen ervaren en vaker vruchtbaarheidsbevorderende behandelingen ontvangen. Uit ons onderzoek blijkt ook dat behandelde vrouwen een verhoogd risico op ongewenste kinderloosheid hebben. Ten tijde van de studie was eenderde van de behandelde vrouwen met actieve kinderwens er nog niet in geslaagd minimaal één levendgeborene te krijgen met een mediane duur van de poging van 40 maanden. Bij het onderzoek naar de ovariële functie hebben wij naar serum hormoon waardes en het aantal antrale follikels op echo gekeken van 119 behandelde lange

vrouwen en 55 onbehandelde lange vrouwen. Wij zagen daarbij dat een aanzienlijk deel van de behandelde vrouwen tekenen van beginnend ovarieel falen hadden, deze vrouwen hadden verhoogde serum FSH waardes samen met verlaagde serum AMH waardes en lage aantallen antrale follikels. Wij concluderen dat behandelde vrouwen een substantieel risico lopen op versnelde uitputting van de follikelvoorraad wat een mogelijke verklaring zou kunnen zijn voor de verminderde vruchtbaarheid van deze vrouwen. Het werkingsmechanisme achter uitputting van de follikelvoorraad bij vrouwen behandeld met hoge doses oestrogeen is echter onbekend en zou mogelijk uit verder onderzoek kunnen blijken.

Hoofdstuk 3

Uit recent onderzoek is gebleken dat lange vrouwen behandeld met hoge doses oestrogeen tijdens de puberteit een verhoogd risico hebben op verminderde vruchtbaarheid en ongewenste kinderloosheid op latere leeftijd. Een dergelijke associatie is niet gevonden bij de behandeling met lage doses oestrogeen ter anticonceptie. Dit wekt de indruk dat er mogelijk sprake zou kunnen zijn van een dosis-respons relatie. In hoofdstuk 3 bestuderen wij daarom het effect van oestrogeen dosis op de vruchtbaarheid door de uitkomsten ter vergelijken van 30 onbehandelde lange vrouwen met 52 lange vrouwen behandeld met 100 µg ethinyl estradiol (EE) en 43 lange vrouwen behandeld met 200 µg EE tijdens de puberteit. Alle onderzochte vrouwen hadden als meisje het Universitair Medisch Centrum Groningen bezocht voor evaluatie van lange gestalte. Opnieuw bevestigen wij dat lange vrouwen behandeld met hoge doses oestrogeen in de puberteit op latere leeftijd een langere duur tot eerste conceptie hebben en vaker vruchtbaarheidsstoornissen ervaren dan onbehandelde vrouwen. Deze studie laat voor het eerst zien dat de mate van verminderde vruchtbaarheid afhankelijk is van de oestrogeen dosis. Wij laten zien dat er met toename van de oestrogeendosis een toename van de tijd tot eerste conceptie is. Als gevolg hiervan zien wij ook dat het aantal vrouwen dat een dokter bezoekt voor vruchtbaarheidsstoornissen toeneemt met toename van de oestrogeendosis. Oftewel, vrouwen behandeld met 200 µg EE ervaren meer vruchtbaarheidsstoornissen dan vrouwen behandeld met 100 µg EE, die weer meer vruchtbaarheidsstoornissen ervaren dan onbehandelde vrouwen. Tot slot hadden vrouwen behandeld met 200 µg EE een verhoogde kans op ongewenste kinderloosheid. Wij hebben dit in deze studie niet kunnen aantonen voor vrouwen behandeld 100 µg EE, mogelijk door een relatief kleine studiegrootte. Wij concluderen dat oestrogeen waarschijnlijk een dosisafhankelijke rol speelt in de eerder gevonden versnelde uitputting van de follikelvoorraad bij behandelde

vrouwen.

Hoofdstuk 4

De behandeling met hoge doses geslachtshormonen ter groeiremming van lange jongens en meisjes tijdens de puberteit is bij vrouwen geassocieerd met verminderde vruchtbaarheid op latere leeftijd. Bij mannen is dit tot op heden niet onderzocht. In hoofdstuk 4 hebben wij daarom de vruchtbaarheid en testiculaire functie bestudeerd van 60 lange mannen die een dergelijke behandeling hadden ondergaan en de uitkomsten vergeleken met 56 lange mannen die geen behandeling hadden ondergaan. Alle onderzochte mannen hadden als jongen het Erasmus Medisch Centrum bezocht voor evaluatie van lange gestalte. Wij laten zien dat er geen nadelige effecten zijn van de behandeling met hoge doses androgeen tijdens de puberteit op de kans op vaderschap op latere leeftijd. Zowel de duur tot eerste conceptie als het risico op vruchtbaarheidsstoornissen was gelijk tussen behandelde en onbehandelde mannen. Bovendien was meer dan 85% van zowel behandelde als onbehandelde mannen met actieve kinderwens erin geslaagd om vader te worden. Deze resultaten staan in duidelijk contrast met de recente bevindingen van verminderde vruchtbaarheid bij vrouwen behandeld met hoge doses oestrogeen tijdens de puberteit. Echografisch onderzoek van de testes liet geen grote afwijkingen zien bij behandelde mannen en semen parameters verschilden eveneens niet van onbehandelde mannen. Wij concluderen dat er op de lange termijn geen nadelige effecten van hoge doses androgeenbehandeling zijn op de spermatogenesis. Testosteronproductie, daarentegen, was significant verlaagd in behandelde mannen. Testosteronspiegels bij behandelde mannen waren nog wel binnen de normaalwaarden maar duidelijk lager dan bij onbehandelde mannen en benaderen klinisch relevante grenswaarden. Aangezien testosteronspiegels zullen blijven dalen met het ouder worden verwachten wij dat onze resultaten in de toekomst van klinisch belang zullen zijn voor de behandelde lange mannen.

Deel 2 - Genetische determinanten van constitutioneel lange gestalte

Hoofdstuk 5

Lengtegroei is één van de meest erfelijke eigenschappen van de mens en heeft een polygenetische opbouw waarbij de uitkomst bepaald wordt door de inbreng van multipele genen en hun interactie met omgevingsfactoren zoals voeding. De groeihormoon (GH)/ insulineachtige groei factor-1 (IGF-1) as speelt een belangrijke rol in onze lengtegroei en de genen van deze as zijn daarom goede kandidaatgenen voor onderzoek naar de genetische determinanten van extreem lange gestalte. In hoofdstuk 5 presenteren wij

een patiënt-controle studie van 166 lange individuen met een lengte van >2 SD en 206 controles met een normaal verdeelde lengte van <2 SD. Wij hebben de rol van genetische variatie in de GH/IGF-1 as in het ontwikkelen van extreem lange gestalte bestudeerd. Zeven bekende polymorphismen in de GH1, GH receptor (GHR), IGF1 en IGFBP3 genen werden onderzocht. Wij laten zien dat het IGFBP-3 -202 C-allel significant vaker voorkwam bij de individuen met lange gestalte dan bij de controles. Tevens was dit C-allel geassocieerd met grotere lengte binnen de groep van lange individuen, dragers van één of twee kopieën van het C-allel waren gemiddeld bijna 0.5 SDS langer dan dragers van beide A-allelen. De genetische variaties verklaarden 5.8% van de IGFBP-3 serumspiegels en deze spiegels waren het hoogst in dragers van het AA genotype. Wat betreft de CA-repeat in het IGF-1 gen zagen wij dat lange mannen vaker homozygoot 192-base paren dragers waren dan controle mannen. Er was echter geen associatie met IGF-1 serumspiegels. Tot slot hebben een associatie gevonden tussen lange gestalte en het haplotype van het GH-1 gen. Wij concluderen dan genetische variaties in de GH1, IGF1 en IGFBP3 genen van invloed zijn op het ontwikkelen van extreem lange gestalte.

Hoofdstuk 6

Polymorphismen in het high mobility group-A2 (HMGA2) gen zijn sterk geassocieerd met variatie in lengte in de algemene populatie. Tot op heden is weinig bekend over het effect van dit gen op extreem lange gestalte. In hoofdstuk 6 beschrijven wij een patiënt-controle studie waarin wij een bekende genetische variatie in het HMGA2 gen bestuderen in 116 lange individuen met een lengte van >2 SD en 103 controles met een normaal verdeelde lengte van <2 SD. Wij laten zien dat het HMGA2 C-allel significant vaker voorkomt bij lange individuen dan bij de controles. Met behulp van een logistisch regressie model hebben wij berekend dat het dragen van het C-allel de kans op extreem lange gestalte duidelijk verhoogd. Tevens repliceren wij de eerder gevonden associatie in de algemene populatie aangezien ook binnen de controles degene met één of twee C-allelen significant langer waren dan de dragers van beide T-allelen. Wij concluderen dat genetische variatie in het HMGA2 gen niet alleen van belang is voor de lengtevariatie in de algemene populatie maar ook een belangrijke rol speelt in de ontwikkeling van extreem lange gestalte.

Hoofdstuk 7

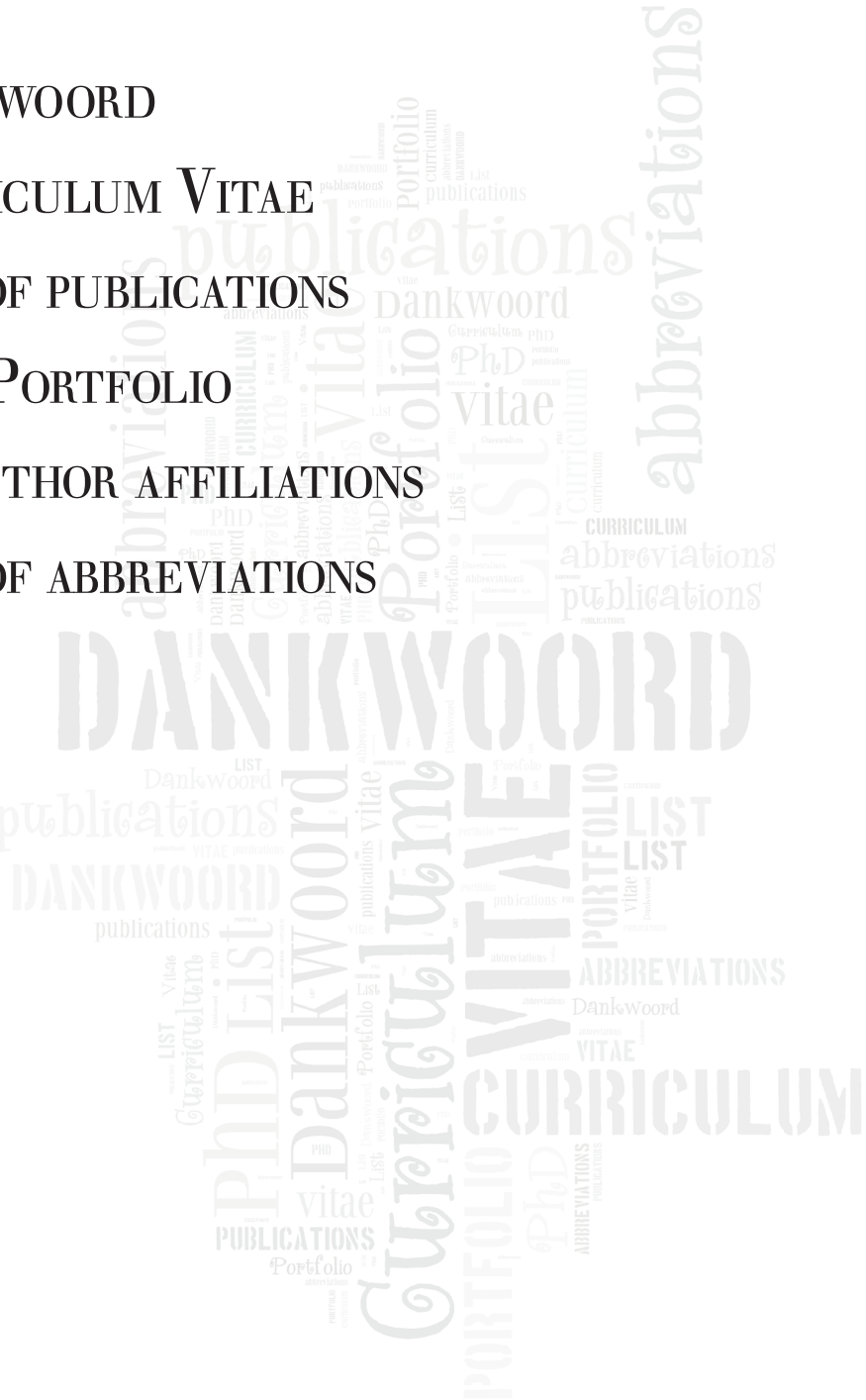
Meerdere genoombrede associatiestudies hebben meer dan 180 loci geïdentificeerd die sterk geassocieerd zijn met lengtevariatie. Deze studies bij elkaar verklaren echter

slechts 10% van de totale variatie van dit phenotype. Om de missende erfelijkheid van lengtegroei te kunnen verklaren is het nuttig om de uitersten van de lengtedistributie te bestuderen en deze te vergelijken met controles uit de normale distributie om op die manier maximale statistische power te verkrijgen. In hoofdstuk 7 beschrijven wij een genoombrede associatiestudie waarin wij genetische variatie in 820 extreem lange individuen hebben vergeleken met 1029 controles van gemiddelde lengte. Wij hebben in deze studie een nieuw genoombreed significant polymorphisme ontdekt in het RBM39 gen, dit gen is betrokken bij de geslachtshormoongebonden transcriptie van DNA. Tevens hebben wij genetische variaties in het ADAMTSL3 gen gevonden die geassocieerd zijn met extreem lange gestalte. ADAMTSL3 is in het verleden meermaals met lengtegroei geassocieerd en is mogelijk van belang in de modulatie van fibrilline-1 functie. Tot slot hebben wij een nieuw locus ontdekt op 6q24.1 waarvoor aanvullend onderzoek noodzakelijk is. Wij concluderen dat deze genoombrede associatiestudie nieuwe en bekende genetische variaties toont die van belang zijn in de ontwikkeling van extreem lange gestalte.

Hoofdstuk 8

Dit hoofdstuk bespreekt onze belangrijkste bevindingen in de context van de huidige literatuur. Wij benadrukken de sterke en zwakke kanten van onze studies, de klinische implicaties van onze bevindingen en onze aanbevelingen voor toekomstig onderzoek.

Chapter 11



Dankwoord

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Curriculum Vitae

Allaird Emile Joseph Hendriks was born on March 10th, 1979 in Rotterdam, the Netherlands. He graduated from secondary school at the Gymnasium Erasmianum in Rotterdam in 1997. In that year he started his medical training at the University of Utrecht School of Medicine. During his training he was an intern at the department of Pediatric Dermatology of the Great Ormond Street Hospital in London, Great Britain and at the Pediatric Intensive Care Unit of the University of Saõ Paulo School of Medicine in Botucatu, Brazil. From October 2000 till October 2001 he was the president of the University of Utrecht Medical Students Association. From October 2002 till March 2003 he worked on a research project on IGFBP-3 promoter polymorphisms in children born small for gestational age at the department of Pediatric Endocrinology at Ste-Justine Hospital of the University of Montréal in Canada under supervision of Dr. C.L. Deal. For this research he received the 2005 Student Research Award of the Wilhelmina Children's Hospital in Utrecht. In 2005 he obtained his medical degree. In September 2005 he started his thesis at the department of Pediatric Endocrinology at the Sophia Children's Hospital of the Erasmus Medical Center in Rotterdam under supervision of Dr. A.M. Boot, Prof. J.S.E. Laven and Prof. S.L.S. Drop. He was awarded a Fulbright scholarship to work from February 2006 till August 2007 as part of this thesis at the department of Pediatric Endocrinology of the Emory University School of Medicine in Atlanta (GA), USA, under supervision of Prof. J.S. Parks. In 2010 he graduated with a Master of Science in Genetic Epidemiology from the Netherlands Institute for Health Sciences in Rotterdam. As of March 2010 he worked as a resident (ANIOS) in Pediatrics in the Neonatology Intensive Care Unit at the Sophia Children's Hospital of the Erasmus Medical Center in Rotterdam. He started his training in Pediatrics in January 2011 at the Medical Center Alkmaar under supervision of Dr. W.W.M. Hack. He is currently continuing his specialist training at the department of Pediatrics of the VU Medical Center in Amsterdam under supervision of Prof. R.J.B.J. Gemke. Emile lives with his fiancée Jennifer Gail Gaultney in Amsterdam.

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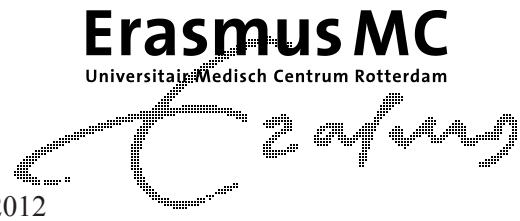
National

1. **AEJ Hendriks**, JSE Laven, WPA Boellaard, FH de Jong, AM Boot, SLS Drop: Vruchtbaarheid na behandeling met hoge doses geslachtshormonen ter groeiremming. *Ned Tijdschr Geneeskd 2012 April;156(14):582-589*
2. **AEJ Hendriks**, T.W. de Vries: Langetermijneffecten van medicatie op de kinderleeftijd. *Praktische Pediatrie December 2011;5(4):248-250.*
3. WJ de Waal, **AEJ Hendriks**, SLS Drop: Evaluatie en behandeling van lange gestalte. *Praktische Pediatrie September 2009;3(3):201-205.*

PhD Portfolio



Erasmus MC Department of Pediatrics

Division of Endocrinology

Research School: Molecular Medicine

PhD period: September 2005 - December 2012

Master of Science in Genetic Epidemiology, NIHES 2008-2010

Promotors: Prof.dr. J.S.E. Laven and Prof.dr. S.L.S. Drop

Copromotor: Dr. A.M. Boot

General academic courses

The Transforming Community Project, Emory University, Atlanta, USA	2007
Race at Emory: Recovering Our Past, Building the Future	

Research skills

Master of Science in Genetic Epidemiology, NIHES	2008-2010
Advanced courses:	
Bayesian Statistics	
Pharmaco-Epidemiology and Drug Safety	
Advances in Population-Based Studies of Complex Genetic Disorders	
Genetic Linkage Analysis: Model-free Analysis	
Statistics for Experimental Biology, Public Health, Emory University	2007
Weekly research meetings, Pediatric Endocrinology, Emory University	2006-2007
Weekly research meetings, Pediatric Endocrinology, Erasmus MC	2005-2010

International conferences

LWPES/ESPE 8th Joint Meeting, New York, USA (oral presentation)	2009
91st Annual Meeting Endocrine Society, Washington, USA (poster presentation)	2009
47th ESPE Meeting, Istanbul, Turkey (poster presentation)	2008
90th Annual Meeting Endocrine Society, San Francisco, USA (poster presentation)	2008
46th ESPE Meeting, Helsinki, Finland (poster presentation)	2007
89th Annual Meeting Endocrine Society, Toronto, Canada (poster presentation)	2007
Annual Meeting SPES, Birmingham, USA (oral presentation)	2006
88th Annual Meeting Endocrine Society, Boston, USA	2006
LWPES/ESPE 7th Joint Meeting, Lyon, France	2005

Symposia and seminars

Section Meeting in Endocrinology, Dutch Pediatrics Society (oral presentation)	2011
Pediatric Grand Round, Sophia Children's Hospital (oral presentation)	2010
Research Meeting Department of Pediatrics, UMCG (oral presentation)	2009
Young Researchers Meeting, Dutch Pediatrics Society (oral presentation)	2009
Annual Research Meeting, Sophia Children's Hospital (oral presentation)	2007, 2009
Gynecology Research Meeting, Erasmus MC (oral presentation)	2005, 2009
Annual Members Meeting "Klub voor Lange Mensen" (oral presentation)	2005

Grants

Scholten-Cordes Foundation study grant	2009
Erasmus MC Trustfund travel grants	2008-2009
Fulbright Fellowship by the Netherland-America Foundation	2005-2006
VSB fund research grant	2005-2006
Foundation "De Drie Lichten" research grant	2005

Other

Peer review of manuscript for Clinical Endocrinology	2006

Co-author affiliations

E. Benyi MD
Pediatric Endocrinology Unit and Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

W.P.A. Boellaard MD
Division of Andrology, Department of Urology, Erasmus MC, Rotterdam, the Netherlands

A.M. Boot MD PhD
Department of Pediatric Endocrinology, UMCG, Groningen, the Netherlands.

M.R. Brown PhD
Pediatric Endocrinology, Emory University School of Medicine, Atlanta (GA), USA

N.J. van Casteren MD PhD
Division of Andrology, Department of Urology, Erasmus MC, Rotterdam, the Netherlands

S.L.S. Drop MD PhD
Department of Pediatrics, Division of Endocrinology, Erasmus MC-Sophia, Rotterdam, the Netherlands

C.M. van Duijn PhD
Department of Epidemiology, Erasmus MC, Rotterdam, the Netherlands

B.C.J.M. Fauser MD PhD
Department of Reproductive Medicine and Gynecology, University Medical Center, Utrecht, the Netherlands

A.M. van Ginneken PhD
Department of Information Technology, Erasmus MC, Rotterdam, The Netherlands

A. Hofman MD PhD
Department of Epidemiology, Erasmus MC, Rotterdam, the Netherlands

F.H. de Jong PhD
Department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands

M. Kayser PhD
Department of Forensic Molecular Biology, Erasmus MC, Rotterdam, the Netherlands

J.S.E. Laven MD PhD
Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Erasmus MC, Rotterdam, the Netherlands

S. Lie Fong MD PhD
Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Erasmus MC, Rotterdam, the Netherlands

F. Liu PhD
Department of Forensic Molecular Biology, Erasmus MC, Rotterdam, the Netherlands

B.A. Oostra MD PhD
Department of Clinical Genetics, Erasmus MC, Rotterdam, the Netherlands

J.S. Parks MD PhD
Pediatric Endocrinology, Emory University School of Medicine, Atlanta (GA), USA

M.A.J. de Ridder PhD
Department of Biostatistics, Erasmus MC, Rotterdam, the Netherlands

F. Rivadeneira MD PhD
Department of Internal Medicine, Erasmus Medical Center, Rotterdam, the Netherlands

J.C. Romijn PhD
Division of Andrology, Department of Urology, Erasmus MC, Rotterdam, the Netherlands

L. Sävendahl MD PhD
Pediatric Endocrinology Unit and Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

A.G. Uitterlinden MD PhD
Department of Internal Medicine, Erasmus Medical Center, Rotterdam, the Netherlands

O. Valkenburg MD
Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Erasmus MC, Rotterdam, the Netherlands

J.A. Visser PhD
Department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands

List of abbreviations

AFC:	Antral Follicle Count
AMH:	Anti-Müllerian Hormone
BMD:	Bone Mineral Density
BMI:	Body Mass Index
CI:	Confidence Interval
CNV:	Copy Number Variation
CTS:	Constitutional Tall Stature
CV:	Coefficient of Variation
DES:	Diethylstilbestrol
DNA:	Deoxyribonucleic Acid
E2:	Estradiol
EE:	Ethinyl Estradiol
EFP:	Early Follicular Phase
FSH:	Follicle Stimulating Hormone
GH:	Growth Hormone
GHR:	Growth Hormone Receptor
GWAS:	Genome Wide Association Analysis
HH:	Hypogonadotropic Hypogonadism
HMGA2:	High Mobility Group-A2
HR:	Hazard Ratio
WE:	Hardy-Weinberg Equilibrium
IBS:	Identity By State
IGF-1:	Insulin-like Growth Factor-1
IGFBP-3:	Insulin-like Growth Factor Binding Protein-3
IOF:	Imminent Ovarian Failure
IUD:	Intrauterine Device
KM:	Kaplan-Meier
LH:	Luteinizing Hormone
MAF:	Minor Allele Frequency
OCP:	Oral Contraceptive Pill
OR:	Odds Ratio
PCOS:	Polycystic Ovary Syndrome
PCR:	Polymerase Chain Reaction
POF:	Premature Ovarian Failure
PTH:	Parathyroid Hormone
PTHrP:	Parathyroid Hormone release Protein
QC:	Quality Control
RS:	Rotterdam Study
SD(S):	Standard Deviation (Score)
SHBG:	Sex Hormone Binding Globulin
SNP:	Single Nucleotide Polymorphism
T:	Testosterone
TTP:	Time To First Pregnancy
WHO:	World Health Organization