Regulation and modulation of growth : insights from human and animal studies

Gool, S.A.van

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Sandy A. van Gool

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A man's reach should extend his grasp,
or what's a heaven for?

Robert Browning, 1855

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Chapter 1

General introduction

General introduction

The blueprint for human postnatal growth is defined by the genetic background of an individual, but is fine-tuned by hormonal, environmental, psychosocial and nutritional factors. As the result of a process called endochondral ossification taking place in the epiphyseal growth plates, long bones elongate and the size of the skeleton increases. Successive phases of growth can be distinguished, each characterized by specific hormonal regulation [1). In the intrauterine phase and neonatal period, up to the age of approximately 3 year, a high but rapidly declining growth velocity is observed. It is assumed that prenatal growth is mainly regulated by insulin-like growth factors (IGFs), insulin and nutrition. From the second semester onward growth hormone secretion plays an important role, and in the first 2-3 years the correlation between the child's length and parental height gradually increases, suggesting a growing influence of genetic factors.

From the age of 3 years to puberty, growth continues at a lower and gradually diminishing rate, predominantly under the influence of thyroxin and growth hormone (GH). During pubertal development, sex steroid secretion and concomitant upregulation of GH-IGF-I signaling causes an increased growth rate, also known as the pubertal growth spurt. In both genders, estrogen-induced maturation and closure of the epiphyseal growth plates at the end of pubertal development is associated with a rapidly declining growth rate and the termination of growth, inevitably determining adult height (2). In contrast, it has also been suggested that growth deceleration precedes epiphyseal fusion (3).

Although many scientific research projects have been dedicated to unraveling the regulatory pathways underlying growth, there are still many remaining questions. Also, the pathophysiological mechanisms underlying the majority of growth disorders are still to be elucidated and more effective modes of treatment need to be developed.

As this thesis touches upon several aspects of growth regulation and modulation, this first introductory chapter is subdivided in three parts. Part A reviews the background of idiopathic short stature (ISS) and growth hormone (GH) treatment. Part B recapitulates evidence from animal studies regarding alternatives for GH treatment. Part C addresses the need for development of an appropriate model system for studying growth regulation in humans.

A. Idiopathic short stature (ISS)

Definition and subcategories

Short stature is one of the most common reasons for referral of a child and his parents to a pediatric endocrinologist for diagnostic evaluation and advice. A large variety of congenital or acquired conditions that can be classified as primary, secondary or idiopathic growth disorders can cause shortness (4). A primary growth disorder, such as achondroplasia, is due to an intrinsic defect in the growth plates of long bones. Secondary growth disorders result from conditions that negatively affect growth plate physiology (e.g. endocrine disorders, chronic illness, malnutrition). In industrialized countries, celiac disease, Turner syndrome and growth hormone deficiency (GHD) are the main causes for short stature. In the majority of cases of short stature, however, an elaborate assessment of the child's medical history, physical examination, laboratory tests, and radiological examinations does not reveal any clue for an underlying pathological mechanism, leaving the patient with the diagnosis ISS, short stature of unknown origin (5).

ISS is a condition defined by an individual's height more than 2 standard deviation (SD) below the corresponding mean height for a given age, sex, and population group (thus a height standard deviation score (SDS) of <-2) without evidence of systemic, endocrine, nutritional, or chromosomal abnormalities (4;6-9). Children with ISS have a normal birth size and body proportions, are not growth hormone deficient and have no psychiatric disorder associated with poor growth. By definition, ISS is a diagnosis of exclusion, as it does not rely on the identification of certain features characteristic for ISS, but rather on excluding all currently known other causes of short stature. Since approximately 80% of the short children referred to a pediatric endocrinologist meets the criteria for ISS (10), the clinical relevance of this entity is evident.

The label ISS presumably unifies a variety of different causes of short stature that are unknown at present. Subdivision of this group in broad categories may therefore seem arbitrary, but is considered useful from a diagnostic and prognostic point of view (11). The most important distinction is between familial and non-familial short stature (FSS and NFSS, respectively), based on how the child's growth pattern relates to his target height. The conditional target height (cTH) for short children, that takes into account the effect of assortative mating and parent-offspring correlations, can be calculated according to the formula of Hermanussen and Cole (12): 0.72 × average of parental height SDS. The lower limit of the target height range is defined as cTH minus 1.6 SD.

A child with FSS grows within the target height range of the family, but is short in comparison with the appropriate reference population. In contrast, NFSS is characterized by short stature for the normal population as well as for the genetically defined familial growth potential. The diagnostic work-up in children with FSS may be limited, since the chance of finding a pathological mechanism underlying short stature is small in those cases, given that no pathological causes for parental short stature are present. However, the probability of a dominant genetic disorder (e.g. hypochondroplasia) is higher in case of one short parent (height SDS <-2.0 SDS) and a more elaborate diagnostic evaluation is then warranted.
A second subdivision can be made according to the tempo of maturation of the child. Girls reaching breast development stage 2 later than the age of 13 years and boys with a testicular volume smaller than 4 ml at the age of 14 years are considered to have delayed puberty in the Netherlands and in many other industrialized countries (11). Information on pubertal onset is usually not available at the moment of diagnostic evaluation, as most short children referred to a pediatric endocrinologist are prepubertal. Therefore, it is impossible to ascertain pubertal delay before the age of 13 years in girls and 14 years in boys. The determination of skeletal age may render additional information, as pubertal delay is more likely in the presence of delayed bone maturation (bone age minus chronological age < 0 years). However, in some children with delayed skeletal age puberty is not delayed, and vice versa.
Whereas the demarcation line between FSS and NFSS is sharply, though arbitrarily defined before puberty, the presence of normal and delayed puberty can only be definitively established in retrospect after the onset of puberty. Still, it is valuable to consider the possibility of delayed maturation from a prognostic perspective. In a child with NFSS and a positive family history of late puberty, the likelihood of delayed pubertal maturation is high and the pediatrician may choose to limit the number of diagnostic tests and follow an expectative line of treatment and advice. In case of normal pubertal timing and development in the parents, a pathological process underlying the clinical presentation of the child is suspected and additional investigations should aim at finding this mechanism.

Impact on psychosocial functioning

Children with short stature may experience psychosocial stress attributed to shortness such as social immaturity, infantilization, low self-esteem, being bullied or not accepted by their peers, and an overprotective parenting style that may hamper development of autonomy (13;14). In most clinic-based studies parents indeed report increased psychosocial stress in their short children (15-19), whereas peers and teachers do not mention a relevant decrease in social competence (15;18). Unfortunately, the experience of the children themselves is usually not

investigated (14). The impression from population-based studies is that children and adolescents with ISS may experience varying degrees of stature-related stress, but generally are functioning within the normal range (20).
Studies on psychosocial well-being of short individuals in adulthood are inconclusive, some reporting a lower chance of getting married, a higher percentage of unemployment and more self-reported problems in social functioning (21-24), whereas others find no difference between short and normal-sized adults (25;26). In the absence of conclusive evidence that ISS has a persistent negative impact on psychosocial functioning, it is important that the potential psychological burden for each patient referred for ISS is individually assessed (13).

Indications for growth-promoting treatment

Once the presence of ISS has been established, various treatment options may be considered (13;27). The decision whether or not treatment is indicated in an individual patient is based on a careful evaluation of auxological, psychosocial, ethical and financial arguments (13;27). Biochemical parameters have not been recognized to justify initiation of pharmacological interventions. Ideally, treatment results in alleviation of psychosocial stress, attainment of a normal adult height and preferably also a normal height during childhood. It should be stressed that a taller stature per se will not necessarily result in an improved quality of life or psychosocial functioning (14). For a short child that is not concerned about or hampered by his height in any way, treatment is usually not recommended. On the other hand, when a child evidently suffers from stress attributed to shortness, treatment can be considered. The patient and parents should be provided with a realistic perspective of the results that can be expected regarding height gain, the variability of clinical outcome, the risks, benefits, costs and possible treatment alternatives.

Growth hormone treatment

Many clinical trials with GH treatment for ISS have been performed since recombinant GH became available in 1985. GH on average increases AH of the majority of ISS children with 3-7 cm after 4-7 years of treatment, compared with historical, placebo-treated or non-treated controls or with predicted adult height before the onset of treatment (27). The effectiveness of GH treatment has been attributed to the notion that most children with ISS have a certain degree of GH insensitivity that cannot be counteracted by endogenous GH secretion, but can be overcome by GH replacement.

The applied dosage is an important determinant for the response to treatment. It has been demonstrated that higher GH dosages result in a more pronounced acceleration of growth velocity and a taller adult height than lower dosages (28;29). The currently known most effective treatment regimen is 1.4 mg/m^2/day (equivalent to 50 µg/kg/day) subcutaneously. This is approximately twice as high as the substitution dose, as assessed by studies on spontaneous secretion (30), but only slightly higher than the regular GH 'replacement' dosage prescribed in GHD in the United States. The long-term outcome of such a regimen has been reported by several authors (reviewed in (27)). The data on the effects of even higher dosages are contradictory. In one study, a dosage of 2 mg/m^2/day (equivalent to 75 µg/kg/day) appeared to accelerate bone maturation and advance pubertal onset (31;32), whereas no such effects were observed in other studies that applied up to 2.8 mg/m^2/day (equivalent to 100 µg/kg/day) (33;34), nor in a recent Swedish study that used a dosage of 1.9 mg/m^2/day (equivalent to 67 µg/kg/day) (35). In the latter three studies the average age at onset of therapy was close to 11 years, while average age in the first study was 8.7 years. Premature advancement of maturation may limit the effectiveness of GH treatment, as the timeframe reserved for growing is limited once puberty has commenced.

Even if corrected for the dosage effect, there is still a large interindividual variation in growth response. It is therefore estimated that there are many other predictive factors, the majority of which still unknown. Factors with a positive influence on growth response are a younger age, larger bone age delay, initial height and midparental height, a higher body weight and a relatively large difference between initial height and cTH at start of treatment. Also, a better response to treatment in the first year is associated with adult height outcome (27). The explained variance of these variables is still rather low (40-60%) (36).

Several parameters can be used for the evaluation of the success of treatment. For assessing the first year's growth response, the change in height SDS, height velocity, height velocity SDS and the change in height velocity may all be useful, provided that these parameters are corrected for the age, pubertal stage, and degree of growth retardation of the individual patient (27). Whether or not treatment was successful in the long term can be objectified with the auxological parameters adult height SDS, adult height SDS minus height SDS at start of GH treatment, adult height SDS minus predicted adult height SDS, and adult height minus conditional target height (27).

The absence of a circumscript defect in the GH-IGF-I axis in most children with ISS, the large inter-individual variation in response to GH-treatment and the high cost of GH have prompted the search for alternatives for GH treatment. An additional reason to do so is that GH treatment is not registered for ISS in the Netherlands and other European countries.

Gonadotropin releasing hormone agonists (GnRHa)

At pubertal onset, a steep increase in the level of circulating estrogen results in acceleration of bone maturation, which preludes epiphyseal fusion and determines a person's adult height. The development of pharmaceutical compounds that can delay or inhibit pubertal development has received increasing interest. Postponing pubertal development would hypothetically result in prolongation of the timeframe reserved for growth, leading to an increased adult height gain. In children with central precocious puberty (CPP), suppression of gonadal sex steroid synthesis effectively halts pubertal development and augments adult height (37;38).
The beneficial effects of GnRHa treatment on height gain in CPP has prompted attempts to apply this treatment strategy in children with short stature without precocious puberty as well. In general, GnRHa monotherapy in children with ISS results in a marginal increase in adult height of 0-4 cm (39-41). The efficacy of GnRHa depends on the duration of treatment. Short term GnRHa (2 years) does not improve adult height, due to a balance between decreased growth velocity and arrested bone maturation, whereas in long term treatment (3-4 years) the decreased but continuous growth in the absence of bone age progression augments adult height with approximately 1 cm per treatment year (42).
The possibility of adding GH to GnRHa can be considered, as the decreased growth velocity observed during GnRHa treatment may limit height gain (43;44). In such a scenario, GnRHa ideally extends the period available for growth while GH simultaneously preserves growth velocity, ultimately leading to a taller adult stature. Combined GH+GnRHa treatment was shown to augment adult height by approximately 4-5 cm in two controlled clinical trials (45;46), but analysis of large databases of GH-treated children that were also treated with GnRHa has revealed no effect (47-49). Combined GH+GnRHa treatment may be considered in children with ISS and a pronounced growth retardation at pubertal onset (27). In the absence of information on the possible adverse effect of sex steroid deprivation on bone mineralization and of postponing puberty on psychosocial well-being, GnRHa treatment, whether or not combined with GH, should not be considered routine treatment for children with ISS (27).

Aromatase inhibitors

Bone age advancement and epiphyseal fusion are caused by the surge in estrogen secretion during puberty (50) as illustrated by unfused epiphyseal plates, ongoing growth into adulthood and extremely tall stature observed in patients with disruptive mutations in the aromatase gene (51-55) or estrogen receptor gene (56). Therefore, it has been postulated that blocking endogenous estrogen synthesis may result in delayed bone maturation, prolongation of the

timeframe reserved for growth and increased adult height (57). Aromatase inhibitors reversibly or irreversibly prevent the conversion of androstenedione and testosterone into estrone and estradiol, respectively.

Several studies have been conducted in order to establish whether aromatase inhibitors can be used as an alternative for GH treatment in children with short stature by various causes. Some studies resulted in no beneficial effects of aromatase inhibitor treatment (58-60), but most reported an increased predicted adult height (61-66). Due to differences in trial design, such as the presence or absence of a control group, inclusion of various types of short stature (GH deficiency, McCune-Albright syndrome, precocious puberty), employment of different classes of aromatase inhibitors or co-treatment with other compounds (GH or testosterone), it is difficult to draw conclusions on the efficacy of aromatase inhibitors. Moreover, only two trials were randomized controlled clinical trials (61;66). Adult height data of most of these studies have not yet been reported. In boys with short stature due to constitutional delay of growth and puberty, letrozole treatment was shown to increase near-adult height, without apparent detrimental effects on bone mineralization (67). However, it was recently reported that aromatase inhibition may predispose to vertebral disc deformities (68).

Estrogen receptors and aromatase activity are ubiquitously present throughout the body, illustrating that estrogen signaling is crucial and inhibition of estrogen biosynthesis may result in adverse effects in various tissues or organs. The clinical phenotype of estrogen-deficient men gives several clues to potential unwanted effects of pharmaceutically induced estrogen deficiency. Obesity, insulin resistance, steatohepatosis, and severely decreased bone mineral density have been described in these patients (52;54;55;69). In ISS boys anastrozole or letrozole treatment did not impair skeletal mineralization (59;61;70), but the bone turnover rate was diminished due to treatment (61). Furthermore, a high number of vertebral body deformities was described after letrozole treatment raising the concern that aromatase inhibition may impair vertebral body strength (68;71). A reduced HDL-cholesterol in the absence of other effects on the lipid profile (61;72), and decreased insulin sensitivity have also been described (72). Theoretically, estrogen deprivation might result in disturbances of cognitive function and fertility, but this has not been documented in human studies so far. A careful exploration of the potential side effects of aromatase inhibition is required before such treatment can be applied in clinical practice (73).

Aromatase inhibition has been tested for the treatment of precocious puberty in girls with McCune-Albright syndrome (58;60), but not for growth enhancement in girls with ISS. Potential adverse effects such as virilization due to hyperandrogenism and the development of ovarian cysts warrant a cautious approach.

B. Manipulation of growth in animal models

Rodent models

Animal studies have provided valuable insights into the process of postnatal growth regulation and modulation. Although growth can be studied in a variety of animals (e.g. goat, rabbit, pig), most information has come forward from studies involving rodent models and this segment will therefore focus on results from studies in the mouse and rat.

GH-IGF-I signaling and growth

The crucial role of GH and IGF-I signaling in the regulation of postnatal growth has been established by studies in various rodent models such as spontaneously mutated mouse models characterized by dwarfism, knockout mice for several constituents of the GH-IGF-I axis, mice transgenically modified to overexpress components of the GH-IGF-I family, and hypophysectomized rats with combined pituitary hormone deficiency.

Dwarf mouse models

Spontaneous mutations in the pituitary-specific transcription factors Pit 1 or Prop-1 result in underdevelopment of subsets of pituitary cells and render the mice deficient for GH, thyroid-stimulating hormone and prolactin (74). This combined pituitary hormone deficiency translates into a growth phenotype characterized by normal body size and weight at birth, but severe growth retardation afterwards (Snell dwarf, Ames dwarf). Similarly, the GH deficient Little mouse (mutation in the GH releasing hormone receptor) shows a progressively impaired growth pattern from the age of two weeks onward. The phenotype of the Little mouse was shown to be rescued by injection with a recombinant adenovirus containing rat GH and a human promoter sequence. This form of gene therapy resulted in elevated levels of GH and IGF-I, and a normalization of body weight and body length as compared with wild-type mice (75), highlighting the important role of GH signaling in postnatal growth.

Transgenic and knockout mice

Additional insights into growth regulation by the GH-IGF-I axis have been gathered from studies in transgenic and knockout mice (76). GH insensitivity due to knockout of the GH receptor/binding protein gene (GHR; Laron mouse) or caused by transgenic overexpression of

an antagonizing GH analog results in decreased IGF-I levels and dwarfism (77-79). In contrast, gigantism is observed in transgenic mice that overexpress GH or hypothalamic GH releasing hormone (GHRH) (80;81).
IGF-I knockout mice have demonstrated that postnatal growth is mainly determined by GH-dependent IGF-I, whereas both hormones also act independently. This was shown by observations of compromised intrauterine and postnatal growth in IGF-I knockouts, but an even more pronounced postnatal growth retardation in IGF-I and GHR double knockouts (82). Partial reduction in IGF-I signaling in heterozygous IGF-I receptor knockout mice leads to an apparently normal phenotype, although there are also clues that a reduced growth potential may be present (82;83). Formation of a ternary complex with IGF binding protein 3 (IGFBP-3) and acid-labile subunit (ALS) normally increases the half-life of IGF-I in the systemic circulation. Overexpression of IGFBP-3 results in pre- and postnatal growth retardation, whereas ALS overexpression results in a moderate delay of postnatal growth. When both ALS and IGFBP-3 are overexpressed, growth inhibition is more pronounced (84). On the other side of the spectrum, transgenic mice overexpressing IGF-I show somatic overgrowth (85).
The findings in GH and IGF-I knockout mice are confirmed by observations in hypophysectomized rats that serve as a model for GHD. A markedly retarded somatic growth is seen in these animals compared with wild-type littermates. Both GH and IGF infusion in these rats results in stimulation of longitudinal growth. However, the effect of GH is more pronounced than that of IGF-I (86).

Representativeness of models for the influence of GH-IGF-I on growth

In the human, disorders or gene mutations leading to GH or IGF-I deficiency or resistance all result in various degrees of shortness, generally similar to the phenotypes observed in rodent counterparts (87). Patients with primary IGF-I deficiency or GH deficiency show a strikingly similar degree of growth retardation (87). Suppletion of GH or IGF-I in these patients results in stimulation of growth, but with GH treatment having a superior effect (88). In summary, both an effect of GH-dependent IGF-I and a separate, direct effect of GH have been demonstrated to be essential for coordinated postnatal growth in humans, which is in agreement with collective conclusions drawn from rodent animal models. Therefore, it can be concluded that rodent animal models are suitable for studying the effects of deregulation of the GH-IGF-I axis and treatment strategies designed for counteracting its effects on postnatal growth.

Sex steroid signaling and growth

In the following paragraphs the results of experiments on steroid signaling and growth will be reviewed. After the growth hormone (GH)-IGF-I axis, sex steroid signaling is the second major determinant of postnatal growth, especially during pubertal development. Many rodent models have been developed and applied to assess the influence of sex steroids on the regulation of growth, amongst which knockout mice for the estrogen receptor α and β, androgen receptor, and aromatase genes, and the ovariectomized or orchidectomized mouse and rat.

Estrogen receptor knockout mice

The actions of estrogen in the human and rodent are mediated by two receptors, the estrogen receptors alpha and beta (ERα and ERβ). Three knockout mouse models have been generated, the partially estrogen-resistant αERKO and βERKO mice (89), and the completely estrogen-resistant double knockout (α/βERKO) mouse (90). The effects of ER inactivation depend on sex and age of the mice (91).
In female mice, axial growth is unaffected in αERKO, βERKO and α/βERKO at all stages of life (92), although during the postpubertal phase a tendency towards slightly increased growth is observed in βERKO (92). During the period of sexual maturation and in adulthood, appendicular growth is decreased in αERKO, but increased in βERKO mice, with a more pronounced effect in adulthood. The phenotype of α/βERKO mice is intermediate between those of αERKO and βERKO in all stadia (92). A direct correlation between appendicular length and IGF-I levels has been noted, with high levels stimulating growth (93). Decreased growth of the axial bones is accompanied by a smaller width of the growth plate (94). Knockout of ERβ does not affect body weight gain, whereas obliteration of ERα or both receptors results in increased body weight (92). In adult αERKO and βERKO mice, a higher BMD is reported than in wild-type littermates, with a more prominent effect in αERKO mice. BMD in double knockouts is comparable to wild-type BMD (92;93). Apart from the growth effects, αERKO and α/βERKO mice also show a distinct ovarian phenotype characterized by enlarged, hemorrhagic and cystic follicles and are anovulatory (89).
Male αERKO and double knockout mice display decreased growth of the axial and appendicular skeleton, narrow epiphyseal growth plates, low IGF-I levels, less body weight, and a decreased bone mineral density (BMD) compared to wild-type littermates (92-94). Ablation of ERβ has no effect on growth parameters in male mice. Male αERKO mice show testicular atrophy, reduced sperm count and viability, altered sexual behavior and decreased fertility (89).

In conclusion, the influence of estrogen signaling on growth in the mouse shows a prominent sexual dimorphism. Signaling through ERα stimulates axial growth in male rats, whereas it has no effect in females. Appendicular growth is repressed via αERKO in both genders. ERβ does not exert an effect on longitudinal growth in both genders, nor on radial growth in males, but it does result in growth retardation in females. As it was demonstrated that estrogen levels are markedly raised in female αERKO and α/βERKO, but normal in males, it has been speculated that hyperestrogenism may inhibit appendicular growth via ERβ signaling.

Aromatase knockout mice

As an alternative model for assessing the role of estrogen signaling on longitudinal growth, estrogen-deficient mice can be considered. Targeted disruption of the aromatase gene has resulted in the development of such a model, the ArKO mouse (95). Aromatase synthesizes estrogen from androgenic precursors. Male and female ArKO mice appear phenotypically normal at birth. Appendicular growth is significantly retarded in adulthood in male, but not in female ArKO mice (96). Effects on axial growth have not been reported, and it might therefore be concluded that such effects are either absent or marginal at most. Both genders display osteoporosis (97). Female ArKO mice have underdeveloped uteri and ovaries, and are sterile due to anovulation (95;98). Male mice are fertile, but to a lesser extent than wild-type littermates (97). Increased abdominal fat deposition and insulin resistance is present in both males and females (99) and it has been postulated that a disturbed androgen to estrogen ratio may promote visceral fat accumulation (99).
The differences between the phenotypes of ERKO and ArKO mice may rely on the fact that partial estrogen signaling is still possible in ERKO mice, whereas the influence of estrogen is completely abolished in the ArKO model. Additionally, the androgen to estrogen ratio is presumably more disturbed in ArKO than in ERKO mice, which may account for phenotypic variations due to elevated androgenic signaling.

Androgen receptor knockout mice

Besides the established role of estrogen in the regulation of growth, there is also compelling evidence that androgens have a unique function. Part of the effect of androgen on the growth plate is probably due to aromatization into estrogens, which is suggested by the presence of aromatase in rat and human growth plate cartilage (100;101). However, the androgen receptor (AR) has been detected in rat (102) and human growth plate cartilage as well (103;104),

suggesting that direct actions of androgen in growth regulation also occur. The observations that androgens accelerate growth in mice, and that a nonspecific ER blocking compound does not attenuate this effect are in support of a direct androgenic effect (105).
The androgen-resistant knockout mouse was generated to study the influence of androgens on bone and metabolism. A normal growth phenotype was observed in these mice. Male ARKO mice had female secondary sexual characteristics, late onset obesity and a marked loss of BMD (106;107). The absence of an impact on growth in ARKO mice suggests that androgen signaling is not crucial for the regulation of longitudinal growth. The observed growth-stimulating effect mentioned before may require supraphysiological levels of androgens.

Gonadectomy and estrogen treatment

Sex steroid deprivation can be effectively induced by gonadectomy. The gonads are the exclusive source of synthesis of androgen and estrogen precursors in rodents, therefore gonadectomy results in complete ablation of sex steroids (108). Ovariectomy (OVX) in rats removes the growth-inhibiting influence of estrogens resulting in augmented longitudinal growth and decreased bone quality (109;110). Administration of estrogen to OVX rats counteracts the growth stimulation induced by estrogen deficiency (111). Treatment of OVX rats with non-aromatizable androgens stimulates longitudinal growth, which demonstrates a direct influence of androgen signaling on growth as well (112). In contrast, the growth pattern of OVX mice is marginally or not altered (113), illustrating different roles for sex steroid signaling in growth regulation in rats and mice.
Orchidectomy (ORCHX) in male rats results in decreased longitudinal growth, impaired body weight gain and osteoporosis (109). The contrasting effects of sex steroid depletion in male and female rats points at the existence of gender-specific patterns of growth regulation.

Pharmacological suppression of sex steroid biosynthesis or action

Instead of gonadectomy, gonadal sex steroid synthesis can also be obliterated by chemical castration with GnRHa. Triptorelin treatment in female prepubertal rats results in enhancement of body weight and length gain, comparable to the observations in the OVX phenotype. In male rats, GnRHa treatment stimulates body weight gain and also slightly improves length gain, which is in contrast with the observed growth retardation after ORCHX (114). It was speculated that GnRHa in rats may interfere with the hormonal regulation of food intake and weight control instead of having an effect on growth regulation (114).

Selective suppression of estrogen signaling can be established by treatment with an aromatase inhibitor. Several studies have analyzed the effects of such treatment on bone health and growth in male rats, but not in females. Young male rats treated with the aromatase inhibitor vorozole display decreased body weight and BMD, but normal femoral length (115). The selective estrogen receptor modulator (SERM) tamoxifen reduces body weight gain, axial and radial growth, and BMD in young male rats (62). These observations are in agreement with the phenotype of castrated male rats, albeit less prominently. In contrast, treatment with the aromatase inhibitor vorozole in male mice was shown to result in augmented body weight and tail length gain, an increased width of the epiphyseal growth plates and elevated GH levels (116). These results in mice are in contrast with the decreased growth patterns that characterize male estrogen receptor or aromatase knockouts that show normal to decreased growth.

Representativeness of models for the influence of sex steroids on growth

Estrogen has been identified as the main regulator of growth during the pubertal phase. Low levels of estrogen initiate the growth spurt at the beginning of puberty, whereas high levels at the end of this period arrest growth and result in epiphyseal fusion both in males and females (2). The growth-inhibiting actions of estrogen have been illustrated by the clinical presentation of male and female patients with estrogen deficiency due to inactivating mutations of the aromatase gene (52;54;55) and in the estrogen-resistant man with a mutation in the ERα gene (56). These patients all share a common phenotype with absence of a pubertal growth spurt, ongoing growth into adulthood, unfused epiphyses and a tall stature.

An *in vivo* model for studies on the effect of sex steroid signaling on growth would ideally recapitulate the hallmarks of human growth regulation. Rodents do not display the typical estrogen-driven signs of maturation as observed in humans. However, even though epiphyseal fusion never occurs in rodents, growth velocity approximates zero at the end of sexual maturation. Female OVX rats show stimulated axial and appendicular growth, and estrogen administration to intact male and female rats inhibits longitudinal and radial growth (110), pointing at an important role of estrogen signaling in growth regulation in the rat similar as in humans.

ERα, ERαβ, and aromatase gene knock-out as well as gonadectomized mice show either retarded or unaffected growth patterns compared with wild-type littermates. The contrast with the observed stimulation of growth in estrogen-devoid human patients implies that growth regulation in mice is markedly different than in humans, and argues against the use of mice models for studying human growth regulation by sex steroids.

C. *In vitro* models for the analysis of growth regulation

The epiphyseal growth plate, localized at the distal ends of long bones, is a complex structure that is crucial for skeletal growth (1;117). At this level, endochondral ossification takes place, during which cartilage is first formed and subsequently replaced by osseous tissue, resulting in bone elongation. The growth plate is a polarized, multilayered structure, that consists of chondrocytes at various stages of differentiation: the resting zone, with relatively quiescent, stem-cell like chondrocytes, the proliferative zone and the hypertrophic zone. Upon unknown triggers, chondrocytes from the resting zone are recruited to undergo proliferation, hypertrophic differentiation and programmed cell death. As a net result of this differentiation program, a cartilaginous scaffold is synthesized, that is subsequently invaded by blood vessels and bone cell precursors and then replaced by bone.
The highly complex process of endochondral ossification is regulated by the interplay between systemically circulating hormones and growth factors produced locally at the level of the epiphyseal growth plate. The exact mechanisms underlying the coordination of growth plate physiology remain to be elucidated in detail. Apart from *in vivo* models as described in part B, various animal-derived and human *in vitro* models have been developed and applied for studying growth regulation and physiology at the level of the growth plate.

Whole bone tissue culture

Explanted, cultured rodent bones such as the tibia or metatarsals can be easily manipulated by the addition of hormones, growth factors, cytokines, or pharmacological compounds to the culture medium. In most studies, the results of metatarsal culture are directly compared with those obtained in similarly designed experiments in cultured (human) chondrocytes. Valuable insights have emerged from this experimental approach. For example, it has been demonstrated that locally produced estrogen increases metatarsal growth by stimulating chondrocyte proliferation and inhibition of apoptosis (118). Inhibition of estrogen activity by the SERM tamoxifen results in growth inhibition (91). Modulation of the androgen receptor does not have an effect on growth (119). Metatarsal cultures have also made clear that glucocorticoids, pro-inflammatory cytokines and parathyroid hormone related protein (PTHrP) inhibit bone growth, possibly by interfering with the phosphoinositide-3-kinase (PI3K) pathway (120-124).
While there are clear advantages of this model, such as the easy accessibility of the explanted whole bone tissue culture, there are also disadvantages. When aiming at the analysis of chondrocyte differentiation, it has to be taken into account that other tissues such as bone and

hematopoietic tissue are also present in this culture system which may influence the behavior of the chondrocytes as well. In addition, species differences may hamper the translation of experimental data obtained in rodents to the regulation of growth in humans.

Growth plate studies

Rats and mice do not display epiphyseal fusion after completion of the sexual maturation phase. In contrast, rabbit growth plates do undergo fusion at the end of sexual development and important knowledge on this phenomenon typically observed in humans has emerged from studies in rabbits. It is hypothesized that epiphyseal fusion is a consequence of senescent changes within the growth plate, a process that is accelerated by estrogen (125). Catch-up growth, that is often seen in children after removal of a growth-inhibiting condition, was also demonstrated in rabbits after termination of growth-inhibiting dexamethasone treatment and was found to be associated with delayed growth plate senescence (126).
Theoretically, human growth plate specimens may also be used for analysis of growth plate architecture at different ages and phases of development, under the condition that a sufficient number of growth plates is available for such studies. However, normal human growth plate specimens are difficult to obtain, as they cannot be biopsied or removed from healthy children. Moreover, single growth plates have limited value in growth studies, as they only represent a specific growth stage corresponding with the donor's age and gender.

Chondrocyte culture

Various animal-derived and human cellular model systems have been developed to study the biomechanics of chondrocyte differentiation. The focus of this paragraph will be on human culture models. Amongst the most often used models are the primary chondrocyte culture, clonal normal cell lines (e.g. HSC-2/8), and transformed clonal cell lines (e.g. C-28/12, T/C-28a2) (127). Although applied for many years, there are many disadvantages to these models. Primary chondrocyte cultures have a low proliferative capacity, that is directly correlated with the age of the donor. Due to ethical considerations, it is difficult to obtain sufficient chondrocytes. Moreover, primary chondrocytes often show a tendency to dedifferentiation.
Clonal normal cell lines descend from one common ancestor and are non-transformed. Many cell-lines are derived from tumors, such as HSC-2/8 that was derived from an aggressive chondrosarcoma. The malignant origin of such cell lines may affect their biological characteristics. In addition, cell lines may display genomic instability or the frequent occasion of random mutations.

Transformed clonal cell lines are genetically modified resulting in immortalization of the cell line. These cell lines may display continuous proliferation in monolayer culture, but the loss of chondrocyte phenotypical characteristics has also been reported, as well as gradual loss of proliferative capacity.
Many of these cell lines have been obtained from articular cartilage, which is not identical to growth plate cartilage, and it is therefore unlikely that such models are representative for processes taking place in the growth plate during endochondral ossification. Some cell lines originate from diseased cartilage, such as osteoarthritic or trauma-damaged cartilage, which has been reported to alter the characteristics of the chondrocytes (127).

Stem cells

Alternatively, much effort is currently put into development of a human model for chondrogenesis using human mesenchymal stem cells (hMSC). hMSC are multipotent cells that can differentiate into a variety of cell types, such as chondrocytes, adipocytes, myocytes, and osteocytes, when exposed to the proper stimuli. hMSCs driven towards chondrogenic differentiation would provide a model that might facilitate a detailed analysis of molecular and biochemical processes underlying growth as it occurs within the human growth plate. It would allow a detailed analysis of even the very first stages of chondrogenic development, when the stem cells are triggered to undergo chondrogenic differentiation.
Some studies have addressed the potential use of hMSC-derived chondrocytes as a model for chondrogenic development and have characterized this model by means of large-scale expression profiling (128-130). Data emerging from these studies are promising, with respect to the aspect that cartilage is indeed formed after appropriate stimulation of hMSCs. However, before cartilage derived from hMSCs can be used as a model for studying chondrogenesis as it occurs in the human growth plate during endochondral ossification, it still remains to be determined whether hMSC-derived chondrocytes adopt a phenotype similar to growth plate or to articular cartilage.

D. Structure and scope of this thesis

In this thesis, we aimed to answer three questions related to the regulation and manipulation of growth. After the introductory ***Chapter 1***, the main body of the thesis is divided into three parts addressing these questions.

The first question we aimed to resolve in part A of this thesis was: what are the long-term results of two novel treatment modalities of children with ISS? Here we report the results of two randomized controlled clinical trials in children with ISS. ***Chapter 2*** describes adult height outcome after high dose GH treatment restricted to the prepubertal period. ***Chapter 3*** presents the effects of combined GH and GnRHa treatment on growth in adolescents with a relatively early pubertal onset.

In part B, we addressed the question: what is the effect of the administration of aromatase inhibitors in the rat? Aromatase inhibitor treatment has been suggested as a possible alternative to currently available growth-enhancing therapeutic regimens for children with ISS, but their efficacy and potential adverse effects have not been studied in a growing and developing model system. The answers to these questions would enable a better estimation of the potential efficacy and adverse effects of aromatase inhibition when considering to apply this type of medication to children. In ***Chapter 4*** the effects are shown of 3 weeks treatment with the steroidal aromatase inhibitor exemestane on longitudinal growth parameters, bone quality and gonadal morphology in female prepubertal rats. ***Chapter 5*** summarizes the results of 6 weeks of exemestane treatment on growth and bone quality in male prepubertal rats.

Part C provides an answer to the question: can mesenchymal stem cells differentiate into chondrocytes with a phenotype resembling the epiphyseal growth plate? ***Chapter 6*** illustrates how human fetal mesenchymal stem cells can be stimulated to undergo chondrogenic differentiation and may serve in future studies as a model for investigating processes taking place in the epiphyseal plate during growth.

Finally, ***Chapter 7*** provides a general discussion on the various elements of this thesis and is followed by a summary in ***Chapter 8***.

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Part A

Chapter 2

High dose growth hormone treatment limited to the prepubertal period in young children with idiopathic short stature does not increase adult height

S.A. van Gool[1], G.A. Kamp[2], R.J. Odink[3], S.M.P.F. de Muinck Keizer-Schrama[4], H.A. Delemarre-van de Waal[5], W. Oostdijk[1], J.M. Wit[1]

[1]Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands.
[2]Department of Pediatrics, Tergooi Hospitals, Blaricum, The Netherlands.
[3]Department of Pediatrics, Catharina Hospital, Eindhoven, The Netherlands.
[4]Subdivision Endocrinology, Erasmus Medical Center/Sophia Children's Hospital, Rotterdam, The Netherlands.
[5]Department of Pediatrics, VU University Medical Center, Amsterdam, The Netherlands (current address: Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands).

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Abstract

Objective: to assess the long-term effect of prepubertal high-dose growth hormone treatment on growth in children with idiopathic short stature (ISS).

Design and methods: 40 children with no signs of puberty, age at start 4-8 years (girls) or 4-10 (boys) years, height SDS<–2.0 SDS and birth length>-2.0 SDS, were randomly allocated to receive GH at a dose of 2 mg/m^2/day (equivalent to 75 µg/kg/day at start and 64 µg/kg/day at stop) until the onset of puberty for at least 2 years (preceded by two 3-month periods of treatment with low or intermediate doses of GH separated by two washout periods of 3 months) or no treatment. In 28 cases adult height (AH) was assessed at a mean (SD) age of 20.4 (2.3) years.

Results: GH-treated children (mean treatment period on high dose GH 2.3 yr (range 1.2-5.0 yr)) showed an increased mean height SDS at discontinuation of treatment compared with controls (-1.3 (0.8) SDS versus -2.6 (0.8) SDS, respectively). However, bone maturation was significantly accelerated in the GH-group compared with controls (1.6 (0.4) versus 1.0 (0.2) yr, respectively) and pubertal onset tended to advance. After an untreated interval of 3-12 yr, AH was -2.1 (0.7) and -1.9 (0.6) in the GH-treated and control group, respectively. Age was a positive predictor of adult height gain.

Conclusion: High dose GH treatment restricted to the prepubertal period in young ISS children augments height gain during treatment, but accelerates bone maturation, resulting in a similar adult height compared to untreated controls.

Introduction

Growth hormone (GH) treatment in children with idiopathic short stature (ISS) has been the subject of many clinical trials. There are essentially four outcome parameters of GH treatment that can be considered: short term growth response (1st year's height velocity), bone age advance, onset and progress of puberty, and increase in adult height (AH). As recently reviewed (1;2), almost all children with ISS respond to GH treatment with an increase in height velocity, even on a relatively low dose. The dose-response curve for the first year's height velocity appears to reach a plateau after a dose of 50 µg /kg/d. No acceleration of bone age advance and pubertal onset and progress has been observed in the dose range of 30-53 µg /kg/d and the most effective dose regimen (50 µg /kg/d) leads to approximately 7 cm adult height gain (1;2).

At the time this study was designed, there were three important issues with respect to GH treatment of children with ISS that awaited resolving. First, it was unknown what the effect would be of a further increase of the GH dosage on growth velocity, bone maturation, puberty and AH. Second, the relative contribution of GH treatment before and during puberty was unclear. Third, there was a need to gain more insight into the factors affecting the growth response, as only a modest part of the inter-individual variation can be explained (3).

In order to address these issues, we started a controlled clinical trial in children with ISS in 1993. We hypothesized that a high GH dose before puberty might be able to bring height within the population's range, as shown for a dose of 0.1 mg/kg/day (4), without undue bone maturation and advance in puberty, and without adverse effects. We limited the period of GH therapy to the years before pubertal onset, primarily to improve the cost-benefit ratio, but also based on studies showing that HSDS at the onset of puberty is a strong predictor of AH in GH deficient children (5;6) and that pubertal height gain on GH treatment was not different between GH treated children and untreated controls (7). In order to improve the predictive power of clinical and biochemical variables we included an elaborate assessment of GH sensitivity.

In three previous papers on this study we reported that high dose GH limited to the prepubertal period increased growth, but also advanced bone age maturation and pubertal development, so that the predicted adult height (PAH) did not change (8), and that biochemical (9) and *in vitro* (10) indices of GH sensitivity had little predictive power for the short-term growth response. In the present paper we report the results on adult height.

Subjects and methods

Patients

This report includes AH data on 28 out of 40 children with ISS who originally enrolled in a multicenter study in The Netherlands from December 1993 to December 1996. Inclusion criteria were: no signs of puberty (G1 in boys and B1 in girls); height at baseline (H_{start}SDS) <-2.0 SDS for Dutch references available at that time (11), age at baseline 4-8 years for girls and 4-10 years for boys; birth length >-2.0 SDS (12); maximum serum GH level more than 10 µg/liter (1 mg = 2 IU, using World Health Organization (WHO) International Reference Preparation 66/217 as standard) after provocation (exercise, arginine, clonidine, L-dopa or glucagon), and a normal sitting height/subischial leg length ratio (between P3 and P97) (13). Screening blood tests and urinalysis were normal. No organic causes of growth failure, primary bone disease, chronic illness, or dysmorphic syndrome were present. Further details of the subjects and data obtained after discontinuation of treatment have been reported previously (8;9).
Three children of Turkish origin were included in the trial, 1 girl and 1 boy in the GH-group, and 1 boy in the control group. H_{start}SDS was calculated using Dutch references (-2.50, -2.73, -3.23, respectively), and their height was also <-2 SDS for references for Turkish children that became available in 1997 (14). For further analyses, SDS values of these and all other children were calculated using references for Dutch children.
The protocol was approved by the medical ethical review boards at the three participating centers (Amsterdam, Rotterdam, and Eindhoven). Before conducting any study-related procedure, written informed consent was obtained from parents and, when appropriate, also from the participants. For AH analysis, written informed consent was obtained from the participants. This clinical trial was registered in the *meta*Register of Controlled Trials (ISRCTN52337368) of the Current Controlled Trials Ltd.

Study design

Forty patients were randomly allocated to receive GH treatment or no treatment (figure 1). Details have been reported previously (8). In short, in the GH treatment group GH responsiveness was assessed during the first year of the study by administering GH in an on/off scheme at a dose of 0.5 or 1.0 mg/m^2/d (equivalent to 19 or 38 µg/kg/day, respectively) during two periods of 3 months, separated by two wash-out periods of 3 months without GH treatment (figure 2). In the second year, long-term GH treatment with 2.0 mg/m^2/d (75 µg/kg/d)

was started and was intended to be given for at least two years. Treatment was discontinued at the first full year visit after the onset of puberty (G2 for boys and B2 for girls), which resulted in a treatment period of 2-5 years on high dose GH (mean 2.3 yr). At discontinuation of GH treatment, the dose was equivalent to 64 µg/kg/d. The GH dose per kg body weight was lower at discontinuation of treatment than at start of the high dose treatment phase due to the fact that body weight shows a larger increase with age than body surface. GH (Genotropin; Pharmacia & Upjohn, Uppsala, Sweden; now Pfizer, New York, USA) was administered subcutaneously, 7 days per week between 6.00 and 8.00 p.m. The measurements at discontinuation of treatment in the GH-group were compared with measurements after attaining Tanner stage 2 (B2 for girls, G2 for boys) in the control group.

Directly after randomization, four patients (two from each group) refused to start the treatment they were randomly allocated to receive and dropped-out (fig 1). In addition, one boy from the GH treatment group was found to have neurofibromatosis and was excluded from the study.

At adult height analysis, 6 patients from the control group could not be motivated to participate. One patient from the GH-group could not be traced and was lost to follow-up. One boy stopped using high dose GH after 1.2 years and could not be motivated to continue according to protocol. However, his growth data are included in this report. Pubertal onset and development were not registered for 1 girl from the control group and her last

Figure 1

Trial design.

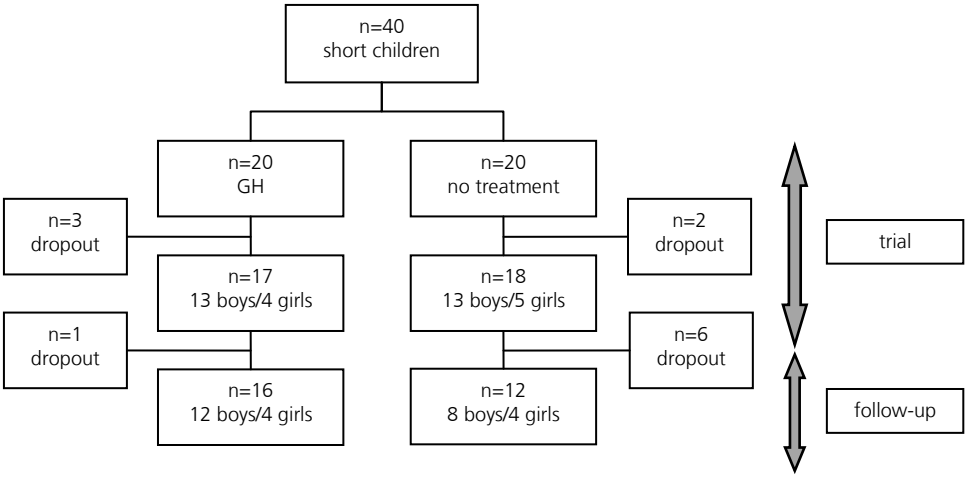
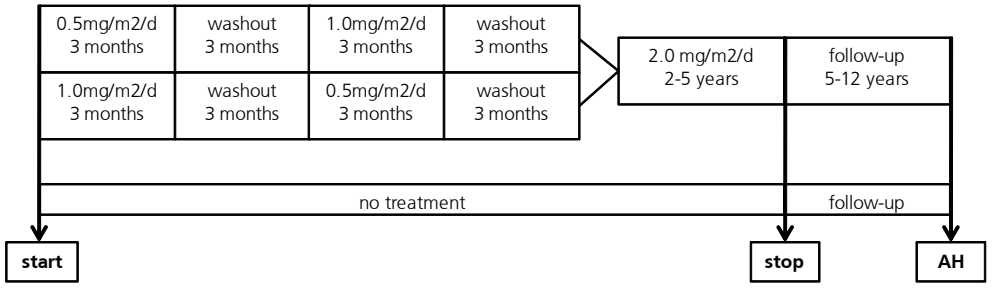


Figure 2

Time-scheme for GH-treatment and control groups. GH doses: 0.5 mg/m^2/d = 19 µg/kg/d; 1.0 mg/m^2/d = 38 µg/kg/d; 2.0 mg/m^2/d = 75 µg/kg/d.



known auxological information at the age of 9.7 yr was used for the analysis at stop.
At follow-up, we took a short medical history, performed a physical examination, assessed bone age (15) and measured height, weight, and sitting height (SH). Leg length (LL) was obtained by subtracting SH from height. Blood was collected for DNA extraction and single-nucleotide polymorphism genotyping (SNP-array) as described before (16) using the Affymetrix Genechip Human Mapping 250K array set. We also assessed the psychosocial status, which will be reported separately.

Outcome parameters

Four outcome parameters were used to evaluate the response to treatment: 1) Adult height SDS (AH SDS); 2) AH minus height at start SDS (AH–H_{start} SDS); 3) AH minus height for bone age at start (AH–H for BA_{start} SDS); and 4) AH minus conditional target height SDS (AH–cTH SDS). Because of the young bone age of most patients at start of the intervention, the predicted adult height (PAH) according to Bayley and Pinneau (17) could not be calculated at start.
To assess the degree of change of growth potential after discontinuation of treatment, we analyzed AH SDS minus predicted adult height at discontinuation of therapy (AH-PAH_{stop} SDS). For both groups, pubertal development at Tanner stage 2 was expressed as SDS for age and gender according to a recent technique (18).
Height and BMI SDS were based on recent Dutch references (19). For calculation of AH SDS, the age of each patient was set at 21 yr, enabling comparison of AH with the height distribution in the normal adult population. For 4 patients, a BA radiograph at discontinuation

of treatment was not available, but bone age was extrapolated from a BA determination closest to this time point (at visit x) using the formula: $BA_{stop} = (BA_{visit\ x}/CA_{visit\ x}) \times age_{stop}$.

A total of 24 out of 28 patients consented to undergo an X-ray of the left hand for automatic determination of the pediatric bone index (PBI), an index for the amount of cortical bone specifically developed for the pediatric population (20). PBI was expressed as SDS based on a Dutch reference cohort. For patients older than 19 yr, SDS values were calculated using references for 19-yr old adolescents.

Parental height SDS was calculated and corrected for the secular trend (in the Netherlands estimated at 4.5 cm/30 yr) as follows: $Height_{father}$ SDS = $[(AH_{father} + 4.5) - 184] / 7.1$ and $Height_{mother}$ SDS = $[(AH_{mother} + 4.5) - 170.6] / 6.5$ (19). Conditional target height (cTH), which is the target height corrected for the effect of assortative mating and parent-offspring correlations, was calculated using the formula: cHT SDS = 0.72 × the average of father's and mother's height SDS (21).

The SH, leg length (LL) and SH/H ratio were expressed in SDS based on Dutch references (22). For calculation of adult SH SDS, LL SDS, and SH/H SDS, the age of each patient was set at 21 yr.

Statistical analysis

The study was designed to compare the effects of high dose GH treatment with those of no treatment on AH. Statistical analyses were performed using the statistical package SPSS version 14.0 (SPSS, Chicago, IL). Results are expressed as mean (SD). Comparisons among treatment and control groups were made using Student's unpaired *t* tests. Possible interactions between the effect of GH treatment on the outcome parameters and the baseline parameters gender, age (age_{start}), height (H_{start} SDS), and bone age delay were analyzed by means of linear regression analysis using ANOVA applied to the whole group of subjects. Possible associations between IGF-I SDS after 3 months or 1 year of treatment with 2.0 mg/m^2 GH and the changes from IGF-I SDS at start of high dose GH were also tested by means of linear regression. The significance level was set at 0.05.

Results

A complete analysis was carried out for the remaining 28 of 40 originally included patients (70%). One female (bone age 13) and one male (bone age 15.5) from the control group had not reached AH, and their predicted AH (17) was used for further analysis. Patient characteristics at start, at stop, and at follow-up are listed in table 1. Age and BMI SDS at

baseline were higher in the GH-treated subjects compared to controls (in the original cohort of 40 patients this was not significant). GH-treated children tended to have more bone age delay than controls. The mean GH treatment period was 3.3 yr (including the first year's on/off scheme), resulting in a mean high dose GH treatment duration of 2.3 yr (range 2.0-5.0 yr, with the exception of 1 boy who stopped after 1.2 years). Children in the control group were seen for a period of 4.9 (1.9) years. AH data were collected at a mean age of 20.4 (2.3) years. The mean period elapsed between treatment discontinuation and AH analysis was 8.5 (1.7) yr (range 3.2-11.7 yr).

Effect on growth, bone maturation and puberty

At discontinuation of treatment, height SDS significantly increased in GH treated children compared to controls (table 1, figure 3), as reported previously (8). Bone maturation in the first two years of treatment was faster in GH treated children compared to controls, both in the original cohort (3.6 yr/2 yrs versus 2.0 yr/2 yrs) and in the cohort available for adult height evaluation (3.1 yr/2 yrs vs. 2.1 yr/2yrs). Over the full trial period bone maturation was also significantly advanced in GH-treated subjects compared with controls (1.6 (0.4) yr/yr versus 1.0 (0.2) yr/yr, respectively, $p<0.001$). PBI SDS was not different between the GH- and control groups (table 1). Madelung deformities or other apparent anatomical anomalies were not detected on the hand X-rays.

AH SDS, AH–H_{start} SDS, AH–H for $BA_{start,}$ and AH-cTH were not significantly different between the GH-treated and control group (table 1), and in both groups 50% of the participants attained an adult height >-2.0 SDS. The percentage of individuals with a height below the target range (cTH SDS – 1.6) decreased from 75% at start to 44% at follow up in the GH-group and from 67% to 27% in controls. The loss of growth potential after discontinuation of therapy (AH–PAH_{stop} SDS) tended to be greater in the treated group compared with controls, but the difference did not reach statistical significance ($p=0.1$).

BMI was significantly higher in the GH group compared with the control group at all stages of the trial. At follow up, BMI in GH-treated subjects was 1.0 SD higher than BMI_{start}, while there was only an increment of 0.1 SD in controls ($p<0.05$).

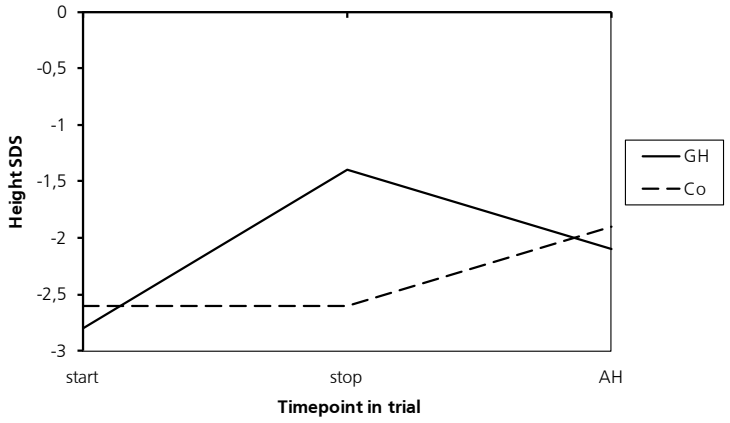
Table 1. Summary of initial and outcome variables (mean (SD)

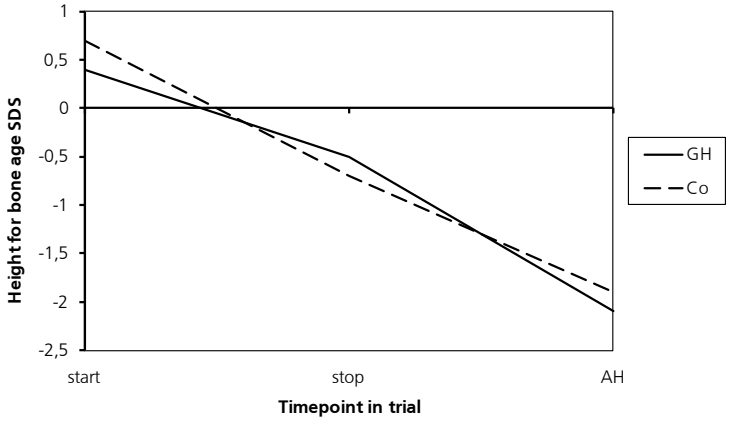
Parameter	GH (n=16)	Control (n=12)	Treatment *vs.* control
Boys/girls	12/4	8/4	
Start treatment			
Age (yr)[a]	8.7 (1.4)	7.0 (1.7)	p=0.009
Bone age delay (yr)	3.0 (1.1)	2.2 (1.3)	p=0.07
H (cm)	118.4 (8.5)	111.5 (10.6)	p=0.06
H SDS	-2.9 (0.6)	-2.5 (0.3)	p=0.09
H for BA SDS	0.4 (1.2)	0.7 (2.2)	p=0.7
cTH SDS	-0.7 (0.5)	-0.8 (0.6)	p=0.8
BMI SDS	-0.6 (0.8)	-1.1 (0.4)	p=0.04
SH SDS	-1.8 (1.0)	-1.9 (0.5)	p=0.1
Leg length SDS	-2.9 (0.7)	-2.3 (0.3)	p=0.007
SH/H SDS	1.7 (2.1)	0.8 (0.9)	p=0.2
Discontinuation of treatment (stop[b])			
Age (yr)	12.0 (1.0)	11.9 (2.0)	p=0.9
Age at T2 (yr)	11.6 (1.2)	12.1 (2.0)	p=0.5
age at B2 (girls)	10.7 (1.2)	11.5 (1.3)	p=0.5
age at G2 (boys)[c]	12.0 (1.0)	12.3 (2.2)	p=0.7
T2 SDS	0.3 (0.7)	-0.2 (1.2)	p=0.2
H (cm)	144.5 (5.6)	135.8 (6.4)	p=0.001
H SDS	-1.3 (0.8)	-2.6 (0.8)	p=0.001
H for BA SDS	-0.5 (0.6)	-0.7 (1.3)	p=0.7
H for BA SDS (stop-start)	-1.2 (0.8)	-1.3 (1.5)	p=0.04
Bone maturation[d]	1.6 (0.4)	1.0 (0.2)	p=0.000
PAH SDS	-1.3 (0.9)	-1.7 (1.1)	p=0.2
BMI SDS	-0.2 (1.0)	-1.4 (0.7)	p=0.003
SH SDS	-0.6 (1.0)	-1.7 (0.6)	p=0.02
Leg length SDS	-1.8 (0.8)	-2.2 (0.6)	p=0.3
SH/H SDS	1.4 (1.0)	1.1 (1.4)	p=0.6
Years from start to stop[e]	3.3 (0.9)	4.9 (1.9)	p=0.02
At adult height			
Age (yr)	21.0 (2.1)	19.6 (2.4)	p=0.1
AH in males (cm)	169.7 (4.2)	168.8 (3.8)	p=0.6
AH in females (cm)	154.6 (5.0)	160.8 (4.5)	p=0.1
AH SDS	-2.1 (0.7)	-1.9 (0.6)	p=0.6
[AH-H at start] SDS	0.7 (0.6)	0.7 (0.6)	p=0.8
[AH-H for BA at start] SDS	-1.6 (1.0)	-1.3 (1.1)	p=0.5
[AH-cTH] SDS	-1.4 (0.8)	-1.1 (0.4)	p=0.4
[AH-PAH at stop] SDS	-0.8 (0.9)	-0.1 (1.3)	p=0.1
BMISDS	0.6 (1.0)	-1.0 (1.2)	p=0.001
PBI SDS	0.5 (0.9)	0.2 (0.7)	p=0.4
SH SDS	-1.2 (1.2)	-1.7 (1.0)	p=0.2
Leg length SDS	-2.1 (0.6)	-1.4 (1.1)	p=0.05
SH/H SDS	1.5 (0.9)	0.4 (1.7)	p=0.04
Untreated interval (yr)	9.0 (1.5)	7.8 (2.1)	p=0.07

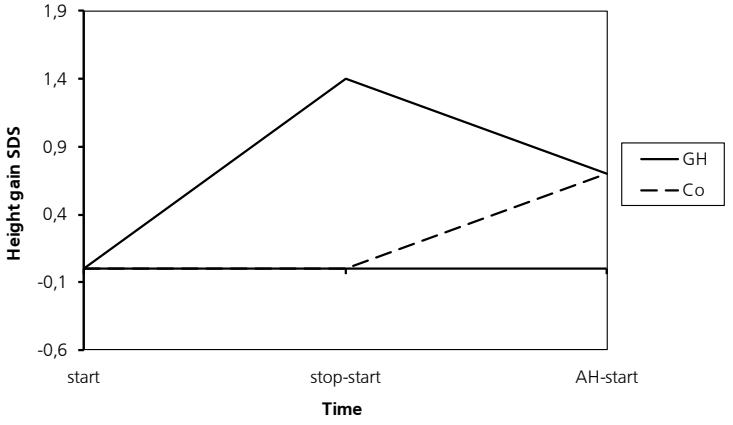
Results are presented as mean (SD). (A)H, (adult) height; BA, bone age; PBI, pediatric bone index; BMI, body mass index; cTH, conditional target height; GH, growth hormone; LL, leg length; PAH, predicted adult height; SDS, standard deviation score; SH, sitting height; SH/H, sitting height/height ratio; T2, tanner stadium 2; TH, target height. [a]Start signifies the start of the on-off scheme. [b]Stop is defined as the moment of discontinuation of GH treatment in the GH-group and the moment of attainment of T2 in the control group. [c]Two boys from the control group had late pubertal onset at the age of 14.2 and 16.0 yr, respectively. [d]Bone maturation calculated for the full trial period. [e]Includes the first year's on-off scheme.

Figure 3

Height SDS, height for bone age SDS, and height gain SDS at start and discontinuation of the intervention, and after reaching adult height (AH).







At baseline, treatment and control groups were found to be slightly disproportionate, with relatively short legs in comparison to sitting height, resulting in a positive SH/H SDS in both groups. At the end of the trial phase, SH/H SDS was similar, but at follow up it was significantly higher in the GH-group compared with controls (p=0.04). Figure 4 shows SH SDS, LL SDS, and SH/H SDS at start and at follow up. GH-treated patients displayed an increased growth of trunk and legs compared with controls during the 4 years after start of the trial phase, whereas controls had more or less stable SH SDS and LL SDS which increased after (more than) 4 years. The GH-treated group had a longer trunk, but shorter legs than controls at follow up.

Our analysis 5 years after inclusion demonstrated significantly earlier pubertal onset in GH-treated subjects (8). However, in our present analysis on 26 out of 28 subjects (missing data on pubertal onset for 1 patient from each group) the difference did not reach statistical significance (p=0.5) (table 1). In boys in the treatment and control groups pubertal onset ranged from 10.3 to 13.6 yr and 9.2 to 16.0 yr, respectively. In girls these ranges were 9.2-12.1 yr and 10.0-12.4 yr, respectively. At the attainment of Tanner stage 2, mean pubertal stage SDS for age (in boys and girls) was 0.3 (0.7) and -0.2 (1.2) in the treatment and control groups, respectively (p=0.2). None of the 12 GH-treated subjects and two boys out of 8 controls had delayed puberty (at 14.2 and 16.0 yrs), while in both groups 50% of patients had at least one parent (most often the father) with a reported late onset of puberty.

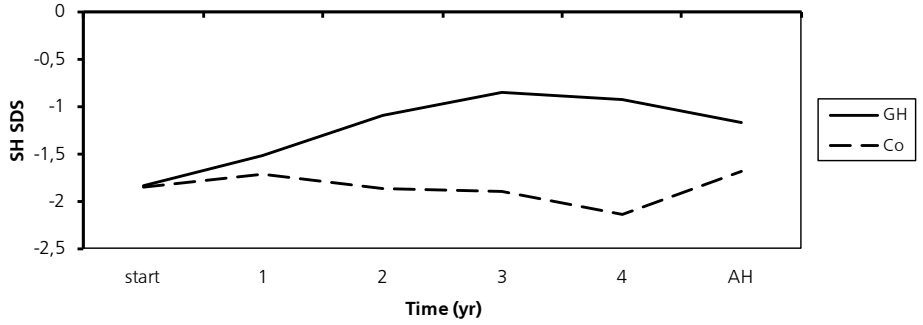
Linear regression analysis of predictors for growth response

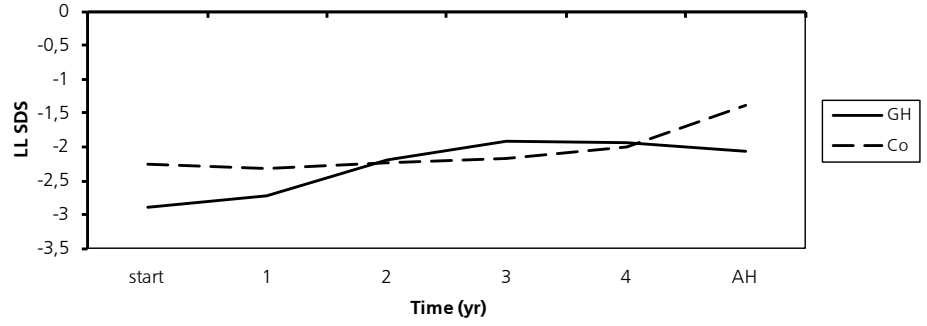
Possible interactions between the effect of GH treatment on the 4 AH outcome parameters and on AH-PAH_{stop} and the baseline parameters gender, age_{start}, H_{start}, bone age delay were analyzed. Age at baseline was a predictor for the treatment effect (GH x age_{start}) expressed as AH (B=0.4, CI=0.03-0.7, p=0.04) and AH–H_{start} (B=0.4, CI=0.06-0.7, p=0.02), but not for the other outcome parameters, thus older children had a better response to treatment. Gender showed a negative interaction with treatment effect (GH × gender) for AH (B=-1.1, CI=-2.3-0.01, p=0.052), with boys showing a larger increase in AH SDS than girls. Height and bone age delay at start were no significant predictors for treatment effect in any of the outcome parameters.

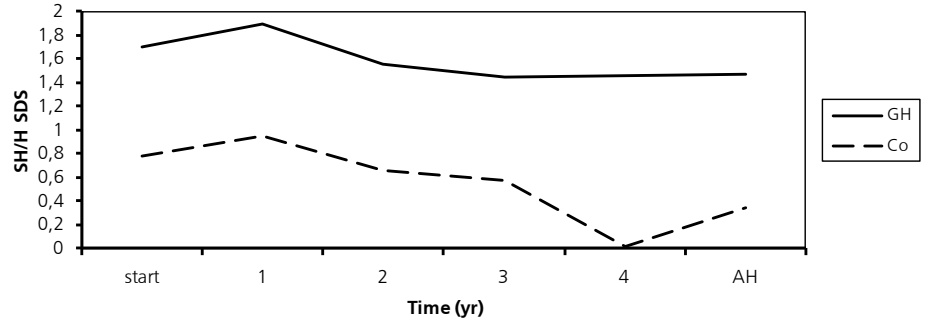
IGF-I SDS after three months of treatment with 2.0 mg/m2 GH was associated with growth potential loss, with higher IGF-I levels resulting in a higher loss of growth potential expressed as AH-PAH_{start} (B=-0.6, CI=-1.2 – (-0.02), p=0.045). The change in IGF-I SDS between start and 3 months of high dose GH treatment showed a trend towards negative interactions with treatment effect expressed as AH SDS (B=-0.5, CI=-1.0-(-0.1), p=0.09) and [AH-$HforBA_{start}$] SDS (B=-1.0, CI=-2.0-(-0.1), p=0.07), with higher changes in IGF-I SDS over 3 months of high dose GH treatment showing lower increases in AH SDS and [AH-$HforBA_{start}$] SDS.

Figure 4

Development of SH SDS, LL SDS, and SH/H SDS during four years after onset of the trial phase and during follow-up until AH. At start and on AH, data of 100% of patients are represented. After 1, 2, 3, or 4 whole years after start of the trial phase, data of 96%, 89%, 89%, and 61% of patients are shown.







Genetic analysis

Informed consent for genetic analysis was obtained from 18 out of 28 patients (11 GH-treated, 8 controls). SNP-array did not detect insertions, deletions or duplications explaining short stature. Mutational analysis was not performed.

Discussion

The long-term results of this first randomized controlled study on the effect of high dose GH treatment restricted to the prepubertal period show that this regimen does not lead to an increased adult height. This confirms our earlier findings after discontinuation of treatment, where we showed that the positive growth response significantly accelerated skeletal maturation and advanced the onset of pubertal development, and did not improve predicted adult height (8). In contrast to retrospective studies (3), where the growth response was inversely associated with age at start of treatment, in our study a younger age at start was associated with a lower adult height.

The lack of effect of this therapeutic regimen can be explained in at least three ways. The most likely explanation is that a high GH dose (approximately 3 times higher than substitution) (23) administered to young children not only leads to faster growth but also results in faster bone maturation. There are only few data on GH treatment of young children with ISS, as in virtually all studies the average age was approximately 11 years. It seems unlikely that the high dose *per se* causes the lack of effect, because children treated with a high GH dosage (67 µg/kg.day) starting at a mean age of 11 years achieve an adult height gain of 1.3 SD, which is slightly more than on 33 µg/kg/day (24). We speculate that the epiphyseal plates of young children may be more sensitive to high doses of GH and/or IGF-I than at later ages. The finding that a higher IGF-I SDS after 3 months of high dose GH treatment was associated with less adult height gain would suggest that circulating IGF-I plays a role in advancing epiphyseal maturation. The report by Cohen et al (2007) that even on a high GH dose (median 98, range 20-346 µg/kg/d) titrated on circulating IGF-I levels of +2SDS, administered to young children (age range 2.9-13.5, mean 7.53), no bone age advance was observed is not necessarily in contradiction with our findings, as the dose range in that study was large, and the children who needed high GH doses to reach the aimed IGF-I level may have been more resistant to GH. The trend towards a worse response to treatment in females may reflect the relatively strong influence of estrogens on bone maturation.

A second explanation is that the effect on bone maturation may be caused by the on-off GH treatment scheme employed during the first year of the trial, that may have primed the epiphyseal growth plate. We cannot exclude this possibility, but consider it less likely than the first explanation. The third possibility, that also cannot be ruled out with certainty, is that the poor result may be due to discontinuation of GH in puberty. The discontinuation of GH may have led to a 'catch-down' phenomenon, as was previously shown in children with SGA, who showed attenuation of growth after discontinuation of GH while puberty (and thus skeletal maturation) was progressing (25). However, the equal predicted adult height in the GH-treated and control groups at discontinuation of the trial phase argues against this hypothesis.

There are two noteworthy limitations of our study. First, the long diagnostic phase that may have been a confounder of the effect of long-term GH therapy. Second, the small size of the cohort. With respect to the latter limitation, we believe that even in this small study group the absence of any effect of treatment makes it very unlikely that this is a false negative result.

Our results imply that there may be an inverse U-shaped relationship between GH dose and adult height gain, if treatment is started at a young age. Dose is positively associated with adult height gain in the range of 25-50 µg/kg/d (2), but in young children higher doses may decrease adult height gain due to accelerated maturation of the epiphyseal plates and possibly also of the GnRH regulatory center, while the effect on growth has reached a plateau. This observation appears in contradiction to the overgrowth and tall adult height of children with pituitary gigantism, but in that condition plasma GH levels are characterized by an elevated baseline without high peaks, while the GH profile on a high GH dose shows one very high peak per day, followed by approximately 12 hours of suppression. During the peak plasma free GH must be considerably higher than in children with pituitary gigantism. Furthermore, the different GH profiles may also have different biological effects, similarly to observations in rodents (26).

The effect of a high GH dosage on pubertal onset in young children is less clear. In the final analysis on 26 children using a novel technique for expressing pubertal stage in SDS (correcting for age and gender) (18), we found a trend ($p=0.2$), but no statistically significant difference between the groups at Tanner stage 2. While this technique enables appropriate correction for the (statistically significant) age difference at start of the trial between the groups, the inability to reach statistical significance may well be related to the limited number of subjects that could be studied at follow up. In the larger group of 35 subjects studied 5 years after inclusion, the age difference at start did not reach significance, some patients had not yet entered puberty at the moment of analysis, and another method (cumulative proportions of patients having entered puberty, and calculation of relative risk) was used. In that analysis the relative risk for early puberty, adjusted for age and sex, was 4.7 (1.4-15.8, $p=0.012$) (8). There are two other observations that can serve as indirect evidence for an effect on puberty onset. First, the

observation that none of the 12 males in the GH treatment group entered puberty late, compared to 2 of the 8 controls. Second, at follow-up the GH-treated subjects had a significantly shorter leg length than controls and a higher SH/H SDS, suggesting earlier exposure to sex steroids. The higher SH/H ratio may also explain the increase in BMI SDS observed in the GH-treated children (27). Unfortunately, the study design during follow-up did not allow for the collection of sufficient data on the progression of puberty.

The untreated controls do not only serve as comparison with the GH treated children, but also to illustrate the natural history of ISS. Up to early adolescence, height SDS remained stable at -2.6, but adult height was 0.7 SD higher than height SDS at start, presumably due to a rather delayed and possibly protracted puberty. A similar pattern was seen for SH and LL SDS. This result confirms our and others' earlier findings (28;29). It also shows that HSDS for BA in young children severely over-predicts adult height. However, the average predicted adult height according to Bayley and Pinneau at discontinuation of the trial was almost identical with the attained adult height, consistent with our previous report (30).

In conclusion, high-dose GH treatment limited to the prepubertal period in young children with ISS has no effect on adult height, probably caused by concomitant advance of bone maturation, and may advance pubertal onset.

Acknowledgements

We greatly appreciate the contributions of H.Visser-van Balen and J. Looij in performing measurements, and the financial support of Pfizer (New York, USA) for this study. We express our gratitude to Hans van der Horst, Annelies van Gool, Simone Sanders, and Anna Rompen from Justus Medical Center (Eindhoven, The Netherlands) for facilitating our study.

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Chapter 3

Final height outcome following 3 years of growth hormone and gonadotropin releasing hormone agonist treatment in short adolescents with relatively early puberty

S.A. van Gool[1], G.A. Kamp[2], H. Visser-van Balen[3,6], D. Mul[4], J.J.J. Waelkens[5], M. Jansen[6], L. Verhoeven-Wind[6], H.A. Delemarre-van de Waal[7], S.M.P.F. de Muinck Keizer-Schrama[4], G. Leusink[8], J.C. Roos[9], J.M. Wit[1]

[1]Department of Pediatrics, Leiden University Medical Center, Leiden, the Netherlands.
[2]Department of Pediatrics, Tergooi Hospitals, Blaricum, the Netherlands.
[3]Department of Developmental Psychology, Utrecht University, Utrecht, the Netherlands.
[4]Subdivision Endocrinology, Erasmus University Medical Center/Sophia Children's Hospital, Rotterdam, the Netherlands (current address: Department of Pediatrics, Juliana Children's Hospital, the Hague).
[5]Department of Pediatrics, Catharina Hospital, Eindhoven, the Netherlands.
[6]Department of Pediatrics, University Medical Center Utrecht/Wilhelmina Children's Hospital, Utrecht, the Netherlands.
[7]Department of Pediatrics, VU University Medical Center, Amsterdam, the Netherlands (current address: Leiden University Medical Center, Leiden, the Netherlands).
[8]Diagnostic Center Eindhoven, Eindhoven, the Netherlands.
[9]Department of Radiology, VU University Medical Center, Amsterdam, the Netherlands.

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Abstract

Objective: To assess final height (FH) and adverse effects of combined growth hormone (GH) and gonadotropin releasing hormone agonist (GnRHa) treatment in short adolescents born small for gestational age or with normal birth size (idiopathic short stature, ISS).

Design and patients: 32 adolescents with Tanner stage 2-3, age and bone age (BA) <12 (girls) or <13 (boys) years, height SDS<–2.0 SDS or between –1.0 and –2.0 SDS plus a predicted adult height (PAH_0) <–2.0 SDS, were randomly allocated to receive GH+GnRHa (n=17) or no treatment (n=15) for 3 years. FH was assessed at the age of ≥18 (girls) or ≥19 years (boys).

Results: FH was not different between treatment and control groups. Treated children had a higher height gain (FH–PAH_0) than controls: 4.4 (4.9) and –0.5 (6.4) cm, respectively ($p<0.05$). FH was higher than PAH_0 in 76% and 60% of treated and control subjects, respectively. During follow-up, 50% of the predicted height gain at treatment withdrawal was lost, resulting in a mean gain of 4.9 cm (range –4.0 to 12.3 cm) compared to controls. Treatment did not affect body mass index (BMI) or hip bone mineral density (BMD). Mean lumbar spine BMD and bone mineral apparent density (BMAD) tended to be lower in treated boys, albeit statistically not significant.

Conclusion: Given the expensive and intensive treatment regimen, its modest height gain results and the possible adverse effect on peak bone mineralization in males, GH+GnRHa can not be considered routine treatment for children with ISS or SGA.

Introduction

Short children with a relatively early onset of puberty often attain a poor final height (FH), due to premature growth acceleration and epiphyseal fusion induced by the pubertal rise in sex steroid levels (1). Growth hormone (GH) treatment increases FH in children with idiopathic short stature (ISS) or a persistent short stature after being born small for gestational age (SGA), particularly when initiated in early childhood (2). The effect of GH therapy is dose-dependent, with lower dosages being less efficacious (3;4), whereas too high doses may accelerate growth velocity and stimulate a rapid progression through puberty, possibly limiting FH gain (5). Once puberty has started, GH treatment may limit FH gain, because by that time the process of epiphyseal maturation has already been set in motion (6).

An alternative for GH treatment is to delay or halt pubertal onset with gonadotropin releasing hormone agonists (GnRHa). However, in many children, GnRHa therapy concomitantly decreases growth velocity to values below the normal prepubertal pace, which may at least in part result from accelerated growth plate senescence induced by prior estrogen exposure (7).

Combined GH and GnRHa treatment in adolescents with short stature has been analyzed in a few studies with limited numbers of patients and conflicting results. None of these were randomized controlled trials (RCT) and final height results are scarce. Height gain defined as the difference between FH and baseline height prediction (PAH_0) was 1.0-10.0 cm (8-13). Treatment response was usually analyzed by comparison with a GH-treated group (12;14), or a not randomly assigned, untreated control group (9;9;15) or no control group at all (8). Moreover, most of the trials focused on girls only and excluded children with a persistent short stature after being born SGA.

In 2001, we reported three years' results of the first RCT investigating GH+GnRHa treatment in short, early pubertal adolescents with ISS or SGA. We concluded that three years of GnRHa treatment was effective in suppressing puberty, while growth velocity was preserved due to addition of GH, resulting in a significant gain in PAH without demonstrable side effects (16). Assessment of the motives of adolescents and their parents to participate in this trial and psychosocial functioning of the participants during treatment were described recently (17;18). Psychosocial functioning in young adulthood will be described elsewhere (Visser-van Balen, submitted for publication). Here we report FH data and analyze possible adverse effects of treatment on bone mineral (apparent) density (BM(A)D) and body mass index (BMI).

Material and methods

Patients

This report includes FH results from 32 out of 40 adolescents with short stature that originally enrolled in a multicenter study in the Netherlands in 1993-96. Inclusion criteria were: chronological age (CA) and bone age (BA) <12 (girls) or <13 years (boys); pubertal stage of G2-3 (boys) or B2-3 (girls); height at baseline H_0<–2.0 SDS for Dutch references (19) or between –1.0 and –2.0 SDS with PAH_0 <–2.0 SDS (according to Bailey & Pinneau, (20)); maximum serum GH level >10 µg/l (1 mg = 2IU, using The first International Reference Population of hGH, MRC London, code 66/217 as standard) after provocation (exercise, arginine, clonidine, L-dopa, or glucagon), and a normal sitting height/subischial leg length ratio (between P3 and P97 (21)). Screening blood tests and urinalysis were normal. No organic cause of growth failure, primary bone disease, chronic illness, or dysmorphic syndrome were present. During the trial phase, fasting glucose and insulin levels remained within the normal range in all children. Markers for bone formation (osteocalcin, alkaline phosphatase, PICP and PIIINP) were not different between treatment and control groups. Details of the subjects and data obtained after withdrawal of treatment were reported previously (16).
The protocol was approved by medical ethical review boards at the four participating centers (Amsterdam, Utrecht, Eindhoven, Rotterdam). Before conducting any study-related procedure, written informed consent was obtained from parents, and, when appropriate, also from the participants. This clinical trial was registered in the *meta*Register of Controlled Trials (ISRCTN82161629) of the Current Controlled Trials Ltd.

Study design

Forty patients were randomly allocated to receive combined treatment with GH+GnRHa or no treatment for a period of three years (see fig.1). GH (Genotropin®, Pharmacia, Sweden; now Pfizer, New York, USA) was given in a dose of 1.33 mg (4 IU)/m^2/day subcutaneously, equivalent to 0.05 mg/kg body weight/day. A 3.75 mg 1-month depot preparation of GnRHa was intramuscularly administered (Triptorelin (Decapeptyl®) Ferring, Malmö, Sweden; after withdrawal by Ferring in 1998, triptorelin from Ipsen, Paris, France was used). This corresponded with a mean Triptorelin dose of 125 µg/kg at start of treatment and 67 µg/kg at discontinuation of treatment.

Randomization was performed separately in children born SGA, defined as birth length <–2.0 SDS (22). Directly after randomization, 4 patients (two from each group) refused to start the intervention they were randomly allocated to receive and dropped out. Additionally, 2 patients dropped out from the control group due to a lack of motivation for visiting the outpatient clinic during the trial phase and another 2 (one in each group) could not be traced and were lost to follow-up.
Adolescents were considered to have attained final height if treatment withdrawal was at least 3 years prior to evaluation and chronological age was greater than or equal to 18 (girls) or 19 years (boys). FH, being the average of four measurements made by a single observer using a Harpenden stadiometer, was determined at an outpatient clinic visit or at a house visit.

Bone mineral density

BMD of the lumbar vertebrae and hip were measured by dual-energy X-ray absorptiometry (DXA) at the moment of FH analysis. DXA-scans were performed at the Department of Radiology of the VU University Medical Center in Amsterdam or at the Diagnostic Center Eindhoven using a Hologic Delphi 4500A or a Hologic Delphi W-type 70991 (Hologic, Waltham, MA, USA), respectively. Cross-calibration with phantoms demonstrated that the DXA machines were comparable, and data from both centers were pooled. Each machine was calibrated using the manufacturer's 'daily quality control' protocol. Z-scores were calculated, using hip reference values based on the National Health and Nutrition Examination Survey from 1988-1991 (NHANES) (23) and Hologic reference values for lumbar spine (L1-L4). To correct for bone size, DXA-derived data were used to calculate lumbar spine BMAD (24). BMAD Z-scores could not be calculated due to the lack of appropriate reference values.

Outcome parameters

Four outcome parameters were used to evaluate response to treatment: 1) FH (SDS); 2) FH minus baseline height (H_0) (SDS); 3) FH minus PAH_0 according to Bayley and Pinneau (20) (cm); and 4) FH minus target height (TH; cm). FH minus PAH_3 (PAH at discontinuation of treatment) was also calculated, to show the difference between final outcome and height prediction at discontinuation of treatment. All SD scores were based on Dutch references (19). For calculation of FH SDS, the age of each patient was set at 21 years, enabling comparison of FH with height distribution in the normal adult population. For 3 patients, a bone age radiograph at baseline

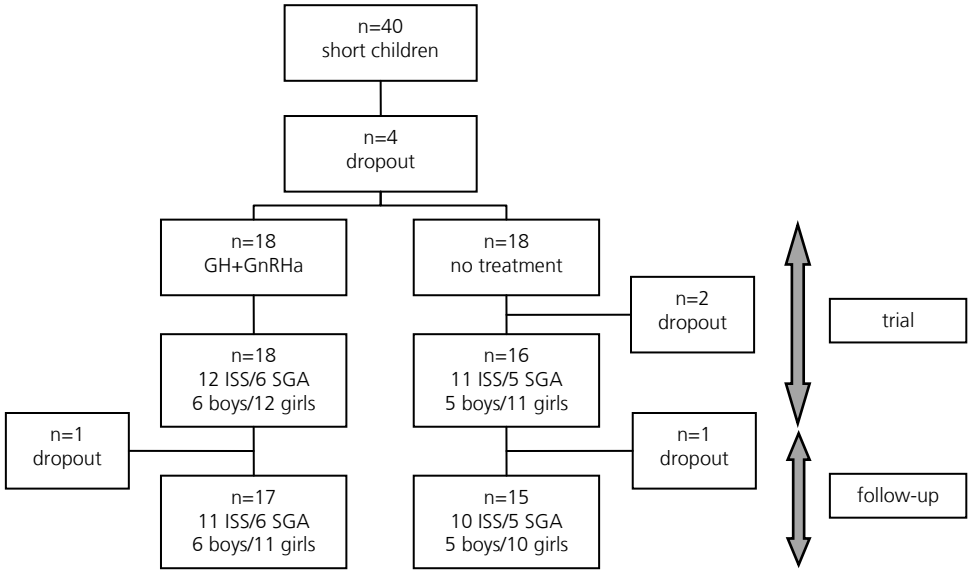
was not available, and BA determination closest to this time point was used and transformed to $BA_{baseline}$ as follows: $BA_{baseline}$ = (BA_{visit}/ Chronological age visit) x $Age_{baseline}$.

TH was calculated as midparental height plus or minus 6 cm (for boys and girls, respectively), plus 3 cm to correct for the average secular trend in the Netherlands (25). One patient's TH could not be calculated, because of adoption from a foreign country and absence of height data of his biological parents.

The following outcome parameters were used to analyze possible effects of GH+GnRHa treatment on bone mineralization and body weight: 1) Lumbar spine BMD (SDS) and BMAD (g/cm^3); 2) Hip BMD (SDS); and 3) BMI (SDS).

Figure 1

Trial design.



Statistical analysis

The study was designed to compare the effects of GH+GnRHa treatment with those of no treatment on final height. Statistical analyses were performed using the statistical package SPSS, version 11.0.1 (26). Results are expressed as mean and SD. Comparisons among treatment and control groups were made using Student's unpaired *t*-tests. Possible interactions between GH+GnRHa treatment and the baseline predictors gender, diagnostic subgroup, age at baseline (age_0), height at baseline (H_0SDS), and bone age delay (CA–BA) were analyzed by means of linear regression analysis using ANOVA. The significance level was set at 0.05.

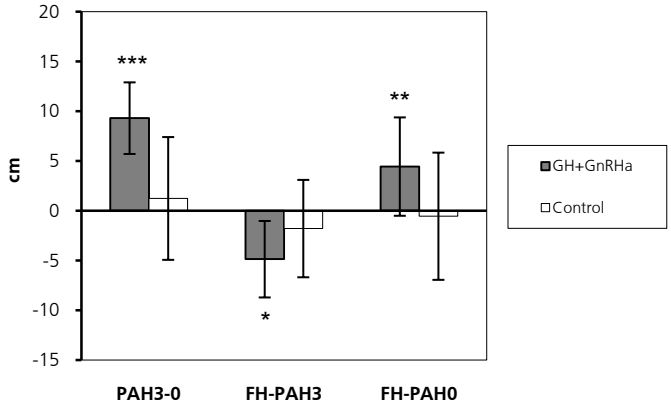
Results

A complete case analysis was carried out for the remaining 32 out of 40 originally included patients (80%). One patient from the GH+GnRHa group discontinued treatment after 2 years due to a lack of motivation, but her near-final height two years after termination of treatment was included in the evaluation. Anthropometric data were not statistically different between treatment and control groups at baseline, but predicted adult height and target height happened to be about 3 cm lower in the treatment group. During the trial phase, clinical (Tanner stage) and biochemical (serum estradiol and testosterone levels) evaluation demonstrated that pubertal development was effectively arrested in the GH+GnRHa group, while puberty progressed in the control group (16). After withdrawal of therapy, pubertal development immediately resumed, with most children from the GH+GnRHa group reaching Tanner V development within 1-2 years and most girls reaching menarche within 1 year. All participants were sexually mature at final analysis.

There was a significant gain in PAH of 9.3 cm after three years of GH+GnRHa treatment, compared with 1.2 cm gain in the control group (fig. 2a). There was no significantly different pattern between the two diagnostic subgroups and genders in changes in height parameters.

Figure 2

Height gain compared with predicted adult height at baseline (FH-PAH_0) and after discontinuation of treatment (FH-PAH_3); in cm. *p<0.1; **p<0.05; ***p<0.001.



Effect on growth

Patient characteristics at start, after treatment discontinuation, and at the moment of FH analysis are listed in table 1. FH data were collected at a mean age of 19.9 (1.8) and 20.9 (1.0) years in girls and boys, respectively. The mean treatment period was 3.0 yr (range 2.8-3.3 yr). The mean period elapsed

between treatment discontinuation and final analysis was 5.7 yr (range 3.6-8.7 yr).

FH SDS [–2.0 (1.0) vs. –2.3 (0.6)] and height gain compared with H_0SDS [0.5 (0.9) vs. 0.3 (0.6)] were not significantly different between treated and control subjects. In the GH+GnRHa group, 53% reached a FH>–2.0 SDS, compared with 33% of control patients (difference not statistically significant). The treated subjects attained a FH closer to TH than controls [–6.0 (7.2) vs. –11.2 (5.7) cm; p<0.05], but mean FH of treated subjects was still significantly lower than TH (p<0.01).

Height gain (FH–PAH_0) was 4.4 (4.9) in the treatment and –0.5 (6.4) cm in the control group (fig. 2b). Of the predicted 9.3 cm gain in PAH (PAH_3–PAH_0) approximately 50% was lost during follow up (fig. 2), resulting in a net gain of 4.9 cm in the treatment group, compared to controls. Seventy-six percent of the GH+GnRHa treated children and 60% of the controls had a FH greater than predicted at baseline.

Height gain was similar in both genders, but there was a difference in the accuracy of the height prediction method between genders. Untreated boys attained a FH 5.5 (4.1) cm lower than predicted at baseline, whereas untreated girls became 1.9 (6.0) cm taller. Treated girls had a FH significantly closer to TH than controls ($p<0.05$), whereas no significant difference existed between treated and untreated boys.

Linear regression analysis of predictors for growth response

Gender was a predictor for the treatment effect (GH+GnRHa∗gender) expressed in FH–TH, with girls showing a better response than boys (regression coefficient B=10.7, CI=1.3–20.2, $p<0.05$; graph not shown). Age at baseline showed a trend towards a positive interaction with treatment effect (GH+GnRHa∗Age_0) in the outcome parameters FH SDS minus H_0SDS (B=0.7, CI= –0.01–1.5, $p<0.1$), and FH SDS minus PAH_0 SDS (B=0.8, CI= –0.1–1.7; $p<0.1$). Baseline height and diagnostic subgroup were no significant predictors for treatment effect in any of the outcome parameters.

Side effects

Lumbar spine and hip BMD were determined in 21 patients (69%), 15 treated and 6 control subjects; the others could not be motivated. There was no apparent difference in lumbar spine and hip BMD SDS between treated and untreated children (fig. 3a, c). Lumbar spine BMD SDS was markedly lower in the six treated boys (–2.5 (1.0) SDS), than in the two controls (–1.1 (0.3) SDS) who consented to undergo this diagnostic procedure (p=0.1; CI=–0.5-3.3). This difference was statistically not significant due to the small sample size of the control group (n=2). Lumbar spine BMAD showed a similar, albeit less prominent pattern (fig. 3b). BMI SDS remained unchanged during and after GH+GnRHa treatment in boys and girls.

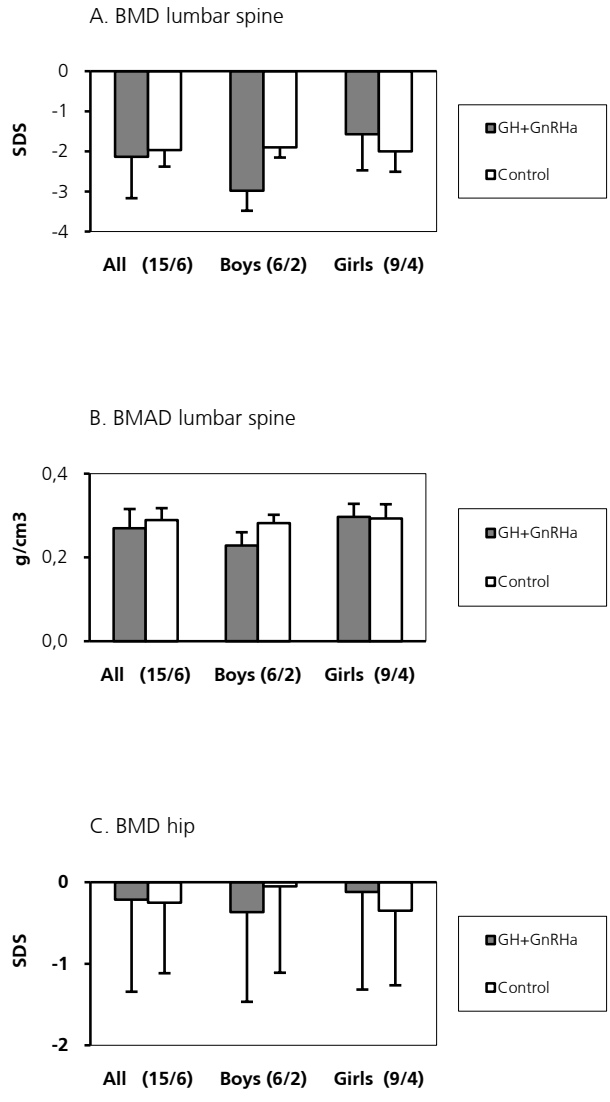
Table 1. Summary of initial and outcome variables (mean (SD))

Parameter	All			Boys			Girls		
	GH+GnRHa (n=17)	Control (n=15)	R_x vs. Co	GH+GnRHa (n=6)	Control (n=5)	R_x vs. Co	GH+GnRHa (n=11)	Control (n=10)	R_x vs. Co
Boys/girls	6/11	5/10							
SGA/ISS	6/11	5/10		2/4	2/3		4/7	3/7	
Start treatment									
Age_0 (yr)	11.6 (0.7)	11.8 (0.7)	ns	11.9 (0.8)	12.5 (0.6)	ns	11.4 (0.6)	11.5 (0.5)	ns
Bone age delay (yr)	0.7 (1.0)	1.0 (0.7)	ns	1.1 (1.0)	1.2 (0.7)	ns	0.5 (0.9)	0.9 (0.8)	ns
H_0 (cm)	135.4 (4.5)	136.1 (4.5)	ns	133.1 (3.8)	138.4 (5.8)	ns	136.7 (4.5)	134.9 (3.4)	ns
H_0SDS	-2.4 (0.5)	-2.5 (0.5)	ns	-2.7 (0.4)	-2.5 (0.5)	ns	-2.3 (0.5)	-2.6 (0.5)	ns
PAH_0 (cm)	157.4 (8.3)	160.0 (10.1)	ns	166.7 (4.9)	170.7 (8.7)	ns	152.4 (5.6)	154.7 (5.6)	ns
PAH_0SDS	-2.7 (0.7)	-2.3 (1.0)	ns	-2.5 (0.7)	-1.9 (1.2)	ns	-2.8 (0.7)	-2.5 (0.9)	ns
TH (cm)	167.4 (10.0)	170.7 (5.8)	ns	176.9 (9.6)	174.9 (4.1)	ns	163.1 (6.9)	168.6 (5.5)	ns
BMI_0SDS	-0.6 (0.9)	-0.5 (1.0)	ns	-0.7 (1.4)	0.0 (0.5)	ns	-0.6 (0.5)	-0.8 (1.0)	ns
Discontinuation of treatment									
Age_3 (yr)	14.9 (0.7)	14.6 (0.7)	ns	15.4 (0.6)	14.9 (0.8)	ns	14.6 (0.5)	14.4 (0.6)	ns
H_3 (cm)	154.6 (5.5)	152.9 (5.4)	ns	159.7 (6.7)	152.1 (5.9)	ns	152.1 (2.5)	153.3 (5.3)	ns
H_3SDS	-2.1 (0.8)	-2.2 (0.4)	ns	-2.7 (0.7)	-2.2 (0.6)	ns	-1.8 (0.7)	-2.1 (0.3)	ns
PAH_3 (cm)	166.7 (8.1)	161.3 (9.5)	P<0.1	174.0 (6.9)	169.3 (12.3)	ns	162.8 (5.8)	157.3 (4.6)	p<0.05
PAH_3SDS	-1.3 (0.9)	-2.1 (1.1)	p<0.05	-1.4 (1.0)	-2.1 (1.7)	ns	-1.2 (0.9)	-2.1 (0.7)	p<0.05
BMI_3SDS	-0.3 (1.0)	-0.4 (1.1)	ns	0.0 (1.1)	-0.6 (1.8)	ns	-0.5 (1.1)	-0.2 (0.4)	ns
At final height									
Age_f (yr)	20.3 (1.6)	20.2 (1.8)	ns	20.4 (0.8)	21.5 (1.0)	ns	20.2 (1.9)	19.5 (1.7)	ns
Final height (cm)	161.8 (6.3)	159.5 (5.7)	ns	165.6 (5.7)	165.2 (5.1)	ns	159.8 (5.8)	156.6 (3.3)	ns
FHSDS	-2.0 (1.0)	-2.3 (0.6)	ns	-2.6 (0.8)	-2.7 (0.7)	ns	-1.7 (0.9)	-2.2 (0.5)	ns
FH-H_0 (SDS)	0.5 (0.9)	0.3 (0.6)	ns	0.2 (0.7)	-0.2 (0.4)	ns	0.6 (1.0)	0.4 (0.6)	ns
FH-PAH_0 (cm)	4.4 (4.9)	-0.5 (6.4)	p<0.05	-1.0. (2.4)	-5.5 (4.1)	p<0.1	7.4 (3.0)	1.9 (6.0)	p<0.05
FH-PAH_3 (cm)	-4.9 (3.8)	-1.8 (4.9)	p<0.1	-8.3 (3.1)	-4.0 (7.4)	ns	-3.0 (2.8)	-0.7 (3.0)	p<0.1
FH-TH (cm)	-6.0 (7.2)	-11.2 (5.7)	p<0.05	-11.8 (6.5)	-9.7 (6.9)	ns	-3.3 (5.9)	-12.0 (5.3)	p<0.05
BMI_fSDS	0.0 (0.8)	0.1 (0.9)	ns	-0.4 (0.9)	0.3 (1.2)	ns	-0.3 (0.5)	0.0 (0.7)	ns

Mean (SD). Rx, treatment with GH+GnRHa; Co, no treatment; ns, no significant difference.

Figure 3

Effect of GH+GnRHa treatment on (a) lumbar spine BMD expressed as Z-score, (b) lumbar spine BMAD expressed in g/cm^3, and (c) total hip expressed as Z-score. Error bars represent standard deviations.



Discussion

We report final height results of the first RCT of combined GH+GnRHa treatment in short boys and girls, that includes a non-treated control group. Treatment resulted in a modest increase of approximately 5 cm compared to controls, as documented by difference between obtained adult height and initial predicted adult height and target height. The reason that the differences in final height SDS as such and the change in height SDS were not statistically significant is probably that the predicted adult height at start, as well as the target height, of the children randomly allocated to the treatment group happened to be approximately 3 cm lower than in controls.

Two previously published studies employed untreated controls as reference group. Saggese *et al.* found an increase in PAH of 6.1 cm after two years of GH+GnRHa in 7 ISS girls compared with controls (27). In contrast, Lanes and Gunczler (9) found no improvement in PAH or FH after 2.5 years of treatment in 10 short boys and girls. Other studies with different experimental designs showed similarly contrasting results on growth, varying from no effect on FH or PAH gain in girls with familial short stature (8) and in ISS boys and girls (14), to 10.5 cm gain in PAH and 10 cm height gain ($FH–PAH_0$) in ISS girls (12). These studies and ours cannot easily be compared due to differences in experimental design. GH+GnRHa trials in short adopted girls showed an increased PAH, $FH–PAH_0$ and FH compared with GnRHa-treated controls (10;13). Mul *et al.* suggested that the relevance of their results in adopted girls might be extrapolated to girls born SGA and those with ISS (10). Indeed, in our trial we found significantly increased PAH, height gain and FH compared with TH in the treated girls.

Approximately 50% of the apparent gain in PAH during treatment was lost during follow up. Initial height prediction overestimated FH in untreated boys, which has also been described by others (28). Height gain in terms of $FH–PAH_0$ is similar in both genders, although the accuracy of height prediction depends on gender. Untreated boys attain a FH considerably lower than predicted at baseline, whereas girls become slightly taller. Prediction at discontinuation of treatment overestimated final height, regardless of gender. A longer duration of combined therapy might further increase height gain, but would delay puberty even longer, up to an age that would far exceed the normal range. This might cause adverse effects on psychological and bone parameters. However, prolonging GH treatment after discontinuation of combined treatment until FH may be a way to fulfil part of the height gain predicted in PAH_3.

Gender did not influence the response to GH+GnRHa in three out of four outcome parameters. Only for FH–TH a better response to treatment was seen in girls. Previously published reports on GH+GnRHa treatment were usually conducted in short girls only, except a few small trials

including both genders (9;14). The inclusion of boys in our trial, might explain a somewhat lower overall response to treatment compared to other studies on GH+GnRHa treatment in short girls. The predictive value of gender on height outcome is limited in this study, since an effect was found in only one outcome measure (FH-TH).

We included an SGA subgroup, even though this category of patients was excluded from the 1996 consensus definition of ISS (29) and from most of the previously published studies. In our opinion the distinction between ISS and idiopathic SGA is arbitrary, due to varying definitions and reference values for length and weight for gestational age. In the ISS consensus, SGA was defined as a birth weight and/or length <–2.0 SDS for gestational age. This definition implies that a child with a birth weight in the lower normal range, may be categorized as ISS in case of an unknown birth length, and as SGA if birth length would be recorded as <–2.0 SDS. Moreover, the Gaussian distribution of birth weight and length in ISS is shifted to the left by 1 SDS (30), suggesting that persistent short stature born SGA and ISS are not separate entities. SGA and ISS children in our study had a similar response to treatment, demonstrating that diagnostic subgroup did not interact with the treatment effect. Similarly, Crowe *et al.* found no difference in response to GH treatment between SGA or ISS groups (31). However, patient numbers in our subgroups were small and larger sample numbers are required to analyze response to definitely confirm or reject our hypothesis that SGA and ISS children respond similarly to combined GH+GnRHa treatment.

Apart from beneficial effects on height gain, GH+GnRHa treatment may also have adverse effects. Palmert *et al.* found an elevated BMI in GnRHa-treated girls with central precocious puberty (CPP) (32). We did not find an increased BMI, neither directly after discontinuation of treatment (16) nor at final height analysis. The stimulatory effect of GnRHa on fat mass induction may have been counterbalanced by an adverse effect of GH in the treated children. The elevated BMI in GnRHa-treated girls from the American trial was possibly associated with precocious puberty itself rather than with the treatment.

A more relevant possible side-effect of GnRHa treatment was described by Yanovski *et al.* (33), who found a significantly decreased lumbar spine BMD after 4 years GnRHa treatment, with or without GH, of children with short stature based on various etiologies. A similar result was reported after 3.4 years of GnRHa treatment in girls with ISS (34). In GnRHa-treated children with CPP, lumbar spine BMD was significantly decreased during treatment (35), but restored to normal values several years after withdrawal of treatment (34;35), suggesting that GnRHa-therapy did not impair peak bone mass in this subcategory of patients. In contrast, Finkelstein *et al.* demonstrated that adult men with a history of delayed puberty had a decreased radial and spinal BMD (36), suggesting that the timing of puberty is an important determinant of peak

bone density in men. BMD achieved during young adulthood might be a major determinant of bone density and a predictor for the risk of osteoporotic fractures in later life.

In our study, there was no apparent difference between treatment and control groups in lumbar spine and hip BMD measured at least 3.5 years after termination of treatment. However, the six treated boys had a lower lumbar spine BMD and BMAD than the 2 untreated boys, albeit statistically not significant. A meta-analysis by Marshall *et al*. demonstrated that with each 1 SDS decrease in lumbar spine BMD, fracture risk increased 2.3 fold (37). Therefore, we believe that there is sufficient reason to be cautious when considering GH+GnRHa treatment in boys. On the other hand, it has been shown that after interruption of skeletal development in childhood, there is still potential for catch-up in BMD, even ongoing into the third decade of life (38;39). Taking this into account, it remains to be seen whether the low lumbar spine BMD in treated boys has clinical repercussions and whether it will restore to normal values.

In girls, there was no difference in lumbar spine and hip BMD between treated and control groups. This may be explained by an immediate rise in estrogen to high levels after treatment withdrawal in girls, favoring bone mineralization. In boys, the post-treatment rise in estrogen levels may be slower and reach a lower maximum, resulting in a later and possibly lesser stimulation of bone mineralization.

Apart from these clinical issues, psychosocial issues should also be taken into account. Analyses of psychosocial functioning during treatment tentatively suggested adverse psychological consequences for the treated adolescents on self-perceived competence of scholastic and athletic ability, and trait anxiety (17). Parents reported some behavioral problems in their children before treatment (18), but did not report changes during treatment. Psychosocial outcome after attaining final adult height was one of the final evaluation criteria of this trial and so far no differences have been observed between treatment and control groups in social circumstances, height-related psychosocial stressors, perceived competence, and psychological distress in young adulthood (Visser-van Balen, submitted for publication).

We conclude that 3 years of GH+GnRHa treatment has a positive, but modest effect of 4.9 cm on final height in short, early pubertal children with ISS or a persistent short stature born SGA, compared with non-treated controls. The costs of long term treatment with GH+GnRHa of short, but otherwise healthy patients, with extremely expensive drugs that require parenteral administration and regular clinic visits, and may have an adverse effect on peak bone mineralization, particularly in males, overshadows the modest benefit of 4.9 cm height gain. We therefore do not recommended this treatment regimen in general. However, treatment may be felt justified in individual patients, particularly girls, with an extremely low adult height prediction, an early pubertal onset and considerable psychosocial problems.

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Part B

Chapter 4

Marginal growth increase, altered bone quality and polycystic ovaries in female prepubertal rats after treatment with the aromatase inhibitor exemestane

S.A. van Gool[1], J.M. Wit[1], T. de Schutter[2], N. de Clerck[2], A.A. Postnov[2], S. Kremer Hovinga[1], J. van Doorn[3], S.J. Veiga[4], L. Miguel Garcia-Segura[4], M. Karperien[5]

[1]Department of Pediatrics, Leiden University Medical Center, Leiden, the Netherlands.
[2]Department of Biomedical Sciences and Department of Physics, University of Antwerp, Antwerp, Belgium.
[3]Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, Utrecht, the Netherlands.
[4]Instituto Cajal, Consejo Superior de Investigaciones Científicas, Madrid, Spain.
[5]Department of Tissue Regeneration, Biomedical Technology Institute, Twente University, the Netherlands.

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Abstract

Background: Aromatase inhibition has been proposed as a potential approach for growth enhancement in children with short stature, but detailed animal studies are lacking.

Aim: to assess the effect and potential adverse effects of aromatase inhibition on growth in female rats.

Methods: Prepubertal Wistar rats received intramuscular injections with placebo (PLC) or the aromatase inhibitor exemestane at a dose of 10, 30 or 100 mg/kg/week (E10, E30, E100) for 3 weeks. A control group was ovariectomized (OVX). Weight and length gain, tibia and femur length, growth plate width, organ weights, insulin-like growth factor I (IGF-I) levels, and histology of the ovaries, uterus, and brain were analyzed. X-ray microtomography of femora was performed.

Results: E100 significantly increased weight gain and growth plate width, but less prominently than OVX. Trabecular number and thickness were decreased in E100 and OVX in the metaphysis and epiphysis. E100 significantly decreased ovarian weight and multiple cysts were seen upon histological evaluation. No significant effects were found on IGF-I levels and brain morphology in E100. E10 and E30 had no effects on growth.

Conclusion: a high dose of exemestane marginally increases axial and appendicular growth in female rats, at the expense of osteopenia and polycystic ovaries.

Introduction

Estrogens play a pivotal role in the regulation of normal skeletal growth and maturation in both boys and girls (1-5). In both genders, estrogen deficiency due to mutations in the aromatase gene (6) leads to the absence of a pubertal growth spurt, ongoing growth into adulthood and potentially tall stature. Based on these clinical observations, it was postulated that estrogen deficiency induced by aromatase inhibition, could be valuable in clinical practice because of its potential growth-enhancing effect (7;8).

Treatment with the nonsteroidal aromatase inhibitor letrozole resulted in an increased predicted adult height in boys with idiopathic short stature (ISS) (9;10) in two clinical trials performed by the group of Dunkel *et al.* In one of those trials, treatment with a combination of letrozole and testosterone increased near-final height in boys with a constitutional delay of puberty (11). There is limited experience with the use of aromatase inhibitors in female patients with short stature, since therapy-induced hyperandrogenism and consequent virilization may be expected, as observed in female aromatase-deficient patients. However, it is uncertain whether treatment with aromatase inhibition limited to the prepubertal period, with the objective to increase longitudinal growth and adult height, would cause persistent virilization and disturbance of reproductive function after cessation of treatment.

In girls with McCune-Albright syndrome (MAS) (12;13) or congenital adrenal hyperplasia (CAH) (14), treatment with the aromatase inhibitor testolactone or letrozole resulted in attenuated height velocity and bone maturation rates, resulting in a poor adult height prediction. These results are in conflict with the potentially growth-enhancing effect of aromatase inhibition in boys with ISS, but may also be the reflection of the complex endocrine deregulation in MAS and CAH patients. The reported clinical trials in girls were uncontrolled, and usually did not include an evaluation of bone mineral density (8).

The broad tissue distribution of aromatase expression in humans underscores the importance of locally produced estrogens (15). Therefore, aromatase inhibition for the purpose of growth enhancement may have side effects on several organ systems. Aromatase-deficient females are diagnosed with 46,XX DSD (disorder of sex development) at birth and show progressive bone age delay, eunuchoid proportions, and reproductive dysfunction based on primary amenorrhea and enlarged, polycystic ovaries in adolescence (6). Bone mineral density has not been studied in these patients, but one could hypothesize that they would develop osteoporosis, as it occurs in adult men with congenital estrogen deficiency (2). Treatment of adolescents girls with a gonadotropin releasing hormone agonist (GnRHa), an alternative way to diminish circulating estrogen levels, indeed resulted in a decreased bone mineral density (16). Moreover, aromatase expression plays an important role in the brain under both physiological and pathological

conditions (17). Aromatase inhibition may also have an impact on the brain, since aromatase expression was found to have a neuroprotective role both under physiological and pathological conditions (17).

In the present article, we report the results of a study in which female sexually immature rats were treated with the steroidal, irreversible aromatase inhibitor exemestane (E) in a slow-release preparation in order to test the hypothesis that aromatase inhibitors can increase longitudinal growth and to evaluate potential side-effects. Treatment was short-term and confined to the period of sexual immaturity in order to mimic the potential treatment strategy in girls with ISS.

We chose to work with exemestane in our experiments, because it can be administered once a week as a slow release preparation to ensure long-lasting aromatase inhibition, in contrast to the other available aromatase inhibitors that have to be administered daily. This facilitates its use in experimental animal models, and it presumably would also increase compliance in clinical practice. A second advantage of exemestane over the other aromatase inhibitors is that it irreversibly inactivates aromatase and completely abolishes estrogen biosynthesis, which can only be overcome by de novo synthesis of aromatase. We applied a slow release depot of exemestane to continuously counteract de novo synthesis of aromatase. Finally, exemestane may exhibit androgenic effects on bone, potentially preventing the loss of bone mineral density (BMD) induced by estrogen deficiency (18), which may be an advantage when treatment with aromatase inhibitors is considered in clinical practice.

In addition to the effects of aromatase inhibition on growth, we also studied possible adverse effects of aromatase inhibition on bone physiology, brain morphology, and morphology of the ovaries and uterus. This study is the first detailed analysis of the impact on growth and potential side effects of aromatase inhibition in a female animal model.

Experimental design

Animals

Weight-matched female Wistar rats (n=30) were purchased from Harlan (Horst, The Netherlands). At the start of the experiments, all rats were 26 days old, and sexually immature, since rats usually enter sexual maturation at the age of 30 days. Animals were kept in a light and temperature controlled room (12 hours light, 20-22°C) with water and food (Hope Farms, Woerden, The Netherlands) ad libitum and a maximum of 3 animals per cage. Experiments were approved by the Committee for the ethical care and use of laboratory animals of the Leiden University.

Five groups consisting of 6 rats were formed. One group was ovariectomized at baseline by the dorsal approach under isoflurane anaesthesia in order to obtain an estrogen-deficient control group (OVX). In previous experiments, no difference in growth phenotype was found between sham-operated and placebo-treated control-group rats (19). Therefore, no sham-group was included in this study. Three groups were treated with the slow-release preparation exemestane (Aromasin® provided by Pfizer, New York, U.S.A) at a dose of 10, 30 or 100 mg/kg body weight/week (coded as E10, E30, E100, respectively). Exemestane was administered by intramuscular injections in alternated hind paws. A control group was treated with vehicle (placebo, PLC). After three weeks of treatment, all rats were sacrificed.

Weekly measurements of nose-anus length, tail length and body weight were performed. At the end of the experimental period, animals were anaesthetized by intraperitoneal Nembutal injection and blood samples were collected by means of cardiac puncture. Serum was separated for insulin-like growth factor I (IGF-I) measurements. Femora were collected for X-ray microtomography (micro-CT). Subsequent *in vivo* fixation was performed by means of transcardiac perfusion with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer supplemented with 75 mM lysine monohydrochloride and 10 mM Na-periodate. After fixation, the rats were decapitated, and the brains were stored in the same fixative for at least 12 weeks. Tibiae of the right hind legs were measured, postfixated for 24 hours, decalcified in 12.5 % EDTA, pH 7.4 for 4 weeks, cut in halves in a sagittal orientation and subsequently processed for paraffin embedding. Ovaries and uterus were weighed and processed for paraffin embedding. The organ weight/total body weight (TBW) ratio was calculated.

Growth plate histomorphometry

For histomorphometrical analysis of the growth plate, paraffin sections (5 µm) perpendicular to the growth plate axis were mounted on APES/glutaraldehyde-coated slides, and deparaffinized in Paraclear® and graded ethanols. They were stained with Haematoxylin/Eosin (H&E) and mounted in Histomount (National Diagnostics, Atlanta, USA). Representative pictures of the proximal tibial growth plate were taken (Nikon DXM 1200 digital camera). Widths of the total growth plate, proliferative zone and hypertrophic zone were measured in ImagePro Plus®. The ratio between proliferative and hypertrophic zone widths (P/H ratio) was calculated.

X-ray microtomography (micro-CT)

To examine the effects of exemestane treatment on whole bone parameters and on trabecular bone in particular, right femora were analyzed by high resolution X-ray microtomography. Micro-CT has been shown to be well suited for the non-destructive visualization and quantitative analysis of bone and calcified tissue (20;21).

a. 'Whole bone' parameters

Isolated whole femora were scanned with a high resolution desktop micro-CT scanner (Skyscan 1076, Kontich, Belgium). In this X-ray micro-CT system, both the X-ray source (focal spot size 5 µm, energy range 20-100 keV) and the detector (CCD camera 2.3kx4k) rotate around the bone. Scans were isotropic and a voxel size of 35×35×35 µm was chosen with 1.0 mm A1 filter. Virtual cross-sections were reconstructed by Feldkamp cone-beam algorithm (22). Further technical details about the scanner have been published elsewhere (23).

The same threshold and reconstruction parameters were used for the reconstruction of all cross-sections. Frequency distribution of the grey levels was analyzed. 3D models were created using Skyscan software packages ANT and CT Analyzer. From the virtual cross-sections the volumes taken by bone as well as the anatomical volume (Archimedean volume) were calculated. Distinction between bone and other tissues was based on thresholding of the grey values in the reconstruction. Analysis of the grey values in the individual slices also resulted in a relative value for total calcium and overall calcium density. Details of these calculations have been reported previously (20). In addition, the length of the femora was measured manually with a caliper.

b. Trabecular bone

For analysis of the trabecular bone, the distal end of the femur was scanned, but for this purpose at a higher resolution than required for the 'whole bone' scans. Therefore, an *in vitro* desktop micro-CT system (Skyscan 1072, Kontich, Belgium) with a rotating stage was used (24). Polychromatic X-rays, with peak energy of 80 kV, were generated by a microfocus X-ray source with a focal spot size of 8 microns. A (1024×1024) 12 bit CCD camera was used as detector. In all experiments the rotation step was 0.9°, acquisition time 6 sec/projection, and total acquisition time about 2 hours. Scanning was isotropic with a voxel size of 13 μm^3.

The growth plate was chosen as a reference for selecting comparable and reproducible regions of interest for analysis of trabecular bone parameters such as trabecular number, trabecular thickness, bone volume and distribution of trabecular thickness. Trabecular bone parameters were studied in two different regions of the femur: the metaphysis (primary ossification center)

and the epiphysis (secondary ossification center). For analysis of the primary ossification center at the proximal side of the growth plate, a transverse reference slice of the growth plate was selected and used to reconstruct a cylinder with fixed dimensions as from 50 slices above the reference slice; the height of the cylinder was 150 slices.

Gonadal histology

Midsagittal sections of the ovaries (5 µm) were stained with Haematoxylin and Eosin (H&E). A minimum of three sections was analyzed per ovary. In H&E stained longitudinal sections (5 µm) of the uterus, endometrial thickness was measured using ImagePro Plus® and the endometrium/total wall thickness ratio (E/T-ratio) was calculated.

Brain histology

Brains were removed from the skulls, rinsed with PBS and cut in coronal sections (30 µm) in a Vibratome. Sections were Nissl-stained with toluidine blue for screening morphometrical analysis of all brain areas. Immunohistochemical staining of astrocytes (anti-GFAP, Glial Fibrillary Acidic Protein, DAKO, Barcelona, Spain; diluted 1:1000), microglia (OX-42, Serotec, Bicister, United Kingdom; diluted 1:300) and reactive astrocytes (anti-vimentin, clone V9, DAKO, Barcelona, Spain; diluted 1:500) was performed. For the morphometric analysis of GFAP immunoreactive astroglia, a quantitative evaluation of the surface density of GFAP immunoreactive cell bodies and cell processes in the hippocampus was performed using a stereological grid, according to the point-counting method of Weibel (25). For each animal, a minimum of two slides was evaluated.

Insulin-like Growth Factor I (IGF-I) assay

Total serum IGF-I levels were measured by specific radioimmunoassay (RIA) after acid Sep-Pak C18 (Waters Associates Ltd., Milford, MA) extraction of 250 µl aliquots, following procedures as described previously (26).

Statistical analysis

The study was designed to analyze the effect of aromatase inhibition on growth and to analyze potential adverse effects on bone, brain and organ development. Comparisons of growth among treatment and control groups were made using one-way analysis of variance (ANOVA). Results are expressed as mean and SD. The significance level was set at 0.05.

Results

Effects on growth and organs

At baseline, there were no significant differences between groups in auxological parameters. Body weight gain, nose-anus length gain and tail length gain, tibia length, growth plate characteristics, and IGF-I levels are summarized in table 1. The effects of aromatase inhibition on organ weights are summarized in table 2. Figure 1 illustrates nose-anus length gain in OVX, PLC and E100. Figure 2 shows growth plate samples from these groups. Results in the E10 and E30 groups were not significantly different from the placebo-group and are therefore not presented.

Body weight gain was significantly increased in both E100 and OVX ($p=0.000$ and $p=0.001$, respectively) compared with PLC. Nose-anus length gain was significantly higher ($p=0.000$) in OVX compared with PLC. Figure 1 illustrates that the increased length gain in OVX was already prominent one week after ovariectomy. A less pronounced increasing nose-anus length gain with a trend towards statistical significance ($p=0.054$) was seen in the E100 group. The difference in nose-anus length gain between OVX and E100 was statistically significant ($p=0.021$). When compared to the rats that received placebo-treatment, for both E100 and OVX no effect on tail length gain was found. Total growth plate width in the E100 group was increased ($p=0.000$), based on equal thickening of both the proliferative and hypertrophic zones compared with PLC. The P/H ratio was not significantly different from controls. Similar effects were found in OVX. The serum IGF-I level was raised in OVX ($p=0.000$), but not significantly in E100, compared to PLC.

In the OVX group, a significant decrease of 15% of the liver/TBW ratio was found ($p=0.042$). The increased thymus/TBW ratio found in OVX ($p=0.000$), was not seen in the E100 group.

Table 1: Effect of aromatase inhibition on growth parameters and epiphyseal morphology

Parameter	PLC	E100	OVX
Tail length gain (cm)	7.2 (0.7)	7.3 (0.4)	7.9 (0.5)
Nose-anus length gain (cm)	5.0 (0.7)	5.7 (0.8)$^{\#\,\wedge}$	6.5 (0.4)***
Body weight gain (g)	96.7 (10.2)	120.6 (7.3)***	115.0 (7.6)**
Tibia length (mm)	30.7 (0.4)	31.2 (0.7)$^{\#\,\wedge}$	31.7 (0.3)**
Femur length (mm)	26.6 (0.4)	27.6 (0.4)*	27.0 (0.2)
Growth plate width (mm)	0.34 (0.02)	0.42 (0.02)***	0.44 (0.02)***
proliferative zone (mm)	0.14 (0.01)	0.18 (0.01)***	0.18 (0.01)**
hypertrophic zone (mm)	0.20 (0.02)	0.24 (0.02)*	0.26 (0.02)**
P/H-ratio	0.70 (0.05)	0.77 (0.1)§	0.67 (0.05)
Serum IGF-I (ng/ml)	0.9 (0.04)	1.1 (0.16)$^{\wedge}$	1.4 (0.25)*

Means (SD). E100 or OVX versus PLC: *p<0.05; **p<0.01; ***p<0.001; $^{\#}$p<0.1.
E100 versus OVX: $^{\wedge}$p<0.05; §p<0.1.

Table 2. Effect of aromatase inhibition on organ weights

Parameter	PLC	E100	OVX
liver	70.9 (10.8)	70.3 (6.3)§	59.7 (11.1)*
thymus	2.9 (0.4)	3.3 (0.5)$^{\wedge\wedge}$	4.0 (0.5)***
kidney	5.5 (0.3)	5.8 (1.1)§	5.1 (0.6)
adrenal gland	0.23 (0.04)	0.19 (0.05)$^{\#\wedge}$	0.23 (0.03)
ovary	0.2 (0.03)	0.1 (0.02)***	
uterus	2.6 (0.8)	2.1 (0.9)$^{\wedge\wedge\wedge}$	0.2 (0.04)***
endometrium thickness (µm)	0.43 (0.10)	0.31 (0.10)$^{*\,\wedge\wedge}$	0.14 (0.05)***
total wall thickness (µm)	0.75 (0.06)	0.57 (0.12)$^{**\,\wedge\wedge}$	0.26 (0.09)***
E/T-ratio	0.57 (0.11)	0.54 (0.11)	0.57 (0.09)

Organ weights as permillage of total body weight. Means (SD). E/T-ratio, ratio between endometrium thickness and thickness of the total uterus wall.
E100 or OVX versus PLC: *p<0.05; **p<0.01; ***p<0.001; $^{\#}$p<0.1.
E100 versus OVX: $^{\wedge}$p<0.05; $^{\wedge\wedge}$p<0.01; $^{\wedge\wedge\wedge}$p<0.001; §p<0.1.

Figure 1

Changes in nose-anus length gain in PLC, E100 and OVX during the experimental period.

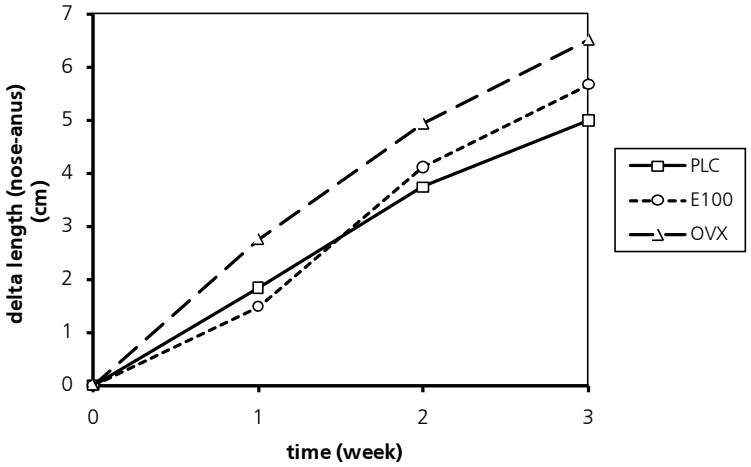
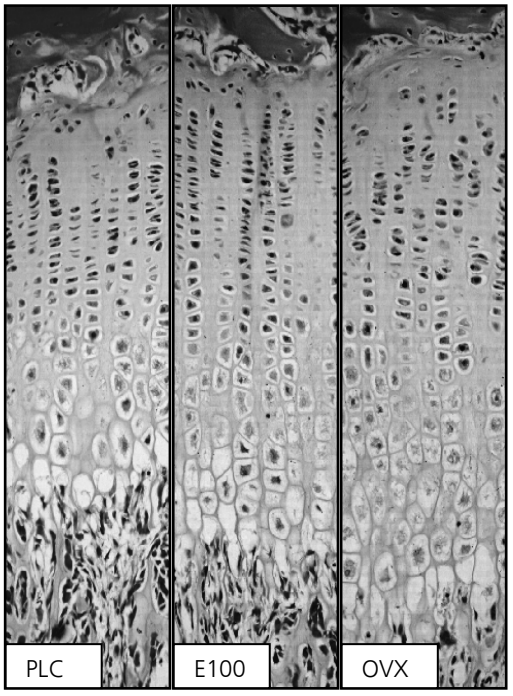


Figure 2

Growth plate samples from PLC, E100 and OVX. Notice the increase in total growth plate width in E100 and OVX.



Effects on bone

a. 'Whole bone' parameters

Femur length was significantly increased in E100, but not in OVX, whereas tibia length was significantly higher in OVX only. Observed changes between groups in calcium density, total calcium, bone volume and anatomical volume did not reach statistical significance (table 3).

b. Trabecular bone analysis

As illustrated in table 4, a clear reduction of trabecular number and trabecular thickness was apparent in both the epiphysis and the metaphysis of OVX and E100 as compared to PLC ($p<0.05$). Figure 3A shows the trabecular volume distribution of trabeculae with a given thickness, corrected for the bone volume that was studied in the epiphysis. Notice that the percentage of thin trabeculae (<5.0 pixels) was increased whereas the percentage of thick trabeculae (>5.0 pixels) was decreased in OVX and E100. A similar, but more pronounced effect on the distribution of trabecular thickness was observed in the metaphysis or primary ossification center (fig. 3B). As expected, the trabeculae in the metaphysis were thinner than in the epiphysis.

Effect on ovaries and uterus

E100 caused a statistically significant decrease of 50% of the ovary/TBW ratio ($p=0.000$). No statistically significant difference in the uterus/TBW-ratio was found, but the ratio was significantly decreased in OVX with 92% ($p=0.000$). Both endometrial thickness and the total thickness of the uterus wall were significantly decreased in E100 and OVX, resulting in normal endometrium thickness/total wall thickness ratios compared with PLC.

Ovaries from placebo-treated controls contained follicles in various stages of development (fig.4A), including secondary follicles, mature Graafian follicles and corpora lutea, the latter confirming the occurrence of ovulation. Similar histological characteristics were seen in the ovaries of E10- and E30-treated rats (data not shown). The ovaries from the E100-treated group also showed different developmental stages of the follicle, but corpora lutea were absent and there were less mature follicles, demonstrating the absence of ovulation (fig. 4B). Instead, large cystic follicles, surrounded by a thin granulosa cell layer or no granulosa at all, were abundantly present.

Table 3. Whole bone parameters

Parameter	PLC	E100	OVX
Va: anatomical volume (mm^3)	277.8 (8.3)	287.3 (7.4)	279.2 (14.5)
Vb: bone volume (mm^3)	95.5 (4.4)	97.1 (5.1)	90.8 (5.1)
Vb/Va-ratio (%)	34	34	33
Total calcium	1.00	0.97	0.85
Calcium density	1.00	0.89	0.85

Means (SD). PLC was taken as a reference for calculating relative total calcium and relative calcium density.

Table 4. Trabecular bone analysis

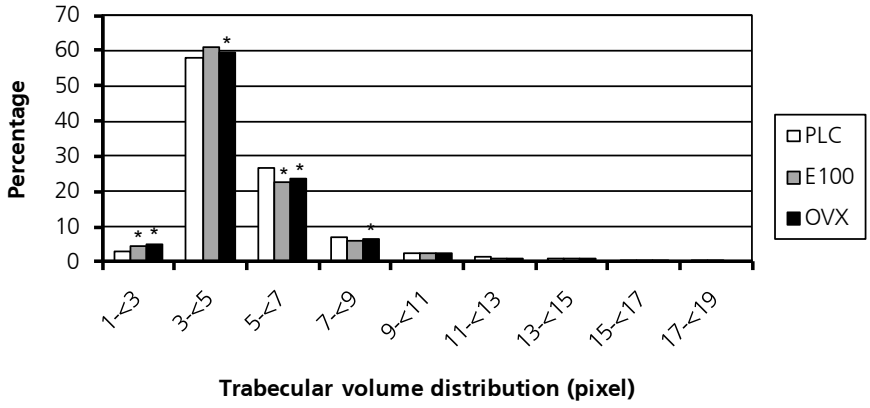
Parameter		PLC	E100	OVX
Epiphysis				
Trabecular bone volume	mm3	25.6 (1.4)	22.4 (1.6)*^	20.2 (1.8)*
Trabecular thickness	µm	143.1 (1.0)	139.4 (2.3)*	139.6 (3.2)*
Trabecular number	1/mm	1.8 (0.0)	1.6 (0.2)* ^	1.4 (0.1)*
Metaphysis				
Trabecular bone volume	mm3	2.5 (0.4)	1.9 (0.3)*	1.8 (0.3)*
Trabecular thickness	µm	85.1 (3.3)	75.9 (2.9)*	78.5 (3.1)*
Trabecular number	1/mm	3.6 (0.5)	3.0 (0.3)*	2.9 (0.4)*

Trabecular bone volume is the volume taken by trabecular bone in the selected region of interest.
Means (SD); *$p<0.05$ as compared to PLC; ^$p<0.05$ E100 versus OVX.

Figure 3

(A) Normalized trabecular volume distribution in the secondary ossification center (epiphysis). Only the volume taken by bone tissue in the epiphysis is taken into account. Notice the increase of trabeculae <5.0 pixels (pixel size = 27.64 µm). (B) Normalized trabecular volume distribution in the primary ossification center (metaphysis). Only the volume taken by bone tissue in the selected cylinder is taken into account. Notice the increase of trabeculae <5.0 pixels (pixel size = 13.82 µm). *p <0.05.

A. Epiphysis



B. Metaphysis

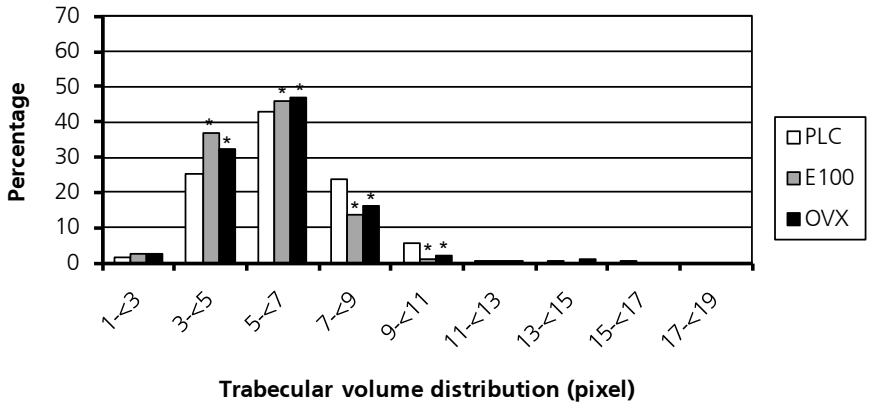
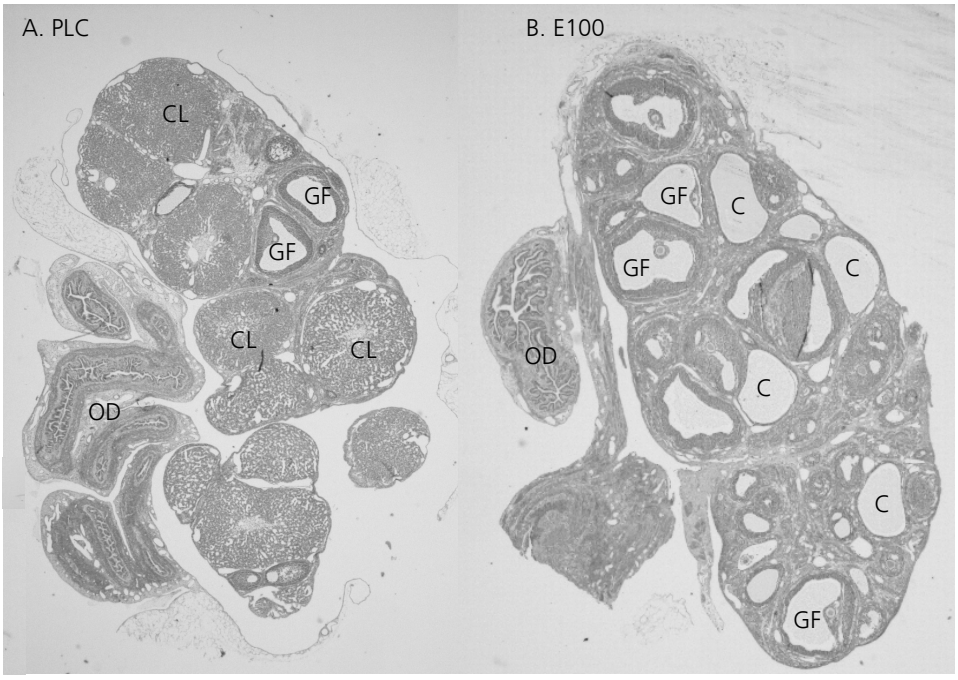


Figure 4

Ovaries of PLC (A) and E100 (B); GF, mature Graafian follicle; CL, corpus luteum; C, cyst; OD, ovarian duct. Magnification 40×. Notice the presence of cysts and the absence of corpora lutea in E100.



Brain histology

Histological examination of the brain was performed on slides of the hilus of the hippocampus area on E100 and placebo-treated animals to assess for possible neurodegenerative effects of the treatments. Since neurodegeneration is associated with gliosis, immunohistochemical staining for several glial markers was performed. There was no statistically significant difference in the surface area of GFAP immunoreactive astrocytes between groups. No reactive astrocytes or reactive microglia were detected after vimentin and OX-42 immunostaining, respectively. In addition, there was no qualitative evidence of neuronal loss or degeneration (data not shown).

Discussion

The aims of this study were to investigate the effect of aromatase inhibition on growth parameters and to study potential side-effects of this treatment on organ weight development, bone, and brain morphology in sexually immature female rats. The main conclusion from this study is that the currently highest available dose of exemestane (100 mg/kg/week) partially inhibits aromatase activity, which causes only a marginal stimulation of length gain and appendicular growth, but also results in the appearance of multiple ovarian cysts and changes in bone architecture consistent with early stage osteopenia.

All the currently available animal models for studying longitudinal growth are in some way flawed since they do not fully represent the hallmarks of human growth, such as an obvious growth spurt at the beginning and epiphyseal fusion with growth arrest at the end of puberty. These processes are both mediated by the exclusive action of estrogen, as illustrated by the clinical phenotype of estrogen resistant or deficient patients (6). Although rodents do not clearly demonstrate these maturational phenomena, growth diminishes after sexual maturation with growth rates approximating zero. Rats tend to respond in a similar fashion as humans to estrogen, with estrogen deficiency stimulating growth and estrogen treatment causing growth arrest (27). In contrast, estrogen receptor (α/β/$\alpha\beta$) and aromatase knockout mice show a normal adult length (28-32), indicating that hormonal regulation of growth in mice is distinctly different than in humans and rats. We therefore feel that the rat is the most appropriate of the available animal models for *in vivo* studies on the impact of chemically induced aromatase deficiency.

To correlate the effects seen in our study to the degree of aromatase inhibition, it would have been valuable to assess serum levels of estradiol, testosterone, dihydrotestosterone (DHT), and exemestane. However, accurate methods for measurement of estradiol in rat serum are lacking (33). Available estradiol assays that are developed for analyzing human serum samples, are insufficiently sensitive for quantification of low estradiol levels (34). Treatment with exemestane even further complicates estradiol detection due to the fact that metabolites of the compound interfere with the available assays for measurement of gonadal steroids (35). To our knowledge, there is currently no laboratory experienced with estradiol serological measurements in exemestane-treated rats.

Alternatively, we used the ovary, a highly estrogen-dependent organ, as a biological readout system for the effect of exemestane. The observed ovarian phenotype characterized by the absence of corpora lutea and the presence of multiple cysts is similar to the ovarian phenotypes of the aromatase knockout mouse (ArKO) (36) and female estrogen receptor alpha and beta knockout mice (αERKO and βERKO) (28). Moreover, aromatase deficient female patients also

have reproductive dysfunction based on primary amenorrhea and enlarged, polycystic ovaries in adolescence (6). The observed changes in ovarian morphology convincingly demonstrate that a reduction in estradiol levels was achieved by the administration of exemestane in our model system.

Hyperandrogenism is likely to occur during treatment with aromatase inhibitors and may eventually lead to virilization, as reported in female patients with aromatase deficiency. In our study, exemestane treatment was applied short-term and was not expected to cause signs of hyperandrogenism. However, when considering a longer treatment period covering the period of sexual maturity, possible signs of virilization, such as masculine sexual behavior and disturbance of reproductive function, should be monitored during and after termination of treatment

The growth phenotype of E100-treated female rats shows similarities with other described animal models for estrogen deficiency. An increased body weight gain was also observed in female αERKO and αβERKO mice, and female ArKO mice (29-31;37), corresponding with an increased deposition of intra-abdominal adipose tissue, high leptin and cholesterol levels, and hyperinsulinism (38). An elevated body mass index has not been described in female patients with aromatase deficiency, but this is probably due to the fact that they have received estrogen treatment. In the present study, fat deposition, food intake and energy expenditure were not measured.

Nose-anus length gain in E100-treated rats was normal, but showed a trend towards a significant increase compared with non-treated controls. In female αERKO, βERKO, αβERKO and ArKO mice, adult length was found to be normal compared to wild-type mice (28-32). However, the βERKO mouse but has a tendency to slightly increased growth during the post-pubertal phase (31), whereas female ArKO mice show an increased length gain peripubertally (29;30). E100 treatment resulted in a marginal increase of the length of the tibia (not significant). Surprisingly, femur length was found to be significantly higher in E100 compared with PLC, in the absence of a similar and more pronounced effect after OVX. All other parameters were more dramatically influenced by OVX than by E100, and we have no explanation for the contrasting effects of OVX and E100 on femur length. However, our knowledge of the timing and regulation of growth in various bones is limited, and has not been studied in detail. Regional differences may exist, and aromatase inhibition may therefore result in various degrees of growth stimulation in different bones.

Increased appendicular growth was found in βERKO mice (31). In contrast, the other knockout mouse models either have normal or decreased growth of the femur and/or tibia (29-31). One must bear in mind that in our study exemestane was administered during a short timeframe, whereas inborn genetic mutations in knockout mice and human patients with estrogen

deficiency or resistance can influence growth during all stages of life, which may explain the differences in growth characteristics.

The OVX group was included as a control group, to be able to compare the results of exemestane-induced and ovariectomy-induced estrogen deficiency. Ideally, there would be a complete absence of circulating estrogen in both E100 and OVX. However, the differences in several parameters between the groups suggest that E100 did not completely abrogate estrogen biosynthesis. OVX induced increased levels of IGF-I, as reported before in ovariectomized rats and mice in other studies (39;40). A raised IGF-I level was also found after E100-treatment, but this effect did not reach statistical significance. Estrogen normally causes thymus atrophy (41), which in our experiments was strongly prevented by OVX and to a lesser extent by E100 (not significant). Similarly, a reduced weight of the liver was found in OVX females, whereas no reduced liver weight was found in E100. Circulating high levels of androgen may have had protective effects on these tissues, either by direct effects on target organs or maybe in part by aromatization to estrogen by residual aromatase activity. Since the gonads are the only organs capable of synthesizing androgen precursors in the rat, ovariectomy results in a combined estrogen and androgen deficiency, ruling out protective effects by circulating androgen in the OVX group.

E100 treatment had a similar, but less prominent effect on growth than OVX. The increase in nose-anus length gain in E100 was delayed compared with OVX, suggesting that at least one week is necessary to establish a steady-state plasma level of exemestane that is able to influence growth. The growth plate and the long bones of the appendicular skeleton seemed most sensitive to aromatase inhibition, resulting in effects similar to those seen after OVX. Axial skeletal growth was less stimulated by E100, compared with OVX. Differences in sensitivity for aromatase inhibition may reflect variability in local estrogen biosynthesis in various tissues resulting in different levels of aromatase inhibition. Second, some organs may be more accessible for exemestane penetration than others. Another possible explanation for the modest effect of E100 on longitudinal growth may be that the dosage was too low to achieve complete inhibition of aromatase activity at the systemic level or at the tissue level. However, preclinical studies have shown that a single subcutaneous dose of 30 mg/kg exemestane reduced ovarian aromatase activity in adult female rats with approximately 80% (E. di Salle, unpublished results). The absence of significant growth effects in E10 and E30 was presumably due to sub-pharmacological serum levels of exemestane.

Studies in mature, female rats have demonstrated that the steroidal aromatase inhibitor exemestane and its principal metabolite 17-hydroxyexemestane significantly prevent bone loss induced by OVX, based on androgenic actions of these compounds on bone. In contrast, the non-steroidal aromatase inhibitor letrozole, that has no structural relationship to androgens,

does not have such protective effects on bone in mature female rats (18;42). Analysis of the trabecular bone in both the epiphysis and metaphysis demonstrated a significant reduction in the number and thickness of trabeculae in E100 and OVX, associated with a non-significantly decreased calcium density in both groups. Loss of bone tissue is a sign of osteopenia, generally considered as the stadium preceding osteoporosis. Similarly, ArKO mice were found to have a decreased peak BMD, prominent osteopenia on plain film radiographs and decreased trabecular volume and thickness (29;30). A low BMD was also found in βERKO and αβERKO mice (31).

In our study, the bone phenotypes of E100 and OVX were very similar. This suggests that there is no apparent bone-protecting androgenic effect of exemestane and its principal metabolite 17-hydroxyexemestane in prepubertal rats treated for 3 weeks, in contrast to what was reported previously about mature rats treated for a period of 16 weeks (18;42). Exemestane was reported to have a very low binding affinity (0.22%) for the androgen receptor *in vitro* and a marginal androgenic activity (only 1% of that of testosterone propionate) *in vivo* in male castrated rats. Since androgen receptor expression is lower in the tibial epiphysis and metaphyseal bone in female rats compared to males, we expect the androgenic potency of exemestane in our model system to be even lower than 1% which makes pronounced androgenic effects of exemestane on growth unlikely. In line with this hypothesis is the fact that no protective androgenic effect of exemestane on bone quality was observed. The discrepancy between our study and the report by Goss *et al.* may be explained by the fact that during bone modeling that occurs in immature rats different regulatory pathways involving androgen signaling may play a role than during maintenance of bone quality in mature rats.

It is not clear whether the osteoporotic changes observed would lead to a permanently worse bone quality. As the growing skeleton is in a constant process of bone modeling, it remains to be seen whether osteoporotic changes induced in this period would render the mature skeleton more vulnerable to osteoporotic fractures. Patients with P-450 aromatase deficiency or estrogen receptor mutation have a low bone mass, which may result from not having achieved a normal peak bone mass during childhood growth and development of the skeleton (43). Estradiol treatment in those patients induced an increase in BMD (6).

The uterus/TBW ratio was decreased after OVX, but not significantly different after E100 treatment, probably due to incomplete aromatase inhibition at the tissue level. Studies in mature cycling rats treated with letrozole also revealed a decreased uterine weight (44). In female patients, aromatase deficiency results in reproductive dysfunction based on primary amenorrhea and enlarged polycystic ovaries in adolescence (45), emphasizing the pivotal role of estrogen in ovarian development (46). Similarly, E100 reduced ovary weight and caused histological changes similar to those seen in polycystic ovary syndrome. It is unknown whether

the effects of temporary estrogen deficiency on the female gonadal tract are reversible and, if not, whether these changes have an impact on fertility or sexual behavior.

In the mammalian nervous system, aromatase expression plays an important role in neural differentiation and plasticity, neuroendocrine functions, and (sexual) behavior (17), both under physiological and pathological conditions. We therefore have assessed whether aromatase inhibition affects morphology of the hippocampus, a brain area that is sensitive to estradiol levels (47). E100 did not result in reactive gliosis, one of the most prominent cellular responses of the central nervous system to neurodegeneration (48). This finding is in agreement with previous studies showing that adult ArKO mice have normal hippocampus morphology, although they are more susceptible to neurodegeneration than their wild-type littermates (49). Thus, although aromatase exerts a protective effect against neurodegenerative stimuli (50-52), its inhibition does not compromise neuronal survival under normal circumstances. However, we cannot exclude that aromatase inhibition may affect other brain regions, especially brain nuclei that develop during sexual maturation (e.g. the medial amygdala) (53).

In summary, partial inhibition of aromatase activity by E100 resulted in marginal stimulation of axial and appendicular growth in female prepubertal rats. Although aromatase inhibition was probably incomplete, pronounced adverse effects were found on ovarian histology and bone architecture, and it remains unclear whether these would have long-term, permanent consequences for reproduction and fracture risk, respectively. Our finding that E100 stimulates growth, albeit marginally, mimics to a certain extent the clinical phenotype in aromatase deficient female patients who exhibit tall stature if untreated (54). Species differences in dependency on estrogen signaling probably underlie the discrepancies found in growth phenotype between the aromatase inhibitor treated rats and mice, and estrogen-deficient patients described in literature. However, considering the potential side-effects on bone physiology and morphology of the ovaries, treatment with aromatase inhibitors in girls in clinical practice should not be advised.

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Chapter 5

Impaired body weight and tail length gain and altered bone quality after treatment with the aromatase inhibitor exemestane in male rats

S.A. van Gool[1], J.M. Wit[1], T. de Schutter[2], N. de Clerck[2], A.A. Postnov[2], S. Kremer Hovinga[1], J. van Doorn[3], S.J. Veiga[4], L.M. Garcia-Segura[4], M. Karperien[5]

[1]Department of Pediatrics, Leiden University Medical Center, Leiden, the Netherlands.
[2]Department of Biomedical Sciences and Department of Physics, University of Antwerp, Antwerp, Belgium.
[3]Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, Utrecht, the Netherlands.
[4]Instituto Cajal, Consejo Superior de Investigaciones Científicas, Madrid, Spain.
[5]Department of Tissue Regeneration, Biomedical Technology Institute, Twente University, the Netherlands.

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Abstract

Background: Estrogen deficiency induced by aromatase inhibitors may be a novel treatment modality for growth enhancement in short children, but may have adverse effects on bone, brain and reproduction.

Aim: To assess growth effects and potential adverse effects of aromatase inhibition in male rats.

Methods: 26 days old prepubertal rats received intramuscular injections with placebo (PLC) or the aromatase inhibitor exemestane at a dose of 10, 30 or 100 mg/kg/week [E10, E30, E100(6)] for 6 weeks completely covering the sexual maturation phase, or with 3 weeks E100 followed by 3 weeks PLC [E100(3)]. Growth parameters and histology of the testis, seminal vesicle and brain were analyzed. Bone architecture was studied with X-ray microtomography.

Results: Exemestane dose-dependently decreased body weight and tail length gain, and liver and seminal vesicle weights, but did not affect nose-anus length gain, growth plate width or radial growth. E100(6) decreased trabecular thickness (epiphysis and metaphysis) and number (metaphysis). Normal IGF-I levels and brain, testis and seminal vesicle morphology were observed. E100(3) resulted in decreased tail length gain only.

Conclusion: Exemestane treatment during sexual maturation did not augment linear growth in male rats, but caused impaired body weight and tail length gain and osteopenia.

Introduction

The phenotypes of estrogen resistance (1) and estrogen deficiency (1-4) are characterized by a lacking pubertal growth spurt, ongoing growth into adulthood and tall stature, demonstrating the crucial role of estrogens in skeletal growth and maturation in both genders (5-8). These clinical observations have led to the hypothesis that estrogen deficiency pharmaceutically induced by aromatase inhibitors may be a novel treatment modality for growth enhancement in children with idiopathic short stature (9;10).

Our limited knowledge on the effects of aromatase inhibition on growth in boys is derived from one clinical trial in patients with idiopathic short stature (ISS) (11) and two trials in boys with constitutional delay of growth and puberty (CDGP) (12;13). Treatment with the nonsteroidal aromatase inhibitor letrozole (either or not combined with testosterone) resulted in an increased predicted adult height and near-final height, without apparent detrimental effects on bone mineralization (11-14). In growth hormone-deficient (GHD) patients treated with growth hormone (GH), co-treatment with the nonsteroidal aromatase inhibitor anastrozole led to an increased predicted adult height, but adult height results have not yet been published (15).

The ubiquitous expression of aromatase in humans underscores the importance of locally produced estrogens (16) and implies that aromatase inhibition may have adverse effects as well. Male patients with congenital estrogen deficiency have been found to suffer from osteoporosis in adulthood (5). Since aromatase has an established neuroprotective function both under physiological and pathological conditions, aromatase inhibition may result in unwanted side-effects on the brain and, as a result, on behavior (17). Finally, estrogen deficiency may result in disorders of the reproductive system.

In this study, the effects of treatment with the steroidal, irreversible aromatase inhibitor exemestane (E) were analyzed in male rats during the phase of sexual maturation in order to test the hypothesis that aromatase inhibitors can increase growth and to evaluate potential side-effects. To mimic the potential treatment strategy in boys with ISS, exemestane treatment was initiated 4 days before the start of sexual maturation and lasted 6 weeks. This treatment regime covered the whole period of sexual maturation in rats

We applied exemestane instead of other aromatase inhibitors. An important advantage of exemestane is that it is available as a slow release preparation that can be administered once a week, in contrast to other inhibitors that require daily dosing. Exemestane completely abolishes estrogen biosynthesis by irreversibly blocking aromatase, which can only be overcome by *de novo* aromatase synthesis. The continuous slow release of exemestane from the injected depot ensures that *de novo* aromatase synthesis is counteracted. In addition, exemestane may exert

androgenic effects on bone that may prevent the loss of bone mineral density (BMD) resulting from estrogen deficiency. We have previously established that exemestane effectively inhibited aromatase activity in female rats, resulting in a marginal growth increase, polycystic ovaries and a decreased bone quality (Van Gool *et al.*, Horm Res, in press). The present study is the first detailed analysis of the impact and side effects of aromatase inhibition on growth in male rats.

Experimental design

Animals

Weight-matched male Wistar rats (n=30), 26 days old and sexually immature, were purchased from Harlan. Animals were kept in a light and temperature controlled room (12 hours light, 20-22°C) with water and food (Hope Farms) ad libitum and a maximum of 3 animals per cage. Rats enter sexual maturation at day 30 and the whole process is completed in 6 weeks. Experiments were approved by the Committee for the ethical care and use of laboratory animals of the Leiden University.

Five groups of 6 rats were treated with placebo (PLC) or with the slow-release preparation exemestane (E) at three different doses: 10, 30 or 100 mg/kg body weight/week (coded as E10, E30, E100, respectively). E100(6) was treated for 6 weeks with E100, covering the complete period in which young male rats usually exhibit fast growth. E100(3) received E100 for 3 weeks, followed by 3 weeks of placebo. This treatment regimen covers only the first half of the period of sexual maturation in male rats. The two groups were included in order to determine whether short- or long-term aromatase inhibition would have the most profound effect on growth. Exemestane (Aromasin®, provided by Pfizer) was administered once per week by intramuscular injections in alternated hind paws.

Nose-anus length, tail length and body weight were measured weekly. At the end of the experimental period, animals were anaesthetized by intraperitoneal Nembutal injection and blood samples were collected by cardiac puncture. Serum was separated for insulin-like growth factor I (IGF-I) measurements. Femora were collected for X-ray microtomography (micro-CT). Subsequent *in vivo* fixation was performed by transcardiac perfusion with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer with 75 mM lysine monohydrochloride and 10 mM Na-periodate. After fixation, rats were decapitated, and brains were stored in the same fixative for at least 12 weeks. Tibiae of the right hind legs were measured, postfixated for 24 hours, decalcified in 12.5 % EDTA, pH 7.4 for 4 weeks, cut in

halves in a sagittal orientation and processed for paraffin embedding. Testis and seminal vesicle were weighed and processed for paraffin embedding.

Growth plate histomorphometry

Paraffin sections (5 µm) perpendicular to the growth plate axis were mounted on APES/glutaraldehyde-coated slides, deparaffinized in Paraclear® and graded ethanols, stained with Hematoxylin/Eosin and mounted in Histomount (National Diagnostics). A Nikon DXM 1200 digital camera was used for photographing the proximal tibial growth plate. Widths of the total growth plate, proliferative zone and hypertrophic zone were measured in ImagePro Plus®.

X-ray microtomography (micro-CT)

To examine the effects of exemestane treatment on whole bone parameters and on trabecular bone in particular, right femora of PLC, E100(3) and E100(6) were analyzed by high resolution X-ray microtomography. Micro-CT has been shown to be well suited for the non-destructive visualization and quantitative analysis of bone and calcified tissue (18;19).

a. 'Whole bone' parameters

Isolated whole femora were scanned with a high resolution desktop micro-CT scanner ((7); Skyscan 1076). In this X-ray micro-CT system, both the X-ray source (focal spot size 5 µm, energy range 40-100 keV) and the detector (CCD camera 2.3kx4k) rotate around the bone. Scans were isotropic and a voxel size of 35×35×35 µm was chosen with 1.0 mm A1 filter. Virtual cross-sections were reconstructed by Feldkamp cone beam algorithm (20). Further technical details about the scanner have been published elsewhere (21).

The same threshold and reconstruction parameters were used for the reconstruction of all cross-sections. Frequency distribution of the grey levels was analyzed. 3D models were created using Skyscan software packages ANT and CT Analyzer. From the virtual cross-sections the volumes taken by bone as well as the anatomical volume (Archimedean volume) were calculated. Distinction between bone and other tissues was based on thresholding of the grey values in the reconstruction. Analysis of the grey values according to previously reported calculations (35) resulted in a relative value for total calcium and overall calcium density. Details of these calculations have been reported previously (18). In addition, the length of the femora was measured.

b. Trabecular bone

For analysis of the trabecular bone, the distal end of the femur was scanned, but for this purpose at a higher resolution than required for the 'whole bone' scans. Therefore, an *in vitro* desktop micro-CT system (Skyscan 1072) with a rotating stage was used (22). Polychromatic X-rays with peak energy of 80 kV were generated by a microfocus X-ray source with a focal spot size of 8 microns. A (1024×1024) 12 bit CCD camera was used as detector. The rotation step was 0.9°, acquisition time 6 s/projection, and total acquisition time about 2 hours. Scanning was isotropic with a voxel size of 13.28 μm^3.

The growth plate was chosen as a reference for selecting comparable and reproducible regions of interest for analysis of trabecular number, trabecular thickness, bone volume and distribution of trabecular thickness. Two different regions of the femur were analyzed: the metaphysis (primary ossification center) and the epiphysis (secondary ossification center). For analysis of the primary ossification center at the proximal side of the growth plate, a transverse reference slice of the growth plate was selected and used to reconstruct a cylinder with fixed dimensions as from 50 slices above the reference slice. The base of the cylinder was a circle and the height was 150 slices.

Gonadal histology

Midsagittal sections of the testis and seminal vesicle (5 µm) were stained with Haematoxylin/Eosin. A minimum of three sections was analyzed per organ.

Brain histology

Brains were rinsed with PBS, cut in coronal sections (30 µm) in a Vibratome and Nissl-stained with toluidine blue for morphometric analysis. Immunohistochemical staining of astrocytes (anti-GFAP, Glial Fibrillary Acidic Protein, DAKO, dilution 1:1000), microglia (OX-42, Serotec, dilution 1:300) and reactive astrocytes (anti-vimentin, clone V9, DAKO, dilution 1:500) was performed. For morphometric analysis of GFAP immunoreactive astroglia, quantitative evaluation of the surface density of GFAP immunoreactive cell bodies and cell processes in the hippocampus was performed using a stereological grid, according to the point-counting method of Weibel (23). A minimum of two slides was evaluated per animal.

Insulin-like Growth Factor I (IGF-I) assay

Total serum IGF-I levels were measured by specific radioimmunoassay (RIA) after acid Sep-Pak C18 (Waters Associates) extraction of 250 µl aliquots, as described previously (24).

Statistical analysis

The study was designed to analyze the effect of exemestane on growth and to assess potential adverse effects on bone, brain and organ development. Comparisons between treatment and control groups were made using one-way analysis of variance (ANOVA). Results are expressed as mean and SD. The significance level was set at 0.05.

Results

Effects on growth and organs

At baseline, there were no significant differences between groups in growth parameters. Body weight gain, nose-anus and tail length gain, tibia length, growth plate characteristics, and IGF-I levels are summarized in table 1. The effects of exemestane on organ weights are summarized in table 2. Figure 1 shows body weight, nose-anus length, and tail length gain for all groups.
Previous experiments in female rats demonstrated that 3 weeks treatment with E100 effectively inhibited aromatase activity, using ovarian development as a bioassay. Treatment with E100 induced polycystic polycystic ovarian syndrome-like features generally known to be due to estrogen-deficiency (chapter 4). In male rats, exemestane treatment did not influence nose-anus length gain. When compared to PLC, the animals that received exemestane showed decreased gains of tail length and body weight in a dose-dependent manner, with E10 having a modest and E100(6) the most pronounced effect. At termination of the experimental period, body weight gain of E100(3) was similar as that of PLC. Exemestane treatment did not affect the lengths of tibia and femur, growth plate width or serum IGF-I levels.
Both E100(3) and E100(6) caused a dose-dependent decrease of the liver/total body weight (TBW) ratio compared to PLC. A normal aspect of the livers was found during macroscopic evaluation. Only E100(6) resulted in a significantly reduced seminal vesicle/TBW ratio, but no apparent morphometrical anomalies were found in the testes or seminal vesicle as revealed by histological examination (data not shown).

Effects on bone

a. 'Whole bone' parameters

No differences were found in femur length, anatomical volume, bone volume, total calcium and calcium density between the groups (data not shown).

Table 1. Effect of aromatase inhibition on growth parameters and epiphyseal morphology

Parameter	PLC	E10	E30	E100(6)	E100(3)
Tail length gain (cm)	12.1 (0.9)	11.1 (0.2)**	10.7 (0.5)***	10.8 (0.5)***	10.9 (0.3)**
Nose-anus length gain (cm)	9.7 (1.1)	9.7 (0.7)	9.6 (0.4)	9.2 (0.5)	9.7 (0.5)
Body weight gain (g)	280.5 (17.9)	268.1 (5.8)	247.9 (14.6)**	236.5 (18.0)***	268.8 (19.1)
Tibia length (mm)	38.3 (0.9)	38.2 (0.3)	37.9 (0.5)	37.8 (0.7)	37.9 (0.7)
Femur length (mm)	33.2 (0.4)	ND	ND	32.9 (0.3)	33.6 (0.4)
Growth plate width (mm)	0.32 (0.02)	ND	ND	0.29 (0.03)	0.33 (0.02)$^{\wedge}$
proliferative zone (mm)	0.18 (0.00)	ND	ND	0.16 (0.02)$^{\#}$	0.19 (0.01)§
hypertrophic zone (mm)	0.14 (0.02)	ND	ND	0.13 (0.02)	0.14 (0.01)
P/H-ratio	1.29 (0.14)	ND	ND	1.23 (0.13)	1.33 (0.10)
Serum IGF-I (ng/ml)	1.5 (0.1)	1.3 (0.3)	1.4 (0.2)	1.5 (0.6)	1.1 (0.1)$^{\# \S}$

Means (SD). ND, not determined; P/H-ratio, ratio between proliferative zone and hypertrophic zone.**p<0.01; ***p<0.001; $^{\#}$p<0.1 (compared to PLC). E100(3) versus E100(6): $^{\wedge}$p<0.05; §p<0.1.

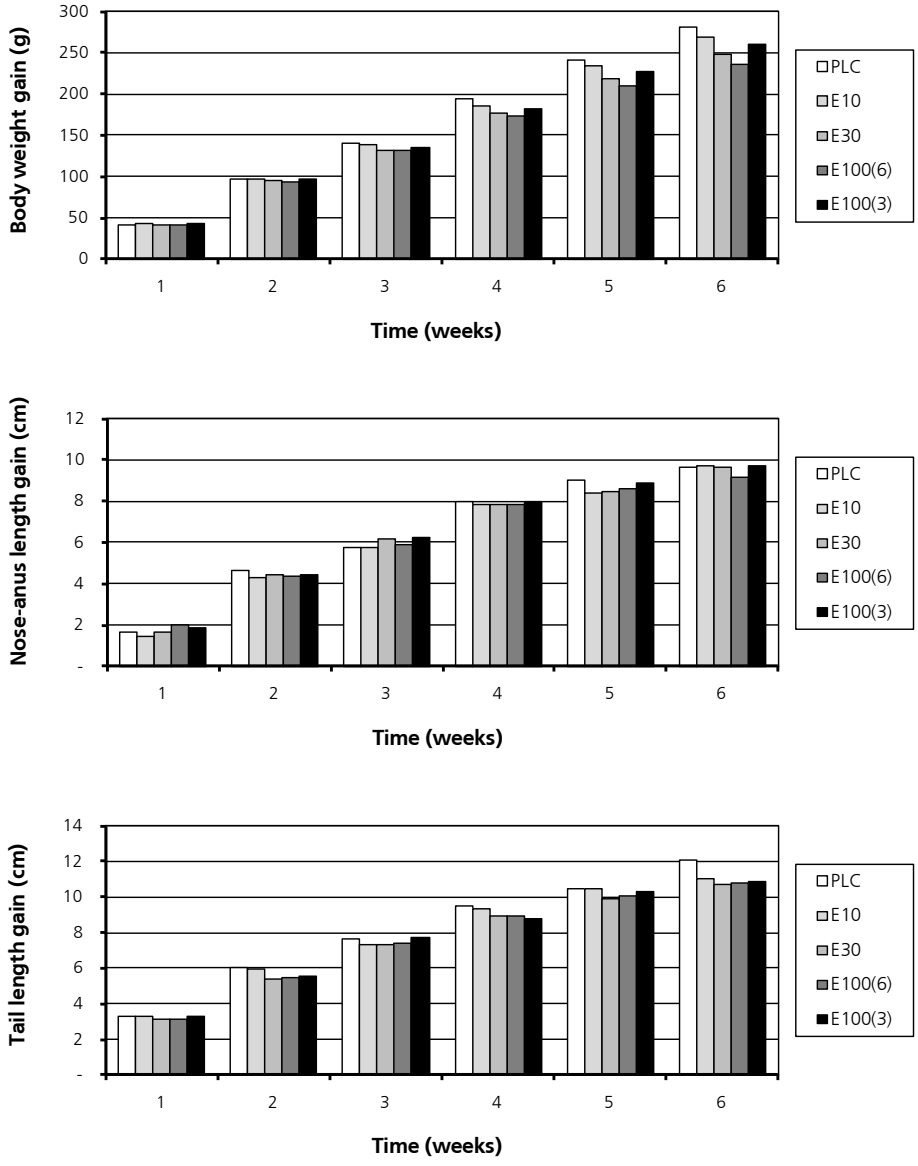
Table 2. Effect of aromatase inhibition on organ weights

Parameter		PLC	E10	E30	E100(6)	E100(3)
liver		48.2 (6.5)	41.7 (2.6)$^{\#}$	41.5 (7.4)$^{\#}$	38.8 (6.1)*	37.8 (6.2)**
thymus		1.5 (0.2)	1.8 (0.3)$^{\#}$	1.6 (0.4)	1.4 (0.2)	1.7 (0.2)§
kidney		4.0 (0.2)	3.6 (0.7)	4.0 (0.3)	3.8 (0.3)*	3.5 (0.6)
testis		4.0 (0.9)	4.5 (0.1)	4.5 (0.2)$^{\#}$	3.7 (0.6)	4.1 (0.4)
seminal vesicle	2.4 (0.4)	2.4 (0.3)	2.2 (0.2)	1.6 (0.2)***	2.4 (0.3)$^{\wedge\wedge\wedge}$	

Organ weights as permillage of total body weight. Means (SD). *p<0.05; **p<0.01; ***p<0.001; $^{\#}$p<0.1 (compared to PLC). E100(3) versus E100(6): $^{\wedge\wedge\wedge}$p<0.001; §p<0.1.

Figure 1

Growth parameters (A) body weight gain, (B) nose-anus length gain, (C) tail length gain.



b. Trabecular bone analysis

E100(6) caused a decline in trabecular thickness in the epiphysis and metaphysis (table 3). In the metaphysis, the number of trabeculae was also decreased. The percentage of small trabeculae was increased after three or six weeks of E100 treatment in both the epiphysis and metaphysis (<5.0 pixel and <7.0 pixel, respectively; figure 2). E100(3) exhibited a normal trabecular number, and a trabecular bone volume intermediate between those of PLC and E100(6) animals.

Brain histology

Gliosis, a hallmark sign of neurodegeneration, was not found in E100(6). The surface area of GFAP immunoreactive astrocytes was not different between groups and reactive astrocytes or reactive microglia were not detected. There was no qualitative evidence of neuronal loss or degeneration (data not shown) caused by exemestane treatment.

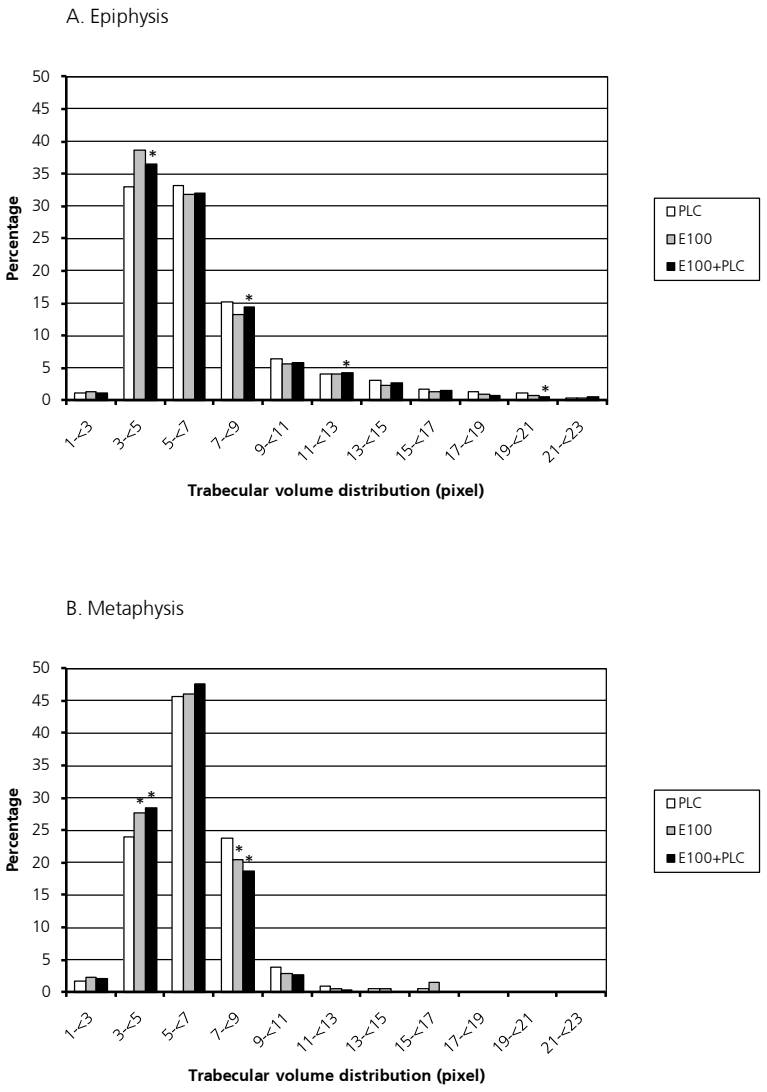
Table 3. Trabecular bone analysis

Parameter		PLC	E100(6)	E100(3)
Epiphysis				
Trabecular bone volume	mm3	48.4 (4.8)	41.0 (3.5)*	43.9 (2.3)
Trabecular thickness	µm	189.1 (9.1)	178.3 (4.0)*	182.1 (5.0)
Trabecular number	1/mm	1.6 (0.3)	1.6 (0.3)	1.7 (0.2)
Metaphysis				
Trabecular bone volume	mm3	1.8 (0.2)	1.4 (0.2)*	1.8 (0.3)^
Trabecular thickness	µm	84.9 (3.1)	81.9 (2.9)	80.6 (3.0)*
Trabecular number	1/mm	2.6 (0.2)	2.1 (0.2)*	2.6 (0.3)

Volume of objects is the volume taken by bone tissue in the selected region of interest. Means (SD);
* $p<0.05$ as compared to PLC; ^ $p<0.05$ E100(3) versus E100(6).

Figure 2

(A) Normalized trabecular volume distribution in the secondary ossification center (epiphysis). Only the volume taken by bone tissue in the epiphysis is taken into account. Notice the increase of trabeculae <5.0 pixels (pixel size= 27.64 µm) in E100(3) and E100(6). (B) Normalized trabecular volume distribution in the primary ossification center (metaphysis). Only the volume taken by bone tissue in the selected cylinder is taken into account. Notice the increase in trabeculae <7.0 pixels in E100(3) and E100(6) (pixel size= 13.82 µm). *p <0.05.



Discussion

The aims of this study were to analyze the effect of aromatase inhibition on growth parameters during the phase of sexual maturation in male rats and to assess potential side-effects of this treatment on organ weight development, bone, and brain morphology. The main conclusion from this study is that exemestane treatment decreased tail length and body weight gain, but also caused alterations in bone architecture that usually occur in early stage osteopenia.

Human growth is characterized by an evident pubertal growth spurt and growth plate fusion at the end of puberty. The clinical phenotype of estrogen-resistant or -deficient patients illustrates that these characteristic features are mediated by the exclusive action of estrogen (25). In contrast, the adult length of estrogen receptor knockout mice is normal, demonstrating a clear difference in hormonal regulation of growth between humans and mice (26-30). For this study, the rat was chosen as an animal model. Although rats do not show a clear growth spurt and lack epiphyseal fusion, their response to estrogen is similar as in humans, with estrogen deficiency stimulating growth and estrogen treatment causing growth arrest (31). We therefore feel that the rat is the most valid of all available animal models for *in vivo* studies on the impact of chemically induced aromatase deficiency.

Serological determination of estradiol levels would have contributed to the interpretation of the effects of exemestane on growth. However, estradiol levels in male rats are very low, and expected to be even lower during treatment with an aromatase inhibitor. The available estradiol assays have been developed for analyzing human serum and are not sufficiently sensitive for quantification of such low levels of estradiol (32). Exemestane treatment further complicates estradiol detection, because the compound and its metabolites interfere with the available assays (33). To our knowledge, there is currently no laboratory experienced with estradiol serological measurements in exemestane-treated rats.

As an alternative to serological evidence, we have demonstrated the effectiveness of exemestane to inhibit aromatase activity in a previous study in female rats (chapter 4) which normally have high serum levels of estrogen during the sexual maturation phase. In this model we used ovarian development as a bioassay for aromatase inhibition. Exemestane at a dose of 100 mg/kg/week resulted in a pronounced decrease of ovarian weight and histological evidence of anovulation and polycystic ovarian syndrome-like features commonly seen in estrogen deficiency. The effects on ovarian development were comparable to ovariectomy letting us conclude that E100 resulted in a near complete inhibition of the aromatization of androgens. Since estrogen biosynthesis is significantly lower in male than in female rats, exemestane is expected to inhibit aromatase activity even more effectively in males.

In contrast with our hypothesis, exemestane treatment did not result in increased linear growth. Instead, although the decreases in nose-anus length gain and appendicular growth did not reach statistical significance, a smaller increment in body weight and tail length gain was found in exemestane-treated rats compared with PLC. Our results are in line with previous reports. Treatment of 6 weeks old male rats with the nonsteroidal aromatase inhibitor vorozole resulted in a decreased body weight gain and bone mineral density (BMD) but had no effect on femoral length (34). Four weeks old male rats treated with the selective estrogen receptor modulator (SERM) tamoxifen led to a declined body weight, nose-anus length, tibial and femoral length, and trabecular BMD (35). Similarly, the growth phenotype of male aromatase knockout (ArKO) mice and estrogen receptor alpha (ERα) and ER$\alpha\beta$ knockout mice are characterized by a lower body weight and retarded axial and radial growth (29;30;36). In contrast with our results and the other animal models of estrogen deficiency or resistance, peripubertal letrozole-treated mice were found to display increased growth (37), which may be attributed to species differences and differences between the aromatase inhibitors employed.

Exemestane has a very low binding affinity for the androgen receptor *in vitro* (0.22%) and a marginal androgenic activity *in vivo* as demonstrated in castrated male rats (1% of the activity of testosterone propionate) (38). It is unlikely that such marginal effects may explain the growth inhibition observed. The serum levels of IGF-I were normal during exemestane treatment, suggesting that the observed effects on growth were not mediated systemically through the GH/IGF-I axis. This observation is in line with the normal IGF-I level reported in a male aromatase-deficient patient (2).

The effects of exemestane treatment appeared to be dose-dependent, E100(6) having the most pronounced impact on growth. E100(3) rats showed a growth phenotype intermediate between those of E100(6) and PLC animals. This could mean that the treatment period of 3 weeks was too short to cause significant effects on growth or that potential exemestane-induced changes already had been undone during the 3 weeks follow-up phase without treatment. Likewise, Karimian *et al.* described a complete catch-up growth of body weight when the rats were allowed to recover after cessation of tamoxifen treatment, whereas the femora and tibia remained shorter than in untreated controls (35). The smaller body weight increment of exemestane-treated rats compared to PLC in our study is consistent with findings in male rats treated with other aromatase inhibitors or with Tamoxifen (34;35;39). A lower food intake or a decreased visceral fat mass have been suggested as possible explanations for the lower weight gain in those studies, but these aspects were not analyzed in our study.

Analysis of the trabecular bone of E100(6) rats demonstrated a significant loss of bone tissue that is generally considered as an early stage of osteoporosis. Impaired skeletal development

and a decreased bone mineral density were also reported in male letrozole-treated rats (34;40) and in tamoxifen-treated rats (35). Similarly, male patients with P-450 aromatase deficiency or estrogen receptor mutations have a low bone mass, probably because they do not achieve a normal peak bone mass during childhood. Moreover, Coleman *et al.* reported on the skeletal effects of exemestane treatment in bone quality in postmenopausal women with a status after complete resection of unilateral breast carcinoma, who were disease-free after 2-3 years of tamoxifen treatment and who were randomly assigned to continue tamoxifen treatment or to receive additional exemestane treatment to complete a total of 5 years of adjuvant endocrine treatment. Two years of exemestane treatment was found to result in a 4% lower lumbar spine BMD and higher levels of bone turnover markers (41). It is unknown whether the observed osteoporotic changes in our study would render the mature skeleton vulnerable to osteoporotic fractures later in life, or whether these changes are reversible.

Impaired liver function has been described in aromatase-deficient males (25), with a wide range of symptoms including variable degrees of impaired glucose tolerance, hyperinsulinemia, dyslipidemia and nonalcoholic hepatosteatosis. Those symptoms can be reversed by estradiol treatment (42;43). In our study, estrogen deprivation by exemestane treatment resulted in a dose-dependent decrease of the liver/TBW ratio, which confirms literature reports showing that the liver is a target organ for estrogen signaling (44;45). This effect seems to be rather persistent since in the E100(3) rats the liver/TBW ratio did not normalize, but 3 weeks may have been too short to allow for a complete recovery of liver weight. Karimian *et al.* also reported a reduced liver weight, without signs of hepatotoxicity in tamoxifen-treated rats (35). The physiological consequences of liver weight reduction in exemestane-treated rats remain unknown, as liver function was not assessed in this study.

Exemestane caused a dose-dependent decrease of the seminal vesicle/TBW ratio, but had no effect on the testis/TBW ratio. E100(3) did not influence seminal vesicle weights. ERα and ERβ expression and aromatase activity have been demonstrated in the reproductive tract of the male rat, implying that estrogen bioactivity is regulated, at least in part, locally (46;47). Our results are not compatible with the increased testis/TBW ratio and normal to increased seminal vesicle/TBW ratio described in adult male anastrozole-treated rats (44). Differences in age of the animals, type and pharmacokinetic properties of the applied aromatase inhibitor, and route of administration may explain the discrepancies. Our results are also in contrast with the normal reproductive tract in male ERβ knockout mice (48) and in aromatase knockout (ArKO) mice (49). There may be species differences in dependency of the reproductive tract on estrogen. The reproductive function was not analyzed in this study and therefore, the consequence of a reduced seminal vesicle weight remains unclear.

Male patients with aromatase deficiency show a variable degree of decreased fertility (25). Testis biopsies of an aromatase-deficient patient have revealed a normal aspect of testicular morphology, with signs of impaired spermatogenesis (43). Semen analysis in another aromatase-deficient patient also identified mild impairment of spermatogenesis (42), which may explain subfertility in these patients. In our rats, a normal testicular morphology was found, but semen analysis was not performed and reproductive disorders based on abnormal spermatogenesis cannot be excluded. Whether discontinuation of treatment reverses such abnormalities remains uncertain. Estrogen replacement in an aromatase-deficient patient did not result in normalization of spermatogenesis (42). Since obesity was very prominent in this patient both before and during estrogen replacement (BMI 29.3 and 32.0, respectively), reproductive disorders may also be secondary to overweight.

Aromatase expression plays an important role in the mammalian nervous system in neural differentiation and plasticity, neuroendocrine functions and sexual behavior (17). It was found to be upregulated and neuroprotective after a neurotoxic or mechanical lesion in the rat and mouse brain (50-52). In our study, no signs of neurodegeneration were observed in the hippocampus, a brain area that is sensitive to estradiol levels throughout the brain (53). This is in agreement with the normal hippocampal morphology in young, adult ArKO mice. We cannot exclude that aromatase inhibition may affect brain nuclei that develop during sexual maturation (e.g. the medial amygdala) (54). Moreover, aromatase inhibitor-treated rats may be more susceptible to neurodegeneration, as was reported for adult ArKO mice (55). Those mice also showed aberrant sexual behavior, which could be reversed by estrogen supplementation (56). In our study, sexual behavior was not studied.

The results of our study contrast with the increased predicted adult height after aromatase inhibitor treatment of boys with ISS, CDGP or GHD (11;13;15). Moreover, male patients with aromatase deficiency or estrogen resistance show a growth phenotype characterized by absence of a pubertal growth spurt, ongoing growth into adulthood, and absence of epiphyseal fusion, resulting in tall stature (25). Presumably, there are marked species differences in the influence of estrogens on longitudinal growth regulation.

The effects of aromatase inhibition by exemestane in the male rats in this study are distinctly different from those observed previously in female rats. The phenotype of the female exemestane-treated rats mimicked the clinical phenotype of aromatase-deficient female patients characterized by augmented growth. In contrast, the male rats in this study showed growth stagnation, whereas male aromatase-deficient patients exhibit a tall adult stature. Although the experimental designs applied in both studies were not completely identical, the distinct discrepancies allow us to conclude that marked gender differences in growth response to aromatase inhibition occur. The osteoporotic changes observed in both male and female rats

demonstrate that estrogen is important in the maintenance of bone quality in both genders. We conclude that six weeks of aromatase inhibition by the steroidal aromatase inhibitor exemestane in male rats causes retardation of tail growth and body weight gain, but has no significant effect on nose-anus length gain or appendicular growth. The adverse effect of treatment on bone quality was prominent, but long-term consequences for bone health and fracture risk remain unclear. The contrasting growth phenotypes of male exemestane-treated rats and human patients with estrogen deficiency or resistance indicate that a species difference in the role of estrogen signaling in growth regulation exists. Therefore, extrapolation of our results to clinical practice does not seem justified.

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Part C

Chapter 6

Human fetal mesenchymal stem cells differentiating towards chondrocytes display a similar gene expression profile as growth plate cartilage

S.A. van Gool[1*], J.A.M. Emons[1*], J.C.H. Leijten[2], E. Decker[3], X. Yu[4], C. Sticht[4], J.C. van Houwelingen[5], J.J. Goeman[5], C. Kleijburg[6], S. Scherjon[6], N. Gretz[4], J.M. Wit[1], G. Rappold[3], M. Karperien[1,2].

[1]Department of Pediatrics; [5]Department of Medical Statistics and Bioinformatics, and [6]Department of Obstetrics, Leiden University Medical Center, Leiden, the Netherlands.
[2]Department of Tissue Regeneration, Twente University, Enschede, the Netherlands. [3]Department of Human Molecular Genetics, and [4]Medical Research Center, Medical Faculty Mannheim, University of Heidelberg, Heidelberg, Germany;
[*]These authors contributed equally to this work

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Abstract

Background: Most studies on growth plate (GP) maturation and fusion have been carried out in animal models not fully representing the human epiphyseal GP.

Aims and methods: We used human fetal bone marrow-derived mesenchymal stem cells (hfMSCs) differentiating towards chondrocytes as an alternative model for the human GP. Our aims were to assess whether chondrocytes derived from hfMSCs are a suitable model for the GP and to study gene expression patterns associated with chondrogenic differentiation.

Results: hfMSCs efficiently formed hyaline cartilage in a pellet culture in the presence of TGFβ3 and BMP6. Microarray and principal component analysis were applied to study gene expression profiles during chondrogenic differentiation. A set of 315 genes was found to correlate with *in vitro* cartilage formation. Several identified genes are known to be involved in cartilage formation and validate the robustness of the differentiating hfMSC model. Other genes like Bradykinin and IFN-γ signaling, CCL20, and KIT were not described in association with chondrogenesis before. KEGG pathway analysis using the 315 genes revealed 9 significant signaling pathways correlated with cartilage formation.

To determine which type of hyaline cartilage was formed, we compared the gene expression profile of differentiating hfMSCs with previously established expression profiles of human articular (AC) and epiphyseal GP cartilage. As differentiation towards chondrocytes proceeds, hfMSCs gradually obtain a gene expression profile resembling epiphyseal GP cartilage, but not AC.

Conclusion: This study validates differentiating bone marrow-derived hfMSCs as an alternative model for the human epiphyseal GP.

Introduction

Growth of the long bones is the result of a tightly orchestrated proliferation and differentiation program called endochondral ossification. In the epiphyseal growth plate of long bones, chondrocytes originating from mesenchymal stem cells subsequently undergo proliferation, hypertrophic differentiation, and programmed cell death before being replaced by bone. At the time of sexual maturation, growth first increases but at the end of puberty epiphyseal fusion and termination of growth occur. Our knowledge on the molecular mechanisms underlying human growth regulation during puberty is limited, although estrogen has been identified as a key regulator of growth plate maturation and fusion (1). Gaining a detailed understanding of growth regulatory processes is essential to facilitate the development of novel strategies for the treatment of various growth disorders.

Commonly used animal models for studying growth plate regulation do not fully represent the human epiphyseal growth plate. For example, rodent growth plates do not fuse at the end of sexual maturation (2), and therefore do not display an important hallmark of human growth plate development. The shortcoming of the mouse model is furthermore demonstrated by the contrast between the marginally affected growth phenotype of the estrogen receptor alpha (ERα) knock out mouse (αERKO) (3) and the prominent growth phenotype of a male patient lacking functional ERα (4), which is characterized by the absence of epiphyseal fusion and continuation of growth into adulthood.

The lack of representative animal models has led to the realization that alternative human models are essential to elucidate the mechanisms involved in growth plate regulation and fusion. However, human growth plate specimens are difficult to obtain, whereas *in vitro* models such as chondrosarcoma cell lines or articular cartilage-derived chondrocyte cultures have limited differentiation capacity, are often difficult to maintain under laboratory conditions or tend to dedifferentiate. Furthermore, articular cartilage and growth plate cartilage have distinct functions and it is therefore questionable whether articular cartilage-derived chondrocytes are representative for epiphyseal growth plate chondrocytes.

Multipotent human mesenchymal stem cells (hMSCs) are a promising *in vitro* model to study chondrogenesis. They have been postulated as an alternative cell source for articular cartilage reconstruction and for studying endochondral ossification as it occurs in the epiphyseal growth plate (5). In this study, we explored the cartilage forming capacity of human fetal (hf)MSCs aiming at the development of an *in vitro* model for the human growth plate. We have chosen human fetal bone marrow-derived MSC for their superior differentiation characteristics compared to adult bone marrow-derived MSCs (6). Efficient cartilage formation was

demonstrated by immunohistochemical analysis and gene expression profiling was applied to identify genetic pathways involved in the differentiation process. In addition, the gene expression profiles of the differentiating hfMSCs were compared with global gene expression patterns of human articular and growth plate cartilage to assess whether differentiating hfMSCs represent either articular or growth plate chondrocytes.

Experimental Procedures

Cell culture

The use of human fetal material was approved by the medical ethical committee of the Leiden University Medical Center and an informed consent was obtained from the women undergoing elective abortion. Cell suspensions of fetal bone marrow were obtained by flushing the long bones of fetuses with M199 washing medium. For the chondrogenic differentiation and microarray analysis, cells derived from a single 22 weeks old fetus were used. MSCs derived from other fetuses were also stimulated to undergo chondrogenic differentiation. Red cells were depleted by incubation for 10 minutes in NH_4Cl (8.4 g/L)/$KHCO_3$ (1g /L) buffer at 4°C. Mononuclear cells were plated at a density of 16×10^4 cells/cm² in M199 culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptavidin (P/S), fungizone, endothelial cell growth factor (ECGF) 20 μg/ml (Roche Diagnostics) and heparin 8 U/ml in culture flasks coated with 1% gelatin according to previously established culture conditions for human fetal MSCs (7). Cultures were kept in a humidified atmosphere at 37°C with 5% CO2. The culture medium was changed twice per week. After reaching near-confluence at passage 4 to 5 (15 population doublings), hfMSCs were harvested by treatment with 0.5 % trypsin and 0.5% ethylene diamine tetra acetic acid (EDTA; Gibco) for 5 minutes at 37°C and replated for chondrogenic differentiation.

In vitro chondrogenic differentiation

hfMSCs (2×10^5 cells/well) were cultured in cell pellets. Pellets were formed by centrifugation of the cells at 1200 rpm for 4 minutes in U-shaped 96-well suspension culture plates (Greiner). To induce chondrogenesis the pellets were cultured at 37°C with 5% CO_2 in 200 μl of serum-free chondrogenic medium consisting of high-glucose (25 mM) Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 40 μg/ml proline (Sigma), 100 μg/ml sodium pyruvate (Sigma, USA), 50 mg/ml ITS (insulin-transferrin-selenic acid) with Premix (BD Biosciences), 1% Glutamax (Gibco), 1% penicillin/streptavidin, 50 μg/ml ascorbate-2-phosphate (Sigma), 10^{-7} M

dexamethasone (Sigma), 10 ng/ml transforming growth factor-β3 (TGF-β3; R&D Systems), 500 ng/ml bone morphogenetic protein 6 (BMP6) and antibiotic and antimycotic mix (0.06% polymixin, 0.2% kanamycin, 0.2% penicillin, 0.2% streptavidin, 0.02% nystatin and 0.5% amfotericin essentially as described by Sekiya *et al.*, 2001. The medium was changed twice per week for 5 weeks.

Histological analysis

Two pellets per time point (after 1, 2, 3, 4, or 5 weeks of chondrogenesis) were used for histological evaluation. Pellets were fixed in 10% formalin, dehydrated by treatment with graded ethanols and processed for paraffin embedding. 5 μm sections were cut using a Reichert Jung 2055 microtome (Leica). For each pellet, only the sections from exactly the center of the pellets were mounted on glass slides. Before histological (toluidine blue) or immunohistochemical staining, sections were deparaffinized in xylene, treated with graded ethanols followed by three washing steps with phosphate buffered saline (PBS).
For immunofluorescence of collagen type II, sections were pre-treated with 10mM citric acid buffer (pH=6) for antigen retrieval. Sections were incubated with a collagen type II monoclonal antibody (clone 3HH1-F9, Abnova) at 1:100 dilution in 1% bovine serum albumine (BSA) /PBS buffer overnight at 4°C. After washing, sections were incubated with Alexa Fluor 488-Goat anti-Mouse IgG1 (Invitrogen, Molecular Probes, diluted 1:1000 in PBS/1% BSA) for 1 hour and protected from light. Sections were counterstained with DAPI and mounted with vectashield.
For collagen type X immunohistochemistry, sections were preincubated with blocking buffer (1% H2O2 in 40% methanol, 60% tris buffered saline) twice for 15 minutes at room temperature, followed by overnight incubation at 4°C with mouse monoclonal antibody against collagen type X in a 1:100 dilution (Quartett). Next, sections were incubated with the secondary antibody biotinylated rabbit-anti-mouse IgG (DAKO) in a 1:300 dilution, followed by incubation with horseradish-peroxidase-conjugated-streptavidine (Amersham Biosciences). Staining was visualized with 3-amino-9-ethylcarbazole substrate in 0.2 mg/ml acetate buffer (pH 5.2) with 0.04% H_2O_2. After counterstaining with hematoxylin, the sections were mounted in Histomount (National Diagnostics). Pictures of the stained pellets were taken with a Nikon DXM 1200 digital camera using standardized settings.

RNA isolation

Total RNA from $2 \cdot 10^6$ undifferentiated hfMSCs derived from the 22-weeks old fetus was extracted with Trizol (Invitrogen). After 1, 2, 3, 4, or 5 weeks of chondrogenesis, 60 pellets (per time point) were pooled and homogenized in 1ml 4M guanidine isothiocyanate solution (Sigma) and RNA was extracted according to the optimized method for RNA extraction from cartilage as described by Heinrichs *et al.* (8). The extracted total RNA was purified using the RNeasy kit according to recommendations of the manufacturer (Qiagen).

Gene expression profiling

High RNA quality was confirmed by capillary electrophoresis on an Agilent 2100 bioanalyzer (Agilent). Total RNA (100 ng) was amplified and labeled using the GeneChip Two-Cycle cDNA Synthesis Kit (Affymetrix) and the MEGAscript T7 Kit (Ambion). For gene expression profiling, labeled cRNA was hybridized in duplicate to Affymetrix Human Genome U133 PLUS 2.0 Array Genechips. All procedures were carried out according to the manufacturer's recommendations. Raw data from Affymetrix CEL files were analyzed using SAS software package Microarray Solution version 1.3 (SAS Institute). Custom CDF version 10 with Entrez based gene definitions (9) was applied to map the probes to genes. Gene annotation was obtained using the Affymetrix NetAffx website (http://www.affymetrix.com/analysis/index.affx). Quality control, normalization and statistical modeling were performed by array group correlation, mixed model normalization and mixed model analysis respectively. The normalized expression values for each gene were standardized by linearly scaling the values across all samples of the time course to a mean of 0 with an SD of 1. Analysis of differential gene expression was based on log-linear mixed model of perfect matches (10). A false discovery rate of a=0.05 with Bonferroni-correction for multiple testing was used to set the level of significance. The raw and normalized data are deposited in the Gene Expression Omnibus database (available at http://www.ncbi.nlm.nih.gov/geo).

Microarray data analysis

The statistical analysis of the microarray data was based on the normalized mean expression values per probe at 6 time points with 2 replications at each time point (12 observations per probe). In order to identify subgroups of probes with similar expression profiles over time, a principal component analysis (PCA) of the covariance matrix was carried out on the mean expression value for each probe at each time point. For each probe, factor scores for principal

components 1, 2 and 3 were obtained by regression analysis of the 12 array results (6 time points in duplicate) for that specific probe to those components. The first principal component corresponded with the general expression level during the whole experiment, whereas the second and third component corresponded with changes over time. Since our interest was to identify genes associated with the changes that occur during differentiation from stem cells towards chondrocytes, we focused our analysis on the second and third component. By construction, these factor scores had a mean of 0 with an SD of 1. Generally, the distribution over the factor scores showed a normal distribution with outliers. We used a cut-off of ±3.29 to select outlying probes. This cut-off would select 0.1% of the probes, if the factors scores would follow a pure normal distribution that could be expected if the data were pure noise. The presence of replications allowed us to assess the statistical significance of the factor scores and to remove probes that were not significant at the α=5% level.
In a separate study we compared the gene expression profiles of human articular cartilage (AC) and epiphyseal growth plate (GP) cartilage. A set of 1818 significant differentially expressed genes was identified, that can be used to discriminate between the two hyaline cartilage subtypes (Leijten *et al.*, manuscript in preparation). All AC (n=5) and GP (n=5) samples were derived from 9 to 17 year old female donors with no history of growth disorders. The gene expression profiles of the stem cells differentiating towards chondrocytes were compared with this list. Principal component analysis (PCA) with Pearson product-moment correlation was performed to compute correlations between the expression profiles.

Pathway analysis

Using sets of probes emerging from PCA, a search for relevant KEGG pathways was performed using the DAVID® Knowledgebase, a publicly available bioinformatics tool for functional annotation (http://david.abcc.ncifcrf.gov).

Quantitative real-time polymerase chain reaction (qPCR)

RNA was transcribed into cDNA using the First Strand cDNA Synthesis kit for qPCR (Roche Diagnostics) according to the manufacturer's protocol. Specific primer sets (available on request) were designed to amplify aggrecan (ACAN), pannexin 3 (PANX3), epiphycan (EPYC), collagen type II (COL2), and type X (COL10), SRY-box 9 (SOX9), WNT11, lymphoid enhancer-binding factor 1 (LEF1), Gremlin 1 (GREM1). β_2-Microglobulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes. Based on the microarray data, the

expression of these housekeeping genes was stable during differentiation of hfMSCs. In order to test donor inter-variation, differentiated MSCs isolated from other fetal donors were used for qPCR analysis as well.

All PCR reactions were performed in triplicate with 5 ng cDNA and according to the manufacturer's protocol of the iQ™ SYBR® Green Kit (Biorad) in a final volume of 25 µl. The cDNA was amplified using the following thermal cycling conditions: one cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 56°C using the mIQ Single-Color—Real-Time PCR System (BioRad Laboratories, Hercules, California, USA). Fluorescence spectra were recorded and the threshold cycle number (Ct) was calculated using the accompanying mIQ-software. For each time point mean Ct was calculated and from this value the fold difference in expression between undifferentiated hfMSCs and differentiating cells using the $2^{-\Delta\Delta Ct}$ method was calculated essentially as described by Schmittgen and Livak, 2008 using β_2-Microglobulin as a reference. For visualization, this value was log-transformed.

Results

Chondrogenic differentiation by hfMSCs

Evaluation of protein and mRNA expression

Pellet cultures were used to induce chondrogenic differentiation of hfMSCs and samples were collected at 1, 2, 3, 4 and 5 weeks of culture. Immunohistological evaluation showed an increasing expression of cartilage markers with time and a gradual morphological change from stem cells to mature and hypertrophic chondrocytes (figure 1). The mean diameter of the pellets increased with time, as well as the amount of glycosaminoglycans, a major constituent of the cartilaginous extracellular matrix. Immunofluorescent staining for collagen type II demonstrated the presence of chondrocytes after 1 week of pellet culture. The expression of collagen type II increased over time. Hypertrophic chondrocytes were first detected after 3 weeks, as evidenced by immunohistochemical staining for collagen type X. These collagen type 10 positive cells were located in a discrete ring-like zone surrounded by collagen type 2 positive chondrocytes. In all stages of differentiation, the chondrogenic core of the pellets was surrounded by a thin layer of two to three undifferentiated cells (figure 1).

From each time point RNA was isolated and subjected to microarray analysis. Changes in gene expression of a subset of genes consisting of both established marker genes for chondrogenesis and differentially expressed genes identified by microarray analysis were validated using qPCR

(figure 2). In concordance with the observations of immunohistological markers of chondrogenesis, microarray data and qPCR showed time-dependent increases in the expression of the cartilage markers collagen type II, and type X, SOX9, and aggrecan mRNA. To further extend this analysis, we randomly selected 7 genes (pannexin 3, epiphycan, WNT11, LEF1, gremlin 1, Dickkopf 1, matrilin) that showed marked regulation over time based on microarray analysis. Again, qPCR demonstrated a strong correlation between the expression patterns revealed by both techniques (results for 5 of these genes are shown in figure 2E-I), providing further support for the robustness of our dataset. Repeating the qPCR analysis using RNA isolated from other fetal donors of MSCs that were stimulated to undergo chondrogenic differentiation rendered similar gene expression patterns as observed in this study (data not shown).

Principal component analysis and KEGG pathway analysis

The sequential changes that occur during chondrogenic differentiation in the hfMSC model were studied with bioinformatics analysis of the microarray data. Using principal component analysis, three components were found to explain 99.6% of the variance within our dataset (figure 3.A). The factor loadings in figure 3.B show that component 1 describes a general level of gene expression, as expected. Component 2 shows to what extent gene expression changed with time during chondrogenic differentiation and

Figure 1

Expression of (A) glycosaminoglycans visualized by toluidine blue staining, (B) collagen type II fluorescence, and (C) collagen type X immunohistochemistry (brown) during 5 weeks of chondrogenic differentiation of hfMSCs to chondrocytes. The top panel shows a magnification of the pellet cultures at week 1 and week 5 stained by toluidin blue demonstrating the change in cell morphology and the deposition of the extracellular matrix. The insets in panel B show higher magnifications of collagen type II positive chondrocytes.

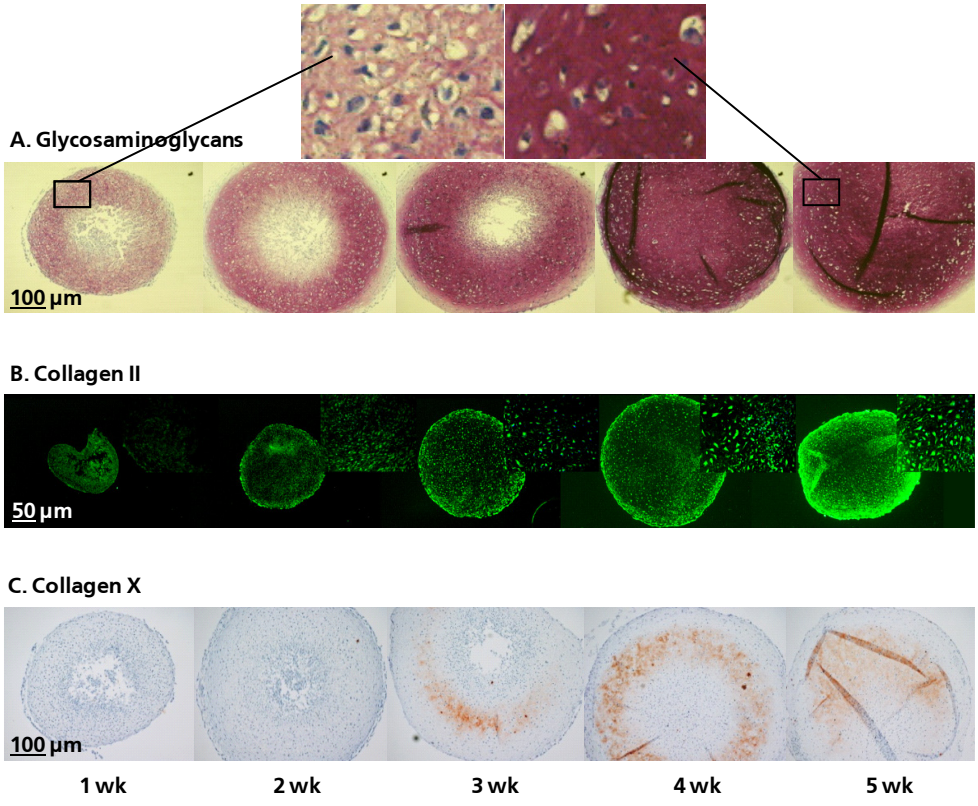


Figure 2

Correlation between qPCR and microarray expression data for (A) aggrecan, (B) collagen II, (C) collagen X, (D) SOX9, (E) pannexin 3, (F) epiphycan, (G) WNT11, (H) LEF1, and (I) gremlin 1 during 5 weeks of chondrogenic differentiation of hfMSCs. qPCR data are expressed as delta delta CT values corrected for the housekeeping gene β2-microglobulin. The primary y-axis (left) indicates the qPCR results as normalized fold expression on a log-scale. The secondary y-axis (right) indicates the microarray analysis results as least square means (lsm).

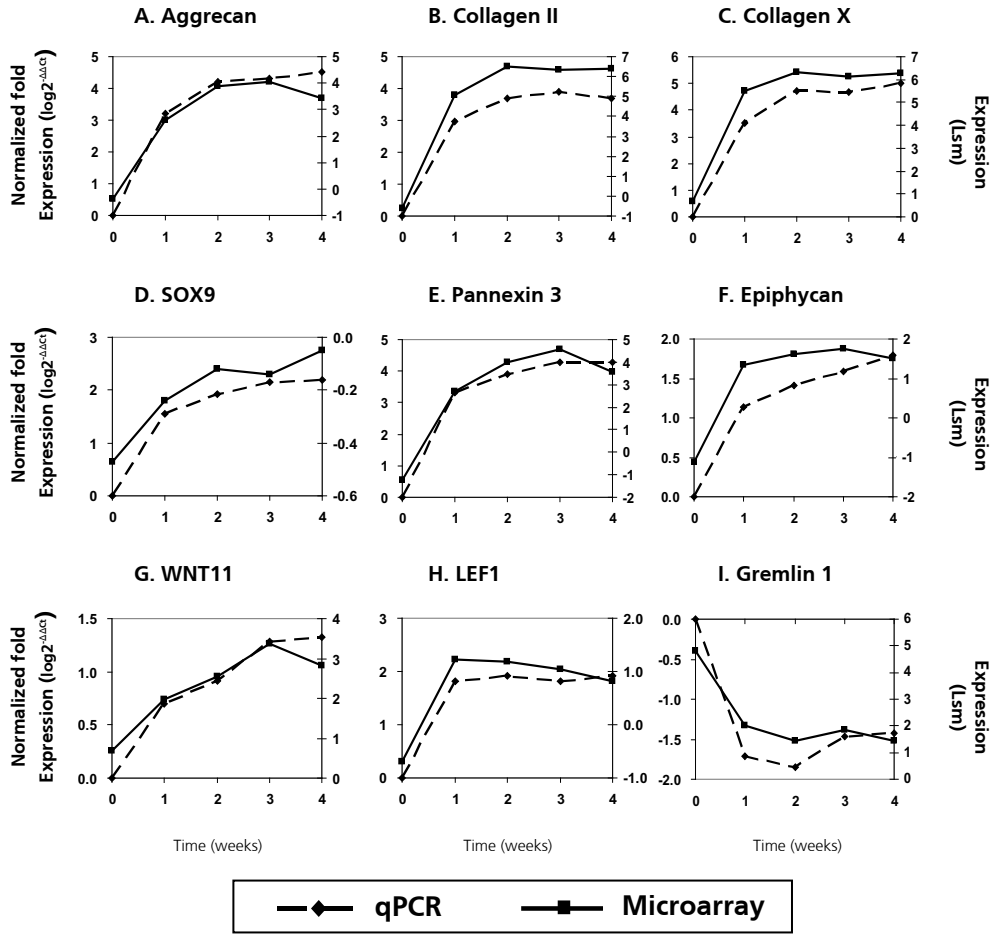


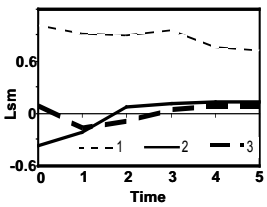
Figure 3

Gene selection based on principal component analysis. A) variance explained by components 1-6 from principal component analysis. B) principal components 1, 2, and 3 as expression profiles. C) selection of probes based on their factor 2 and 3 scores. D) scatterplot view of gene data in respect to their correlation (factor score) to prinicpal components 2 and 3. Subgroups 1, 2, 3, and 4 are represented by blue, green, yellow, and pink dots, respectively. Side-placed graphs depict the gene expression profiles for genes found in the four subgroups.

A. Variance explained by PCA

Component	Variance (%)	Cumulative variance (%)
1	95.16	95.16
2	3.24	98.40
3	1.15	99.55
4+5+6	0.45	100

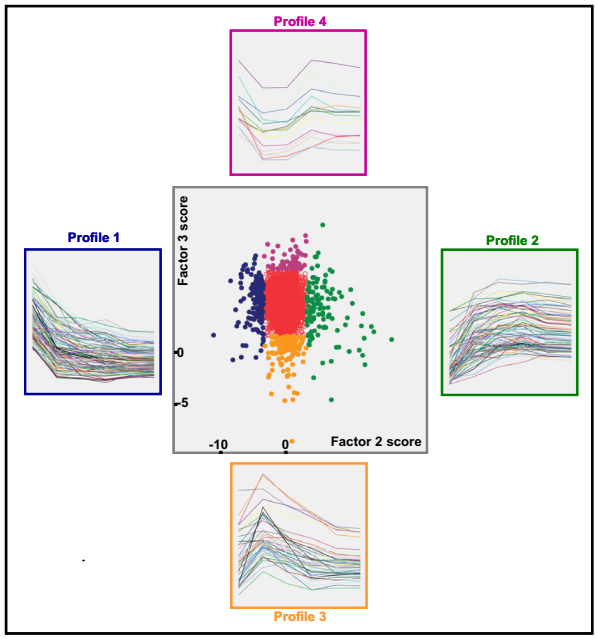
B. Principal components



C. Subgroup definitions

Subgroup	Factor 2 score	Factor 3 score	Nº. of probes	Sign. probes
1	≤-3.29		149	146
2	≥3.29		118	105
3	<-3.29	≤-3.29	135	49
4	>3.29	≥-3.29	64	15

D. Expression profiles



component 3 signifies whether there was an additional, short term elevation or dip in expression around 2 to 3 weeks of differentiation. Since components 2 and 3 were most likely to contain genes associated with the loss of stem cell characteristics or the gain of a chondrocyte phenotype, we focused on those components.

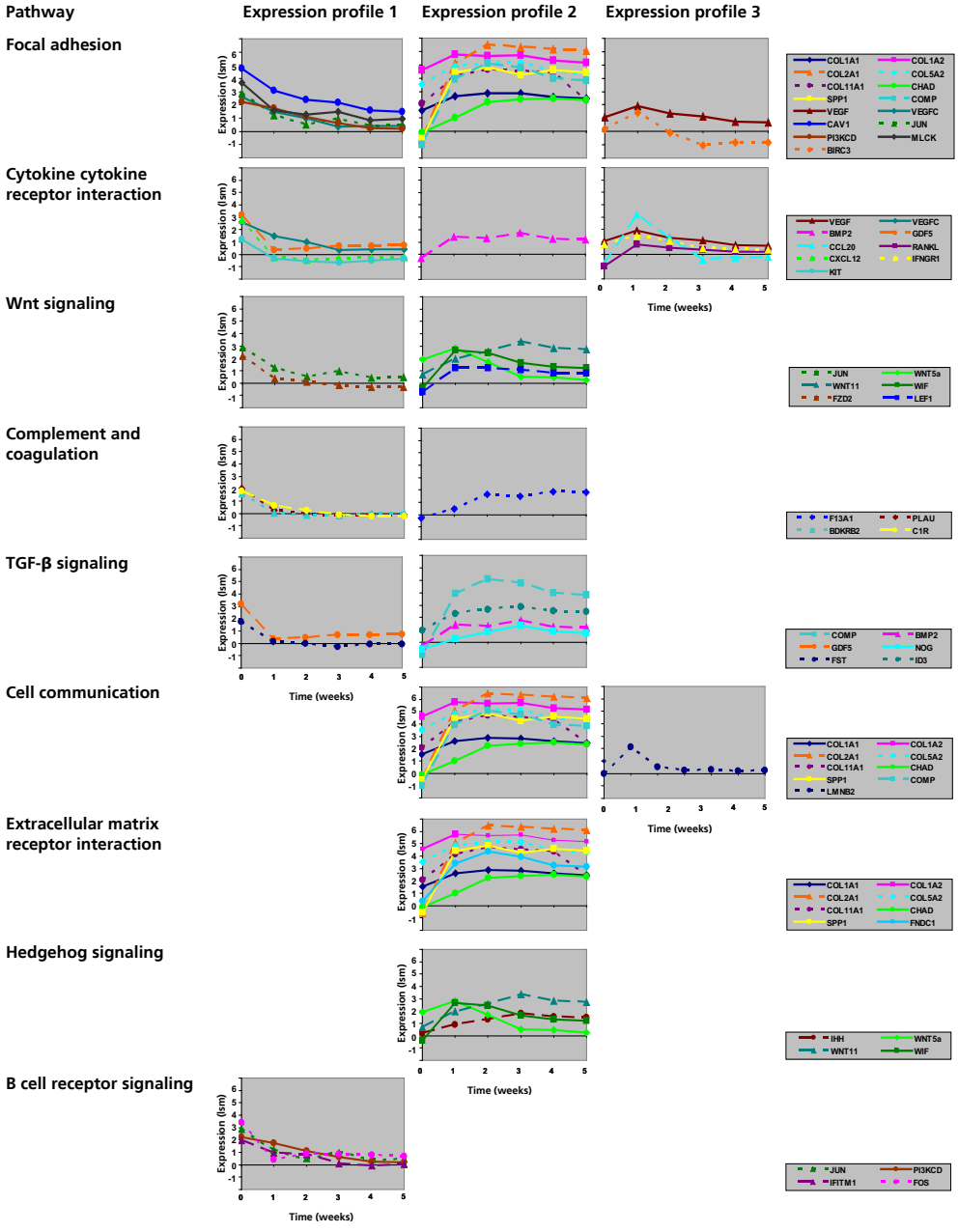
Using the ±3.29 cut-off in combination with a 5% significance test, we distinguished four subgroups of probes. The precise definitions and the resulting numbers of these subgroups are given in figure 3.C. The scatter plot in figure 3.D illustrates that the numbers of probes in subgroups 1 and 2 are much larger than the 9 probes (0.05%) that would have been expected under purely random selection. Moreover, in these two subgroups nearly all probes in the first selection are significant at the 5% level, suggesting that the number of false discoveries in these two groups is quite small. More noise is presumably present in the smaller subgroups 3 and 4 based on factor 3 scores.

The profiles of the selected probes demonstrate that subgroup 1 containing the largest number of probes (n=146) describes a peak of expression on t_0 followed by a decrease in expression thereafter. In contrast, the second largest subgroup of probes (n=105) in profile 2 demonstrates increasing expression levels from t_0 onward. The smaller subgroups 3 and 4 demonstrate lower levels of expression with profile 3 (n=49) showing a short-term increase in expression at t_1 followed by decreases thereafter and profile 4 (n= 15) displaying a short-term expression dip between t_1-t_2.

A total of 83 out of 315 probes could not be annotated and was discarded from further analysis. The remaining 232 probes that could be matched to genes (supplementary table 1) were used to identify 9 KEGG pathways that were significantly associated with chondrogenic differentiation and contained 39 genes. (figure 4). Some genes were present solely in one pathway (n= 23), but others were found in 2 (n= 6) or 3 (n= 10) pathways (table 1). Three functional groups of genes were recognized: 1) growth factor (GF) and GF-related genes; 2) genes associated with the extracellular matrix; and 3) genes associated with signal transduction, cell cycle, and cell survival. In supplementary table 2, we have listed the top hits of upregulated genes as identified by the microarray analysis at each time point compared to undifferentiated hfMSCs.

Figure 4

KEGG signaling pathways significantly associated with chondrogenic differentiation of hfMSCs. For each pathway, genes showing the same distinct expression profile during 5 weeks of chondrogenic differentiation are depicted as groups.

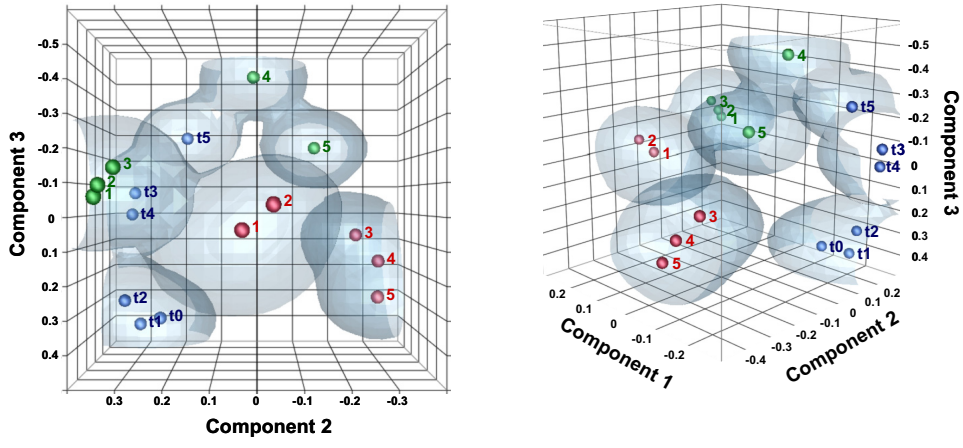


Gene expression fingerprinting for cartilage subtype

Histological and gene expression analyses showed that the differentiating hfMSCs acquire a hyaline cartilage phenotype. Two major types of hyaline cartilage can be distinguished, namely articular and epiphyseal cartilage. In order to serve as a model for the epiphyseal growth plate, differentiating hfMSCs should obtain a growth plate signature. To test this, we compared the expression profiles of the differentiating hfMSCs with previously established profiles of human articular and growth plate cartilage (AC and GP, respectively, Leijten *et al.*, in preparation). In a three-dimensional schematic representation, samples of AC and GP plot in two different groups (figure 5). As expected, AC, GP, and undifferentiated hfMSCs (hMSC_t0) plotted as distinct entities in a three-dimensional space. As differentiation progressed, the expression profile of the hfMSCs changed, and the differentiating chondrocytes gradually acquired a fingerprint resembling GP, but not AC. This analysis demonstrated that the hfMSCs differentiating towards chondrocytes acquired a GP cartilage-like phenotype.

Figure 5

Three-dimensional overviews of gene expression data in respect to their correlation (factor score) to principal components 1, 2, and 3. Red dots, samples of articular cartilage; Green dots, samples of growth plates; Blue dots, differentiation time-range of hfMSCs (t0, undifferentiated hfMSCs; t5, mature chondrocytes). Dots indicate the mean factor score for all genes on the Affymetrix chip on the three principal components for one cartilage sample. Clouds represent the spread around the mean factor score in three dimensions.



Discussion

The present study was conducted in order to determine whether fetal bone marrow-derived MSCs are a representative human model for studying processes taking place in the epiphyseal growth plate and to identify associated signaling pathways. It has been reported that human bone marrow-derived MSCs display a better chondrogenic differentiation capacity than those derived from other sources, with fetal being superior over adult MSCs (6). Consequently, it seems appropriate to use fetal bone marrow-derived MSCs as a model for chondrogenesis. However, fetal MSCs are not easily obtained, due to ethical and legal considerations, and as a consequence, adult bone marrow-derived MSCs have been used in many previous studies (11-18).

Chondrogenic differentiation occurred in our *in vitro* model, as illustrated by the progressive increase in expression of the chondrocyte markers collagen type II and X and the cartilaginous matrix constituent glycosaminoglycan over time. Interestingly, hypertrophic differentiation as evidenced by an increase in cell size and positive staining for collagen 10 was only observed in a discrete zone of the pellet which was surrounded at both sites by chondrocytes positive for collagen type 2.

Further confirmation of chondrogenesis was obtained by analysis of mRNA expression of cartilage markers, which, in addition, also validated our microarray results. Similar gene expression patterns were obtained during chondrogenic differentiation of fetal MSCs derived from other donors. This suggested that the selected 22-weeks old fetal MSC-donor was representative for fetal bone marrow in general.

Matrix mineralization was not observed after 5 weeks of differentiation, suggesting that the matrix was not ready for mineralization or that environmental stimuli necessary to induce this process were absent.

Microarray analysis generated a multidimensional dataset of differentially expressed genes for each time point. Several methods for analyzing such complex data have been reported, many based on presence/absence analysis, which starts with the list of differentially expressed genes and applies a strict but arbitrary cut-off for differential expression of individual genes (13;18). Misinterpretation of the data can easily occur, since genes are assumed to be independent, whereas it is more likely that sets of correlated genes play a role in complex biological processes (19). Genes that are not considered differentially expressed, but that do play a role in important signaling pathways, may be wrongfully eliminated. Another analysis strategy applied by many groups is to report on *a priori* selected pathway(s) of interest, thereby disregarding the relative importance of this pathway in view of other potentially co-regulated or interacting pathways.

Alternatively, we have applied PCA with restrictive criteria as a statistical selection method for identification of gene expression profiles associated with the acquisition of chondrocyte characteristics or the loss of a stem cell phenotype. Since biological replicates were not included in this study, such a stringent approach was necessary in order to minimize potentially false-positive results.
The gene expression data generated with this analytic approach are consistent with previous reports on *in vitro* cartilage formation by adult mesenchymal stem cells. We therefore conclude that PCA is a suitable and unbiased analysis tool for data reduction in multidimensional and complex microarray experiments. Using this method, 232 genes were identified to be significantly associated with chondrogenic differentiation, 39 of which were present in 9 significantly enriched KEGG pathways. These 39 genes could be classified in three major functional groups that are discussed in the following sections.

Growth factor (GF) and GF-related genes

Growth factors from the transforming growth factor β (TGF-β), Wnt, Hedgehog and VEGF families have been recognized as major regulators of endochondral bone formation in embryos and postnatally (20;21). Expression of some members of these families changed over time in our *in vitro* model suggesting their involvement in chondrocyte differentiation from fhMSCs.
BMP2 and its downstream effector ID3 are upregulated early in differentiation consistent with previous reports on the importance of BMP signaling in chondrogenesis (22;23). Growth and differentiation factor 5 (GDF5), previous reported as stimulator of chondrocyte proliferation (24), was highly expressed at the earliest time point observed and downregulated thereafter. A similar expression profile was found for the BMP inhibitor follistatine (FST) that was previously shown to be expressed by proliferative, but not by hypertrophic chondrocytes (25).
Previous *in vitro* studies have demonstrated that BMP2 interacts with Wnt and hedgehog family members and their downstream effectors, indicating that functional crosstalk between regulatory pathways occurs during chondrogenesis (23;26). Such interactions may have taken place in our *in vitro* model as well, since several genes out of the Wnt and Hedgehog family were affected during differentiation, e.g. WNT5a, WNT11, FZD2, WIF1 and IHH. IHH expression reached a maximum after 3 weeks of differentiation, a time point at which the first collagen type X positive hypertrophic chondrocytes were detected using immunohistochemistry. IHH is recognized as an important regulator of hypertrophic chondrocyte differentiation.
The superfamily of cytokines was another major group of regulatory factors for which involvement in chondrogenesis was suggested. Several genes in this group were changing

significantly over time, e.g. CXCL12, CCL20, interferon-γ (IFN-γ), IFN-γ receptor IFNGR1, interferon-induced transmembrane protein 1 (IFITM1) and the cytokine RANKL (receptor activator for nuclear factor κ B ligand). To our knowledge a role for CCL20, IFITM1, IFN-γ and its receptor IFNGR1 in chondrogenesis has not been described before.
Vascular endothelial growth factors (VEGF and VEGFC), originally described to promote epiphyseal vascularization prior to endochondral ossification, also regulate *in vitro* chondrogenesis (27-29). Both the expression of VEGF and VEGFC were significantly changing at early time points in our model.

Genes associated with the extracellular matrix

Progression of chondrogenic differentiation depends on the coordinated expression of ECM components and on cell-matrix interactions (20). Expression of several genes involved in focal adhesion, cell-matrix communication, ECM receptor interaction and matrix remodeling changed significantly over time in our *in vitro* model of chondrogenesis.
The expression of cartilaginous ECM proteins, such as collagens (COL1A1, COL1A2, COL2A1, COL5A2, COL11A1), chondroadherin (CHAD), cartilage oligomeric matrix protein (COMP), secreted phosphoprotein 1 (osteopontin, SPP1), and fibronectin type III (FNDC1), was upregulated. Apart from a structural role in the extracellular matrix, FNDC1, SPP1 and CHAD also function as integrin ligands and regulate cell-matrix signaling by binding to the cell surface plasma membrane protein integrinT. Aggregation of integrins in focal adhesions is induced by activity of myosin light chain kinase (MLCK) (30). Upon ligand-integrin binding, signaling complexes are activated that mediate downstream effectors of integrin signaling such as phosphoinositide-3-kinase (PI3K) (31), thereby stimulating cell proliferation (32). We observed early downregulation of integrin signaling-related proteins such as PI3K and MLCK.
Members of the complement and coagulation family of proteins were expressed during *in vitro* chondrogenic differentiation. Coagulation factor XIII (F13A1) expression was upregulated, while other genes like complement component I (C1R), urokinase-type plasminogen activator (PLAU) and bradykinin receptor B2 (BDKRB2) were downregulated during differentiation in our model. These proteins are associated with matrix mineralization (33-35) or matrix degradation in the growth plate (36-38).

Genes associated with signal transduction, cell cycle, and cell survival

Based on differential expression, several genes involved in the regulation of cell survival and proliferation and signal transduction were identified in our *in vitro* model of chondrogenesis. Caveolin 1 (CAV1), a multifunctional scaffolding protein located at cell surface caveolae, regulates TGF, Wnt, cytokine and VEGF signaling by modulating their downstream signaling cascades such as the JAK/STAT, β-catenin/LEF1, MAPK/ERK and PI3K/AKT signaling pathways (39-42). The anti-apoptotic baculoviral IAP repeat containing 3 (BIRC3) gene has been shown to increase the survival of cultured human chondrocytes (43). Phosphoinositide-3-kinase (PI3K), proto-oncogene KIT, transcription factor JUN and FOS are all associated to the regulation of cell proliferation (44;45). Expression of the nuclear envelope protein lamin B2 (LMNB2) was first upregulated and later in differentiation downregulated in our model. Constantinescu et al suggested a role for LMNB2 in suppressing differentiation of undifferentiated embryonic stem cells (46).

Conclusion

Many genes identified in this study were previously reported in association with chondrogenesis, validating the robustness of differentiating hfMSC as a model for cartilage formation. The implication of bradykinin and IFN-γ signaling, CCL20, and KIT are novel findings. Discrepancies between our results and reports by others may rely on differences between the source, and chondrogenic capacity of MSCs used, the experimental conditions for inducing chondrogenesis, and the gene expression analysis methods (13;14;17). Developmental genes essential for chondrogenic differentiation (e.g. SOX genes, IGF-I) were not identified, in line with other reports (15). Marginal changes in the expression of these genes may be sufficient for inducing major effects, but too subtle to be detected by most gene expression analysis methods, including PCA. Alternatively, changes occurring within the first days of differentiation may have been unnoticed due to the chosen time interval of analysis.

This study has demonstrated for the first time that bone marrow-derived hfMSCs acquire an epiphyseal GP-, rather than an AC-like gene expression signature during differentiation towards chondrocytes. This conclusion is based on comparison of expression profiles of the differentiating hfMSCs with human growth plate cartilage and human articular cartilage. These findings confirm the growth plate-like nature of the differentiating fhMSCs, indicating its validity as an alternative model for the human epiphyseal growth plate. Although a native growth plate is present in rodent animal models, these growth plates are not fully representative for the human growth plate particularly in puberty. The *in vitro* model developed in this study can potentially be used as an alternative for animal models. It is readily accessible

for genetic manipulation and might be used for unraveling the molecular mechanisms underlying growth regulation in the human epiphyseal plate during puberty. As such it may find its use in the development of novel treatment strategies for various growth disorders aimed at intervening in growth plate maturation and fusion

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Supplementary Table 1. List of genes selected with principal component analysis

Affymetrix ID	Gene code	Gene title
205856_at	SLC14A1	solute carrier family 14 (urea transporter), member 1 (Kidd blood group)
205911_at	PTHR1	parathyroid hormone receptor 1
219148_at	PBK	PDZ binding kinase
213182_x_at	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)
206737_at	WNT11	wingless-type MMTV integration site family, member 11
203868_s_at	VCAM1	vascular cell adhesion molecule 1
218730_s_at	OGN	osteoglycin (osteoinductive factor, mimecan)
200665_s_at	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
223484_at	C15orf48	chromosome 15 open reading frame 48
206315_at	CRLF1	cytokine receptor-like factor 1
205497_at	ZNF175	zinc finger protein 175
204724_s_at	COL9A3	collagen, type IX, alpha 3
219410_at	TMEM45A	transmembrane protein 45A
218391_at	SNF8	SNF8, ESCRT-II complex subunit, homolog (S. cerevisiae)
210538_s_at	BIRC3	baculoviral IAP repeat-containing 3
201487_at	CTSC	cathepsin C
219134_at	ELTD1	EGF, latrophilin and seven transmembrane domain containing 1
212551_at	CAP2	CAP, adenylate cyclase-associated protein 2 (yeast)
206421_s_at	SERPINB7	serpin peptidase inhibitor, clade B (ovalbumin), member 7
219837_s_at	CYTL1	cytokine-like 1
210220_at	FZD2	frizzled homolog 2 (Drosophila)
207064_s_at	AOC2	amine oxidase, copper containing 2 (retina-specific)
218542_at	CEP55	centrosomal protein 55kDa
206423_at	ANGPTL7	angiopoietin-like 7
231227_at	---	Transcribed locus, strongly similar to WNT-5A protein precursor
229494_s_at	CD63	CD63 molecule
223734_at	OSAP	ovary-specific acidic protein
206614_at	GDF5	growth differentiation factor 5 (cartilage-derived morphogenetic protein-1)
205713_s_at	COMP	cartilage oligomeric matrix protein
230372_at	---	Transcribed locus, PREDICTED: similar to hyaluronan synthase 2 [Pan troglodytes]
1563724_at	SACS	Spastic ataxia of Charlevoix-Saguenay (sacsin)
203499_at	EPHA2	EPH receptor A2
1556499_s_at	COL1A1	collagen, type I, alpha 1
219230_at	TMEM100	transmembrane protein 100
206790_s_at	NDUFB1	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7kDa
204825_at	MELK	maternal embryonic leucine zipper kinase
212565_at	STK38L	serine/threonine kinase 38 like
1554997_a_at	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase & cyclooxygenase)
204894_s_at	AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)
203886_s_at	FBLN2	fibulin 2
203153_at	IFIT1	interferon-induced protein with tetratricopeptide repeats 1
242517_at	KISS1R	KISS1 receptor
1552340_at	SP7	Sp7 transcription factor
203963_at	CA12	carbonic anhydrase XII

1554950_at	AGC1	aggrecan 1 (chondroitin sulfate proteoglycan 1, large aggregating proteoglycan)
232451_at	---	MRNA; cDNA DKFZp564I0816 (from clone DKFZp564I0816)
227705_at	TCEAL7	transcription elongation factor A (SII)-like 7
1570574_at	GPR177	G protein-coupled receptor 177
218273_s_at	PPM2C	protein phosphatase 2C, magnesium-dependent, catalytic subunit
224735_at	CYBASC	cytochrome b, ascorbate dependent 3
239787_at	KCTD4	potassium channel tetramerisation domain containing 4
226281_at	DNER	delta-notch-like EGF repeat-containing transmembrane
218839_at	HEY1	hairy/enhancer-of-split related with YRPW motif 1
214710_s_at	CCNB1	cyclin B1
231798_at	NOG	Noggin
204595_s_at	STC1	stanniocalcin 1
209189_at	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
203297_s_at	JARID2	Jumonji, AT rich interactive domain 2
230137_at	TMEM15	transmembrane protein 155
208078_s_at	SNF1LK	SNF1-like kinase
217989_at	DHRS8	dehydrogenase/reductase (SDR family) member 8
229125_at	ANKRD3	ankyrin repeat domain 38
205141_at	ANG	angiogenin, ribonuclease, RNase A family, 5
204712_at	WIF1	WNT inhibitory factor 1
1552960_at	LRRC15	leucine rich repeat containing 15
225155_at	SNHG5	small nucleolar RNA host gene (non-protein coding) 5
204351_at	S100P	S100 calcium binding protein P
1569372_at	TUBB2B	Tubulin, beta 2B
205097_at	SLC26A2	solute carrier family 26 (sulfate transporter), member 2
204881_s_at	UGCG	UDP-glucose ceramide glucosyltransferase
203434_s_at	MME	membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase)
1568574_x_at	SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I)
206908_s_at	CLDN11	claudin 11 (oligodendrocyte transmembrane protein)
1556153_s_at	NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
210643_at	TNFSF11	tumor necrosis factor (ligand) superfamily, member 11, RANKL
203305_at	F13A1	coagulation factor XIII, A1 polypeptide
213791_at	PENK	proenkephalin
242324_x_at	CCBE1	collagen and calcium binding EGF domains 1
213338_at	TMEM15	transmembrane protein 158
213139_at	SNAI2	snail homolog 2 (Drosophila)
217979_at	TSPAN13	Tetraspanin 13
215420_at	IHH	Indian hedgehog homolog (Drosophila)
229645_at	C18orf51	chromosome 18 open reading frame 51
218717_s_at	LEPREL1	leprecan-like 1
238332_at	ANKRD2	ankyrin repeat domain 29
205828_at	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)
209395_at	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)
204337_at	RGS4	regulator of G-protein signalling 4
201939_at	PLK2	polo-like kinase 2 (Drosophila)
228844_at	SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5
218468_s_at	GREM1	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)

201467_s_at	NQO1	NAD(P)H dehydrogenase, quinone 1
224482_s_at	RAB11FIP	RAB11 family interacting protein 4 (class II)
206239_s_at	SPINK1	serine peptidase inhibitor, Kazal type 1
213492_at	COL2A1	collagen, type II, alpha 1
1552737_s_at	WWP2	WW domain containing E3 ubiquitin protein ligase 2
204162_at	KNTC2	kinetochore associated 2
213622_at	COL9A2	collagen, type IX, alpha 2
202497_x_at	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3
206309_at	LECT1	leukocyte cell derived chemotaxin 1
1556427_s_at	LOC2210	similar to hypothetical protein
201762_s_at	PSME2	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)
201795_at	LBR	lamin B receptor
209946_at	VEGFC	vascular endothelial growth factor C
210432_s_at	SCN3A	sodium channel, voltage-gated, type III, alpha
206439_at	DSPG3	dermatan sulfate proteoglycan 3
203498_at	DSCR1L1	Down syndrome critical region gene 1-like 1
202912_at	ADM	adrenomedullin
221729_at	COL5A2	collagen, type V, alpha 2
1555345_at	SLC38A4	solute carrier family 38, member 4
210095_s_at	IGFBP3	insulin-like growth factor binding protein 3
201601_x_at	IFITM1	interferon induced transmembrane protein 1 (9-27)
205483_s_at	ISG15	ISG15 ubiquitin-like modifier
1554685_a_at	KIAA119	KIAA1199
221019_s_at	COLEC12	collectin sub-family member 12
240448_at	KIAA080	KIAA0802
200790_at	ODC1	ornithine decarboxylase 1
206932_at	CH25H	cholesterol 25-hydroxylase
205352_at	SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1
228640_at	---	CDNA clone IMAGE:4800096
205051_s_at	KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
204731_at	TGFBR3	transforming growth factor, beta receptor III (betaglycan, 300kDa)
221823_at	C5orf30	chromosome 5 open reading frame 30
1554736_at	ARHGAP	Rho GTPase activating protein 29
217997_at	PHLDA1	pleckstrin homology-like domain, family A, member 1
226907_at	PPP1R14	protein phosphatase 1, regulatory (inhibitor) subunit 14C
223235_s_at	SMOC2	SPARC related modular calcium binding 2
202403_s_at	COL1A2	collagen, type I, alpha 2
204469_at	PTPRZ1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1
223614_at	C8orf57	chromosome 8 open reading frame 57
212850_s_at	LRP4	low density lipoprotein receptor-related protein 4
202965_s_at	CAPN6	calpain 6
223316_at	CCDC3	coiled-coil domain containing 3
200974_at	ACTA2	actin, alpha 2, smooth muscle, aorta
213293_s_at	TRIM22	tripartite motif-containing 22
222020_s_at	HNT	neurotrimin
210609_s_at	TP53I3	tumor protein p53 inducible protein 3
201739_at	SGK	serum/glucocorticoid regulated kinase

217995_at	SQRDL	sulfide quinone reductase-like (yeast)
204682_at	LTBP2	latent transforming growth factor beta binding protein 2
201195_s_at	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
206764_x_at	MPPE1	metallophosphoesterase 1
213060_s_at	CHI3L2	chitinase 3-like 2
205334_at	S100A1	S100 calcium binding protein A1
209955_s_at	FAP	fibroblast activation protein, alpha
204035_at	SCG2	secretogranin II (chromogranin C)
217875_s_at	TMEPAI	transmembrane, prostate androgen induced RNA
203879_at	PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide
202709_at	FMOD	fibromodulin
1554737_at	FBN2	fibrillin 2 (congenital contractural arachnodactyly)
205941_s_at	COL10A	collagen, type X, alpha 1(Schmid metaphyseal chondrodysplasia)
202727_s_at	IFNGR1	interferon gamma receptor 1
226930_at	FNDC1	fibronectin type III domain containing 1
207001_x_at	TSC22D3	TSC22 domain family, member 3
206960_at	GPR23	G protein-coupled receptor 23
203666_at	CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
204320_at	COL11A	collagen, type XI, alpha 1
203058_s_at	PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2
205870_at	BDKRB2	bradykinin receptor B2
201464_x_at	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)
226989_at	RGMB	RGM domain family, member B
229740_at	LOC6430	PP12104
203304_at	BAMBI	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)
218899_s_at	BAALC	brain and acute leukemia, cytoplasmic
224348_s_at	H19	H19, imprinted maternally expressed untranslated mRNA
209560_s_at	DLK1	delta-like 1 homolog (Drosophila)
222162_s_at	ADAMTS	ADAM metallopeptidase with thrombospondin type 1 motif, 1
206115_at	EGR3	early growth response 3
1562094_at	MGC269	Hypothetical protein MGC26963
216952_s_at	LMNB2	lamin B2
210948_s_at	LEF1	lymphoid enhancer-binding factor 1
1563466_at	MYLK	Myosin, light polypeptide kinase
212689_s_at	JMJD1A	jumonji domain containing 1A
205347_s_at	TMSL8	thymosin-like 8
204967_at	SHROOM	shroom family member 2
218009_s_at	PRC1	protein regulator of cytokinesis 1
212067_s_at	C1R	complement component 1, r subcomponent
1560259_at	RORA	RAR-related orphan receptor A
206432_at	HAS2	hyaluronan synthase 2
1561065_at	ANKRD6	Ankyrin repeat domain 6
1555800_at	ZNF533	zinc finger protein 533
219747_at	C4orf31	chromosome 4 open reading frame 31
1558636_s_at	ADAMTS	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)
227497_at	---	CDNA FLJ11723 fis, clone HEMBA1005314
1555527_at	COL9A1	collagen, type IX, alpha 1

202768_at	FOSB	FBJ murine osteosarcoma viral oncogene homolog B
204221_x_at	GLIPR1	GLI pathogenesis-related 1 (glioma)
204774_at	EVI2A	ecotropic viral integration site 2A
206157_at	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta
202643_s_at	TNFAIP3	tumor necrosis factor, alpha-induced protein 3
234994_at	KIAA191	KIAA1913
227475_at	FOXQ1	forkhead box Q1
219334_s_at	OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A
218986_s_at	FLJ20035	hypothetical protein FLJ20035
228382_at	FAM105	family with sequence similarity 105, member B
205523_at	HAPLN1	hyaluronan and proteoglycan link protein 1
224967_at	UGCG	UDP-glucose ceramide glucosyltransferase
213817_at	---	CDNA FLJ13601 fis, clone PLACE1010069
212900_at	SEC24A	SEC24 related gene family, member A (S. cerevisiae)
1552619_a_at	ANLN	anillin, actin binding protein
224609_at	SLC44A2	solute carrier family 44, member 2
203755_at	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)
1555724_s_at	TAGLN	transgelin
202450_s_at	CTSK	cathepsin K (pycnodysostosis)
213861_s_at	FAM119	family with sequence similarity 119, member B
213248_at	LOC2213	hypothetical protein LOC221362
203570_at	LOXL1	lysyl oxidase-like 1
230407_at	---	Transcribed locus, strongly similar to strawberry notch homolog 1; MOP-3
209567_at	RRS1	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)
210512_s_at	VEGF	vascular endothelial growth factor
205289_at	BMP2	bone morphogenetic protein 2
203065_s_at	CAV1	caveolin 1, caveolae protein, 22kDa
203758_at	CTSO	cathepsin O
205476_at	CCL20	chemokine (C-C motif) ligand 20
207826_s_at	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
205479_s_at	PLAU	plasminogen activator, urokinase
201136_at	PLP2	proteolipid protein 2 (colonic epithelium-enriched)
203764_at	DLG7	discs, large homolog 7 (Drosophila)
209160_at	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)
207977_s_at	DPT	dermatopontin
205125_at	PLCD1	phospholipase C, delta 1
207980_s_at	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
204475_at	MMP1	matrix metallopeptidase 1 (interstitial collagenase)
1556209_at	CLEC2B	C-type lectin domain family 2, member B
205830_at	CLGN	calmegin
219295_s_at	PCOLCE2	procollagen C-endopeptidase enhancer 2
205907_s_at	OMD	osteomodulin
206869_at	CHAD	chondroadherin
223836_at	KSP37	Ksp37 protein
204948_s_at	FST	follistatin
240955_at	PANX3	pannexin 3

Supplementary Table 2. List of top hits of upregulated genes at each time point with their fold change in expression compared to undifferentiated hfMSCs

TOP upregulated genes of pellets isolated at 1 week of differentiation compared to undifferentiated hfMSCs

Symbol	Gene title	Fold change
COL2A1	collagen, type II, alpha 1	48,69295
COMP	cartilage oligomeric matrix protein	29,52449
SPP1	Secreted phosphoprotein 1 (osteopontin)	29,24743
COL10A1	collagen, type X, alpha 1	26,33897
S100P	S100 calcium binding protein P	23,64239
ANKRD38	ankyrin repeat domain 38	21,03949
H19	H19, imprinted maternally expressed untranslated mRNA	20,74613
COL9A3	collagen, type IX, alpha 3	17,44117
OMD	osteomodulin	14,81684
CCL20	chemokine (C-C motif) ligand 20	14,58234
PANX3	pannexin 3	14,0997
SP7	Sp7 transcription factor	10,99764
WIF1	WNT inhibitory factor 1	7,854743
FNDC1	fibronectin type III domain containing 1	7,754044
MMP3	matrix metallopeptidase 3	7,723496

TOP upregulated genes of pellets isolated at 2 weeks of differentiation compared to undifferentiated hfMSCs

Symbol	Gene title	Fold change
COL2A1	collagen, type II, alpha 1	127,526
COMP	cartilage oligomeric matrix protein	63,39943
COL9A3	collagen, type IX, alpha 3	56,65738
COL10A1	collagen, type X, alpha 1	43,11545
S100P	S100 calcium binding protein P	39,35336
SPP1	Secreted phosphoprotein 1 (osteopontin)	38,08394
H19	H19, imprinted maternally expressed untranslated mRNA	37,46208
PANX3	pannexin 3	35,24063
OMD	osteomodulin	26,66117
ANGPTL7	angiopoietin-like 7	20,79869
AGC1	aggrecan 1 (chondroitin sulfate proteoglycan 1)	17,50114
FMOD	fibromodulin	16,41947
ANKRD38	ankyrin repeat domain 38	14,97306
FNDC1	fibronectin type III domain containing 1	14,939
SP7	Sp7 transcription factor	12,00436

TOP upregulated genes of pellets isolated at 3 weeks of differentiation compared to undifferentiated hfMSCs

Symbol	Gene title	Fold change
COL2A1	collagen, type II, alpha 1	124,5769
PANX3	pannexin 3	56,73114
COMP	cartilage oligomeric matrix protein	55,02747
COL9A3	collagen, type IX, alpha 3	52,95049
S100P	S100 calcium binding protein P	47,56261
COL10A1	collagen, type X, alpha 1	42,84764
H19	H19, imprinted maternally expressed untranslated mRNA	37,78425
SPP1	Secreted phosphoprotein 1 (osteopontin)	29,09326
OMD	osteomodulin	28,90007
ANGPTL7	angiopoietin-like 7	24,41976
AGC1	aggrecan 1 (chondroitin sulfate proteoglycan 1)	21,56025
FMOD	fibromodulin	19,79998
SMOC2	SPARC related modular calcium binding 2	18,17641
SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	16,55767
SP7	Sp7 transcription factor	14,70519

TOP upregulated genes of pellets isolated at 4 weeks of differentiation compared to undifferentiated hfMSCs

Symbol	Gene title	Fold change
COL2A1	collagen, type II, alpha 1	86,82906
COL10A1	collagen. type X, alpha 1	43,4907
COL9A3	collagen, type IX, alpha 3	31,28265
SPP1	Secreted phosphoprotein 1 (osteopontin)	30,3413
S100P	S100 calcium binding protein P	25,19413
PANX3	pannexin 3	17,31646
COMP	cartilage oligomeric matrix protein	16,64807
ANGPTL7	angiopoietin-like 7	14,37924
H19	H19, imprinted maternally expressed untranslated mRNA	13,72816
FMOD	fibromodulin	10,87799
OMD	osteomodulin	10,24159
AGC1	aggrecan 1 (chondroitin sulfate proteoglycan 1)	9,153789
CAPN6	calpain 6	8,080906
SMOC2	SPARC related modular calcium binding 2	7,830802
SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	7,085708

TOP upregulated genes of pellets isolated at 5 weeks of differentiation compared to undifferentiated hfMSCs

Symbol	Gene title	Fold change
COL2A1	collagen, type II, alpha 1	118,4129
COL10A1	collagen, type X, alpha 1	43,15333
COL9A3	collagen, type IX, alpha 3	64,24298
SPP1	Secreted phosphoprotein 1 (osteopontin)	36,69967
S100P	S100 calcium binding protein P	45,26042
PANX3	pannexin 3	36,55029
COMP	cartilage oligomeric matrix protein	49,01692
ANGPTL7	angiopoietin-like 7	24,0925
H19	H19, imprinted maternally expressed untranslated mRNA	27,29474
FMOD	fibromodulin	22,08534
OMD	osteomodulin	22,77907
AGC1	aggrecan 1 (chondroitin sulfate proteoglycan 1)	19,0998
CAPN6	calpain 6	9,836288
SMOC2	SPARC related modular calcium binding 2	16,58001
SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	13,48765

Chapter 7

General discussion

General discussion

The aim of this thesis was to investigate the long-term results of two novel treatment modalities in children with ISS (part A), to study the effect of the administration of aromatase inhibitors in the rat (part B), and to assess whether mesenchymal stem cells can differentiate into chondrocytes with a phenotype resembling the epiphyseal growth plate. In this chapter, the results of our findings are discussed in the perspective of current knowledge. Considerations with regard to clinical practice will be presented, the role of model systems in growth studies will be debated, and recommendations for future research will be given.

A. What are the long-term results of two novel treatment modalities in children with ISS?

Despite almost three decades of clinical trials with GH treatment in ISS, many questions have remained with respect to the optimal treatment regimen. The two clinical trials reported in this thesis have contributed to the discussion about optimizing GH treatment strategies in children with ISS. The first trial was designed to study the effect of high dose GH treatment restricted to the prepubertal period in young children with ISS. In the second trial, GH was combined with GnRHa treatment in adolescents with ISS and a relatively early-timed pubertal onset. In both of our studies, subjects were randomly allocated to receive treatment or to participate in a non-treated control group. Non-treated controls did not only serve for comparison with GH-treated subjects, but also illustrated the natural history of ISS. We have reported both interim data (1-3) and adult height data (this thesis) of both trials.

As mentioned in ***Chapter 2***, at the time this study was designed, there were three important unresolved issues with respect to GH treatment of children with ISS. First, it was unknown what the effect would be of a high GH dosage on growth velocity, bone maturation, puberty and adult height. Second, the relative contribution of GH treatment before and during puberty was unclear. Third, there was a need to gain more insight into the factors affecting the growth response, as only a modest part of the inter-individual variation can be explained (4)

Besides these questions on GH monotherapy, there was an additional question that was approached in ***Chapter 3***: what would be the effect of combined treatment with GH and GnRHa on adult height?

Relationship between GH dose and growth response.

Most clinical trials with GH in ISS have used a GH dosage of 40-50 µg/kg/day, or the equivalent dosage corrected for body surface (1.2-1.4 mg/m^2/day). As reviewed in chapter 1, adult height gain on such a dosage is close to 7 cm (5-7). While there is little doubt that lower dosages are less efficacious, leading to an adult height gain of 3-4 cm (8;9), the effect of higher dosages is unclear.

Results of two trials in which high dose GH treatment was applied have been reported. In the first trial (10) there were three study arms: 1) a conventional dose of 40 µg/kg/day; 2) a titration arm in which GH dose was titrated to achieve an IGF-I SDS at the mean level of the normal range; and 3) a titration arm in which GH dose was titrated to achieve an IGF-I SDS level 2 SD above the mean of the normal range. In the last group the growth response was best, but required on average a 2.5 times higher dosage than used in the second group. In the other study (11) two doses were compared: 33 and 67 µg/kg/day. These doses led to a change in height SDS of 1.0 and 1.4, respectively, in comparison to 0.2 SDS in untreated controls. Thus, in both studies a high GH dose had a positive effect on adult height gain.

In contrast, we showed in ***Chapter 2*** that high dose GH treatment restricted to the prepubertal period, and administered to young children, increased height gain during treatment, but also accelerated bone maturation and ultimately did not lead to an increased adult height. Cohen and co-workers did not observe bone age advancement after treatment with a high GH dose (median 98 µg/kg/d, range 20-346 µg/kg/d) titrated on circulating IGF-I levels of 2 SDS (10). However, the dose range in that study was large, and the children who needed high GH doses to reach the aimed IGF-I level may have been more resistant to GH. Also in the study of Albertsson-Wikland no advance of skeletal maturation was observed (11).

We speculate that the difference between our results and those of Cohen *et al.* and Albertsson-Wikland *et al.* can be explained by a difference in age at start of GH therapy. The mean age at start of GH treatment in our study was 8.7 years, considerably younger than the mean age of approximately 11 years in the other studies. The observation in retrospective studies of an inverse association of age at start of GH treatment and height gain (4) is not necessarily in contradiction with our hypothesis, as the average age in those retrospective studies is also around 11 years.

The discrepancy between the negative effect of high dose GH treatment in our trial and positive effects on height gain in other studies may rely on an increased sensitivity of the epiphyseal growth plates of younger children in comparison with those of older children to high doses of GH and/or IGF-I. In favor of the hypothesis of age-dependent sensitivity to GH was our finding that a younger age was associated with a lower height gain. Cruickshank and co-workers have

described that GH administration to rats was found to have no effect on GHR expression and to decrease IGF1R expression in the epiphyseal growth plates of prepubertal rats, whereas expression of both receptors was found to be upregulated at the age of sexual maturation, a phase associated with fast growth (12). They hypothesized that the dissociation between GHR expression and exogenous GH at a young age may reflect an inherent mechanism to prevent excessive proliferation and prevent overgrowth of the bone (12).
The combined results of our and other studies in young children imply that at such a young age GH dose may be positively associated with adult height gain in the range of 25-50 µg/kg/d, but that higher doses may decrease adult height gain due to accelerated epiphyseal maturation, while at the same time the effect on growth reaches a plateau after a certain dose.

The relative contribution of GH treatment before and during puberty

An alternative explanation for the attenuated adult height outcome after high dose GH in our study may be the discontinuation of treatment at the onset of puberty, which theoretically may have abolished therapy-induced height gain. Such a catch-down phenomenon has been described in children with persistent short stature born SGA when GH treatment was discontinued 1-3 years before the onset of puberty (13). Those children showed a marked reduction in height velocity and height SDS in the post-treatment follow-up period and a decrease in adult height prediction, suggesting that GH treatment should at least be continued until pubertal onset.
At the time our study was initiated, there were several arguments in favor of the hypothesis that growth enhancing strategies should be designed to improve prepubertal growth. One of these arguments was that height at onset of puberty is positively correlated with adult height in GHD or short SGA children (14-16). Another argument was that prolonged GH treatment during puberty might not be needed, since height gain during the pubertal growth spurt is primarily determined by sex steroid signaling, possibly in combination with an increased secretion of GH and IGF-I. Since children with ISS by definition have a normal GH secretion, one could argue that GH levels should be sufficient at the time of increased sex steroids. A third argument was that Rekers-Mombarg *et al.* demonstrated in previous studies that pubertal height gain was not different between GH-treated and non-treated GHD children (17).
In our study described in ***Chapter 2***, GH treatment was discontinued at the onset of pubertal development. Already at that time, predicted adult height of the GH-treated group was found to be similar to that of the non-treated controls, arguing against the catch-down growth hypothesis as an explanation for the absence of enhanced adult height.

Another topic, that is related to the question whether GH should be continued or not during puberty, is whether GH influences the timing of pubertal onset and the tempo of pubertal development. On regular dosages most studies have not been able to document that GH advances pubertal onset and tempo, but in our study (chapter 2), using a higher dosage, there are at least reasons to suspect such an effect. We believe that this may be related to age as well. In the final analysis on 26 children using a novel technique for expressing pubertal stage in SDS (correcting for age and gender) (18), we found a trend (p=0.2) towards a younger age at Tanner stage 2 in the GH-treated groups compared to controls. While this technique enables appropriate correction for the (statistically significant) age difference between the groups at start of the trial, the inability to reach statistical significance may well be related to the limited number of subjects that could be studied at follow up.
In the larger group of 35 subjects studied 5 years after inclusion, the age difference at start did not reach significance, some patients had not yet entered puberty at the moment of analysis, and another method (cumulative proportions of patients having entered puberty, and calculation of relative risk) was used. In that analysis the relative risk for early puberty, adjusted for age and sex, was 4.7 (1.4-15.8, p=0.012) (2).
There are two other observations that can serve as indirect evidence for an effect on pubertal onset. First, the observation that none of the 12 males in the GH treatment group entered puberty late, compared to 2 out of 8 controls. Second, at follow-up the GH-treated subjects had a significantly shorter leg length than controls and a higher SH/H SDS, suggesting earlier exposure to sex steroids. Unfortunately, the study design during follow-up did not allow for the collection of sufficient data on the progression of puberty.
The higher SH/H ratio may also explain the increase in BMI SDS observed in the GH-treated children, as shown previously in GH deficient children (19). Up to early adolescence, height SDS remained stable at -2.6, but adult height was 0.7 SD higher than height SDS at start, presumably due to a rather delayed and possibly protracted pubertal development. A similar pattern was seen for SH and LL SDS.
In conclusion, high-dose GH treatment limited to the prepubertal period in young children with ISS has no effect on adult height, probably caused by concomitant advance of bone maturation, and may advance pubertal onset.

Factors affecting the growth response

The study described in ***Chapter 2*** has not contributed to the identification of factors that can predict growth response to GH therapy. In our study only age was a (positive) predictor of treatment effect. In the first part of the study, it was shown that plasma IGF-I during treatment

was positively associated with growth response in the first year (2), but in the final analysis we have demonstrated that short-term growth is not necessarily predictive for long-term success.
In the last decades several other studies have investigated the possible predictors of growth response, either on first year height velocity or adult height gain. These were recently reviewed (20). Data from the large KIGS cohort have shown that positive predictors for adult height after GH treatment include midparental height and the first year growth response (expressed as studentized residual), while age is a negative predictor (4).

The effect of a combined treatment with GH and GnRHa on adult height

In ***Chapter 3*** we have shown that 3 years of treatment with GH and a GnRH agonist in short children with a relatively early puberty increases adult height, with a mean gain of 5 cm. This result confirms the results of previous less well controlled clinical trials. However, this positive effect apparently has a price. Postponing puberty in adolescents has some negative impact on psychosocial status (21), and in boys there are indications that it decreases bone mineral density of the lumbar spine.

Conclusions

We conclude that a regimen of high dose GH started at an early age in children with ISS, and restricted to the prepubertal phase, is not effective, and therefore not to be advised. The combination of GH and GnRHa in young teenagers with short stature and relatively early puberty can be considered in selected cases, but its effect is modest and bone mineral density may be at risk in males.

B. The effect of the administration of aromatase inhibitors in the rat

The main reason to perform the studies described in ***Chapters 4 and 5*** was that aromatase inhibitors (AIs) would be a logical class of drugs for growth enhancement in boys who are short at the onset of puberty. Clinical trials had already been planned, whereas at the same time there was uncertainty about the short- and long-term adverse effects of these drugs, as locally produced estrogens are important in many tissues. With our studies we hoped to gain more insight into the effect of aromatase inhibition on growth, as well as on potential adverse results of such treatment in several tissues.

An unexpected outcome of our studies was that the effect on growth was strongly dependent on gender. Female rats became larger after treatment, while male rats became shorter. The growth attenuation observed in male rats was not consistent with the growth phenotype of male aromatase-deficient or estrogen-resistant patients. However, the negative effects of exemestane treatment on growth and BMD in male rats are in line with similar findings in male 6 weeks old rats treated with the nonsteroidal aromatase inhibitor vorozole (22) and in 4 weeks old male rats treated with the selective estrogen receptor modulator tamoxifen (22;23). Growth attenuation was also observed in male aromatase knockout (ArKO) mice and estrogen receptor alpha (ERα) and ER$\alpha\beta$ knockout mice (24-26). We must therefore conclude that the male rat is not a suitable model to study the effect of AIs on growth.

With respect to side effects, in both genders a negative effect on bone quality was observed, which was not unexpected based on the pivotal role of estrogens in bone metabolism (27;28). In female rats there was a negative effect on the ovaries, leading to abnormalities similar to those observed in girls and women with the polycystic ovary syndrome (PCO). Such a phenotype has also been reported in female aromatase-deficient patients. These adverse effects argue against the use of aromatase inhibitors in girls with ISS, at least until additional research has proven beyond doubt that the observed ovarian changes are reversible and do not negatively affect fertility later in life. The development of the male genital tract was not impaired by aromatase inhibitor treatment, and no adverse effects were seen on brain morphology in both genders.

In the meantime, more data have become available about the long-term effect of AIs in adolescent boys with short stature. In children with GH deficiency, the addition of AIs (anastrozole) to GH treatment had a modest positive effect on predicted adult height (29). In children with ISS, AIs alone (letrozole) for 2 years increased adult height by 5.9 cm (30). Initial worries that letrozole would lead to abnormalities of the thoracic vertebrae (31), have recently been confirmed (32). MRI-analysis of the vertebral spine demonstrated that letrozole-treated boys with ISS developed vertebral deformities, whereas no such findings were reported in the non-treated control group (32).

Although the clinical trial with letrozole in boys with ISS showed a modest positive result on predicted adult height (30), the Finnish investigators emphasized that aromatase inhibition should be considered an experimental form of treatment, and that more studies had to be performed before AIs could be applied in clinical practice on a routine basis (33). The recent identification of vertebral body deformities potentially induced by estrogen depletion further justifies a reluctant attitude towards this type of treatment.

C. Development of an *in vitro* model for growth plate physiology: do mesenchymal stem cells differentiating into chondrocytes show a phenotype resembling the epiphyseal growth plate?

In ***Chapter 6*** we have shown that human fetal MSCs can indeed differentiate into chondrocytes, and that the gene expression profile resembles more the profile of epiphyseal chondrocytes than that of articular chondrocytes. We have therefore concluded that this model is suitable to further study the role of the numerous genes that are associated with the transition from immature chondrocytes in the resting zone, towards actively replicating chondrocytes in the proliferative zone, and finally to hypertrophic chondrocytes.
We believe that this model can serve for various purposes. First, the model can be used to study the effect of various growth factors and hormones on chondrocyte differentiation and may assist in increasing the understanding of the interplay between those various factors. This may result in the identification of new drug targets and the development of new drugs. Second, the model can be used to assess whether genes that seem to be associated with growth, for example the genes encountered in genome-wide association studies (GWAS) on height (34-36) are indeed expressed during chondrocyte differentiation. Similarly, SNP-array studies in children with short or tall stature have shown deletions and duplications in various chromosomal regions, disabling various genes associated with growth regulation. The search for candidate genes, as detected in these studies, can be facilitated by checking the role of the essential genes that orchestrate chondrocyte differentiation.

D. Conclusion and suggestions for future studies

The results of the two clinical trials included in this thesis have shown that a high GH dosage in young children with ISS restricted to the prepubertal phase is not efficacious, and we believe that even if GH had been continued during puberty, the result would not have been better. The treatment course of 3 years of GH plus GnRHa is more effective (5 cm height gain), but may have undesirable side effects. This indicates that for the treatment of ISS, in countries where GH is registered for this indication, a dosage of 40-50 µg/kg/day or 1.2-1.4 mg/m2/day may still be the best option. However, the pros and cons of GH treatment in ISS are still heavily debated among pediatric endocrinologists and legislators, since the modest gain in adult height is counterbalanced by a very long and intensive treatment (daily injections for 4-8 years), high costs, and little effect on psychosocial status. Future studies in this area could focus on better

tools to assess psychosocial consequences of short stature, and on developing psychological counselling programs for short children who seem to suffer from their shortness.

The results of the animal studies on AIs show that for the analysis of drugs that intervene with sex steroid signaling, rodents are not an optimal model. The role of sex steroids in rodent growth appears very different from that in the human. Still, such studies can serve to collect information about possible side effects of steroids and their antagonists. As it is uncertain which other animal models are better suited, well-controlled clinical trials on AIs in children and adolescents are needed before their use in clinical practice can be propagated.

The *in vitro* model of MSCs differentiating into epiphyseal chondrocytes can be used in future studies aimed at a better understanding of the physiology of the epiphysis, and to assess the influence of various growth factors and growth-modifying drugs.

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Chapter 8

Summary

Summary

Postnatal growth in humans is programmed by a person's individual genetic blueprint and fine-tuned by hormonal, environmental, psychosocial and nutritional factors. For decades, scientists have been determined to identify regulatory mechanisms associated with growth and to unravel the pathophysiological processes underlying growth disorders. Increased insight into those mechanisms and processes is fundamental for the development of novel treatment strategies for enhancing growth in children with idiopathic short stature. This thesis consists of three parts, in which several aspects of growth modulation and regulation are investigated.

Part A describes the long-term results of two randomized controlled clinical trials in which novel treatment modalities for children with ISS were analyzed. At the time these clinical trials were initiated, there were several unresolved issues with respect to the optimal growth hormone treatment strategy for children with ISS:

(1) What is the effect of a high GH dosage on growth velocity, bone maturation, puberty and adult height?
(2) What is the relative contribution of GH treatment before and during puberty?
(3) Which factors can predict the individual growth response to GH treatment?
(4) Can height gain be optimized by co-treatment with a GnRH agonist?

Chapter 2 describes that high dose GH treatment restricted to the prepubertal period, and administered to young children, increased height gain during treatment, but simultaneously accelerated bone maturation and ultimately did not lead to an increased adult height. Moreover, there were indications that high dose GH treatment may have led to an advanced pubertal onset. Our results are in contrast with data from other groups that have reported a positive effect of high dose GH treatment on adult height in older children. We speculate that the discrepancy between our and other trials may rely on an increased sensitivity of the epiphyseal growth plates of younger children in comparison with those of older children to high doses of GH (and/or IGF-I). The finding of a similar predicted adult height in treated and untreated groups at the time of discontinuation of treatment (at pubertal onset) makes it unlikely that prolonged GH treatment during pubertal development would have led to better adult height results. Age at onset of treatment was found to show a positive correlation with height outcome, which is in contrast with data from other trials that have identified a negative correlation between age at onset and adult height. Again, an explanation for this difference

may reside in the considerably younger age at onset of treatment in our trial. Apart from age at onset, our study did not identify other predictive factors for growth response to GH therapy. We concluded that a regimen of high dose GH started at an early age in children with ISS, and restricted to the prepubertal phase, was not effective and therefore not to be advised.

Chapter 3 demonstrates that the addition of a GnRHa to GH treatment in children with ISS and a relatively early onset of puberty increased adult height, especially in girls. However, postponing pubertal development may have a negative impact on psychosocial development in young children, and there were also indications of a lower bone mineral density of the lumbar spine in treated boys. These aspects require additional investigation. In the meantime, combined GH and GnRHa treatment should not be routinely prescribed for growth stimulation in children with ISS, but it may be considered in strictly selected individual cases, especially in girls with an extremely low predicted adult height, an early onset of puberty, and considerable psychosocial problems associated with being short.

Part B assesses the effect on growth and the potential side effects of estrogen depletion by treatment with an aromatase inhibitor (exemestane) on growth in an experimental animal model. Aromatase inhibition resulted in a sexual dimorphic response in young rats. In female rats, exemestane treatment resulted in augmented length and weight gain, longer femurs and an increased growth plate width, as illustrated in ***Chapter 4***. However, adverse effects on genital development were found, including uterus atrophy and polycystic ovaries. The observed effects on growth and genital development are consistent with the phenotype of female aromatase-deficient patients. As long as it is uncertain whether the genital abnormalities are reversible or whether long term effects on fertility and reproduction are to be expected, aromatase inhibitors should not be considered in the treatment of girls with ISS.

In male rats, a decreased weight gain was found, without effects on axial and appendicular growth and genital development (***Chapter 5***). In contrast, the growth phenotype of male estrogen-deficient or estrogen-resistant patients is characterized by the absence of a pubertal growth spurt and ongoing growth into adulthood resulting in a tall stature. Also, clinical trials with aromatase inhibitors in boys with ISS or short stature due to constitutional delay of growth and puberty have demonstrated a positive effect of estrogen depletion on adult height. The difference between these data and our results may rely on species differences. It suggests that the rat, at least the male rat, is not a suitable animal model for studying the effect of aromatase inhibition on growth.

In both male and female rats, we found evidence of osteopenic changes of the bone due to aromatase inhibitor treatment, which may precede the development of osteoporosis. In aromatase-deficient patients, osteoporosis has also been described. Recently, MRI-analysis of

the spine in boys treated with an aromatase inhibitor revealed the occurrence of vertebral body deformities. These collective data urge for a reluctant attitude towards the application of aromatase inhibitors in growth-enhancing treatment strategies in children with ISS. Additional research is necessary to identify the pathological mechanisms underlying the observed changes in bone quality.

In **part C** the role of mesenchymal stem cells differentiating towards the chondrogenic lineage as a potential new *in vitro* model for growth plate physiology is evaluated. Development of alternative model systems is required, as the available animal models for growth studies are all hampered in the sense that they do not fully represent the human growth pattern, and species differences sometimes hamper the interpretation of results from animal experiments. In ***Chapter 6*** it is shown that human fetal MSCs can indeed differentiate into chondrocytes, and that the gene expression profile bears more resemblance to the profile of epiphyseal than to that of articular chondrocytes. This model is suitable to further study the role of the numerous genes that are associated with growth plate maturation and fusion. This may aid in the identification of new angles for the development of novel treatment strategies as alternatives to regular GH therapy for enhancing growth in children with ISS.

In ***Chapter 7*** the major findings of this thesis are summarized and critically reviewed in the perspective of current literature. It is discussed that high dose GH or combined GH and GnRHa treatment are no routine alternatives to standard GH treatment schedules. Results from rat studies also do not support the use of aromatase inhibitors in growth enhancement strategies. Future studies may need to ameliorate the tools to assess psychosocial consequences of short stature and to develop better methods for counselling short children who suffer from being short.
Finally, the limited role of animal models for the analysis of human growth regulation and modulation has urged the development of an alternative human model system. Using a model of human fetal mesenchymal stem cells differentiating towards the chondrogenic lineage may render new insights into human growth regulation and may aid the development of new methods for growth modulation.

Chapter 9

Samenvatting

Samenvatting (summary in Dutch)

De blauwdruk voor de lengtegroei van de mens wordt gedefinieerd de genetische achtergrond van het individu, maar wordt eveneens beïnvloed door hormonale, psychosociale, omgevings- en voedingsfactoren. Wetenschappers proberen al geruime tijd de regulatiemechanismen die betrokken zijn bij lengtegroei te identificeren. Er gaat ook veel aandacht uit naar het ontrafelen van de pathofysiologische processen die leiden tot groeistoornissen. Een beter begrip van deze mechanismen en processen is essentieel en kan een eerste stap zijn naar de ontwikkeling van nieuwe vormen van behandeling voor groeibevordering bij kinderen met onbegrepen kleine gestalte, ook wel 'idiopathic short stature' (ISS) genoemd. In de drie delen van dit proefschrift worden verschillende aspecten van groeimodulatie en groeiregulatie bestudeerd.

In **Deel A** worden de resultaten gepresenteerd van twee gerandomiseerde klinische studies waarin het effect van nieuwe, potentieel groeibevorderende behandelvormen voor kinderen met ISS is bestudeerd. In de tijd dat deze studies werden opgestart, waren er enkele onopgeloste kwesties met betrekking tot de optimale behandelstrategie met groeihormoon (GH) voor deze categorie patiënten:

(1) Wat is het effect van een hoge dosis groeihormoon op de groeisnelheid, botrijping en volwassen eindlengte?
(2) Wat is de relatieve bijdrage van GH behandeling voor en tijdens de puberteit?
(3) Welke factoren kunnen de individuele groeirespons op behandeling voorspellen?
(4) Kan de lengtewinst worden verbeterd door toevoeging van een puberteitsremmer, een 'gonadotropin releasing hormone agonist' (GnRHa), aan de standaard GH behandeling?

In ***Hoofdstuk 2*** wordt beschreven dat behandeling van jonge kinderen met ISS met een hoge dosis GH in de periode voorafgaand aan de puberteit resulteerde in een toename van de voorspelde lengtewinst gedurende de behandelfase, maar ook in een versnelde botrijping, waardoor de uiteindelijke volwassen eindlengte niet groter was dan bij onbehandelde patiënten. Er waren tevens aanwijzingen dat een hoge dosis GH een vervroegde start van de puberteit veroorzaakte. Onze resultaten komen niet overeen met die van andere studies waarin oudere kinderen werden behandeld met een hoge dosis GH en een positief effect op de eindlengte werd gevonden. Wij speculeren dat deze verschillende bevindingen mogelijk berusten op een verhoogde gevoeligheid voor GH (en/of 'insulin-like growth factor I' (IGF-I)) van de groeischijven van jonge kinderen vergeleken met die van oudere kinderen. Er werd geen

verschil gevonden tussen de GH-behandelde en de onbehandelde controlegroep in de voorspelde volwassen eindlengte ten tijde van het afronden van de behandelfase (bij aanvang van de puberteit). Om deze reden denken wij dat het voortzetten van GH behandeling gedurende de puberteit niet zal leiden tot een grotere lengtewinst. In onze studie vonden we dat de lengtewinst bij jongere kinderen minder groot was dan bij oudere kinderen. Andere studies vermelden een tegenovergesteld effect, namelijk een afname van de lengtewinst met toename van de leeftijd bij aanvang van GH behandeling. De verklaring voor deze discrepantie tussen onze en andere studies ligt misschien wederom in het feit dat de gemiddelde leeftijd in onze studie aanmerkelijk lager was dan in de andere bekende studies. In onze studie konden er geen andere voorspellende factoren voor lengtewinst worden geïdentificeerd. We concluderen dat behandeling van jonge kinderen met ISS met een hoge dosis GH in de periode voorafgaand aan de puberteit niet effectief is en adviseren daarom om deze behandeling niet te implementeren in de kliniek.

Hoofdstuk 3 vermeldt dat behandeling met een combinatie van GH en een GnRHa bij kinderen met ISS en een relatief vroege puberteit resulteerde in een toegenomen volwassen eindlengte, met name bij meisjes. Echter, het uitstellen van de puberteitsontwikkeling zou een negatieve invloed kunnen hebben op de psychosociale ontwikkeling van kinderen in deze leeftijdscategorie. Daarnaast werden er aanwijzingen gevonden voor een afgenomen botdichtheid van de lumbale wervelkolom bij behandelde jongens. Aanvullende studies zijn nodig om dergelijke ongewenste effecten nader te bestuderen. Gecombineerde behandeling met GH en GnRHa zou niet standaard toegepast moeten worden in de dagelijkse praktijk, maar kan wel overwogen worden in individuele gevallen, met name bij meisjes met een extreem kleine voorspelde eindlengte, een vroege puberteit en zwaarwegende psychosociale problematiek gerelateerd aan het klein zijn.

Deel B bestudeert het effect op groei en de mogelijke bijwerkingen van oestrogeendepletie ten gevolge van behandeling met een aromataseremmer (exemestane) in de rat. Er werd een geslachtsafhankelijk effect van aromataseremming gevonden. In vrouwtjesratten leidde behandeling met exemestane tot een toename van lengte- en gewichtsgroei, langere femora en een toegenomen dikte van de groeischijf, zoals beschreven in ***Hoofdstuk 4***. Er werden echter tevens atrofie van de uterus en polycysteuze ovaria gezien. Zolang onduidelijk is of deze effecten reversibel zijn en of deze leiden tot vruchtbaarheidsproblematiek, is het onverstandig om aromataseremmers toe te passen in de behandeling van meisjes met ISS. De gevonden effecten van exemestane-behandeling in vrouwtjesratten zijn vergelijkbaar met het fenotype van vrouwelijke aromatase-deficiënte patiënten.

In mannetjesratten werd geen toename gezien van de lengtegroei, maar wel een verminderde gewichtsgroei (***Hoofdstuk 5***). Er waren geen nadelige effecten op de genitale ontwikkeling. Dit groeipatroon is duidelijk anders dan dat van mannelijke oestrogeenresistente of -deficiënte patiënten, dat wordt gekenmerkt door het ontbreken van de groeispurt in de puberteit, een ook op de volwassen leeftijd continu doorgaande groei en daaruit volgende grote lengte. Eveneens zijn onze resultaten in tegenspraak met het groeibevorderende effect van aromataseremming bij jongens met onbegrepen kleine gestalte of jongens met constitutionele vertraging van de groei en ontwikkeling. Speciesverschillen liggen mogelijk ten grondslag aan deze contrasterende resultaten. Dit suggereert dat de rat, en in het bijzonder de mannelijke rat, niet een optimaal model is voor het bestuderen van het effect van aromataseremming op de lengtegroei.
Bij zowel mannetjes- als vrouwtjesratten werd als gevolg van de behandeling met exemestane een afname van de botkwaliteit gevonden, passend bij osteopenie, een voorloperstadium van osteoporose. Bij aromatasedeficiënte patiënten is osteoporose eveneens beschreven. Onlangs zijn de resultaten verschenen van een MRI-studie waarin werd gekeken naar de wervelkolom van jongens met een kleine gestalte die waren behandeld met aromataseremming. Deze studie toonde aan dat aromataseremming resulteerde in afwijkingen van de wervellichamen. Meer onderzoek is geïndiceerd om het precieze pathologische mechanisme hierachter te identificeren. Op basis van momenteel beschikbare gegevens adviseren wij dat men terughoudend dient te zijn met aromataseremming voor de behandeling van kinderen met een kleine gestalte.

In **deel C** wordt de rol van een nieuw *in vitro* model voor het bestuderen van de fysiologie van de groeischijf beschouwd. De ontwikkeling van alternatieve modellen voor het bestuderen van groeigerelateerde processen is essentieel, aangezien de huidige beschikbare (dier)modellen geen perfecte weergave van het humane groeipatroon geven en speciesverschillen de interpretatie van proefdierstudies kunnen bemoeilijken. In ***Hoofdstuk 6*** wordt beschreven hoe mesenchymale stamcellen (hMSCs) werden gestimuleerd tot differentiatie in de chondrogene richting. De aldus verkregen kraakbeencellen bleken een genexpressiepatroon te vertonen dat meer gelijkenis vertoonde met dat van groeischijfkraakbeen dan dat van gewrichtskraakbeen. Dit model is geschikt voor het bestuderen van talloze genen die betrokken zijn bij de rijping en sluiting van de groeischijf en dergelijk onderzoek kan leiden tot het vinden van nieuwe invalshoeken voor de ontwikkeling van groeibevorderende behandelingen als alternatief voor GH behandeling bij kinderen met ISS.

Tenslotte zijn in ***Hoofdstuk 7*** de belangrijkste bevindingen van dit proefschrift samengevat en besproken in de context van de bestaande literatuur. Behandeling met een hoge dosis GH of GH gecombineerd met een GnRHa zijn geen alternatieven voor standaard GH behandeling. Dierexperimentele studies in de rat pleiten ook tegen een rol voor aromataseremmers in behandelingen gericht op groeibevordering. Toekomstig onderzoek zou zich kunnen richten op het verbeteren van methoden voor het objectiveren van de psychosociale gevolgen van klein zijn en op een betere psychosociale begeleiding van kinderen die lijden onder hun kleine gestalte.

Tot slot wordt besproken dat de beperkte waarde van diermodellen voor het bestuderen van humane groeiregulatie en -modulatie heeft benadrukt dat alternatieve humane modellen ontwikkeld moeten worden. Studies met het model van humane foetale mesenchymale stamcellen die zich differentiëren tot chondrocyten kunnen nieuwe inzichten opleveren op het gebied van humane groeiregulatie, hetgeen de ontwikkeling van groeibevorderende behandelvormen zou kunnen bevorderen.

Curriculum vitae

Curriculum vitae

The author of this thesis was born on October 13th, 1974 in Uitgeest, the Netherlands. She attended secondary school at the 'Gymnasium Felisenum' in Velsen-Zuid and passed her exam in 1993. From 1993-2001, she studied Medical Biology at the University of Amsterdam. During her study, she performed two research projects at the Department of Pediatrics of the Academic Medical Center Amsterdam (AMC) on human gallbladder mucin (Dept. of Pediatric Gastroenterology and Nutrition; Prof. dr. H.A. Büller and dr. A. Einerhand), and on carnitin biosynthesis (Dept. of Genetic Metabolic Disorders; Prof. dr. R. Wanders and dr. R. Ofman). In September 2001, she received her Medical Biology degree (*cum laude*). From 1998-2001 she studied Medicine at the University of Amsterdam from 1998-2001 and received a Scholarship for Excellent Students on a double degree programme (Medicine and Medical Biology). She obtained her medical degree in September 2003 and subsequently worked for 6 months as a pediatric resident at the Zaandam Medical Center (Mr. M. Westra). In April 2004, she started as a research fellow at the Department of Pediatrics of the Leiden University Medical Center (Prof. dr. J.M. Wit, dr. M. Karperien and dr. W. Oostdijk). Her application for an AIOSKO program sponsored by NWO (the Netherlands Organization for Health Research and Development), a combination of pediatric residency and PhD studentship, was honored. During her research period, she performed working visits to the Instituto Cajal in Madrid, Spain (Prof. dr. L.M. Garcia-Segura and Dr. S. Veiga), the University of Veterinary Medicine (Prof. Böck) and the Pediatric Department of the University Hospital of Vienna, Austria (Prof. dr. G. Haeusler). In July 2009, she started her clinical training in pediatrics at the Leiden University Medical Center (Prof. dr. H.A. Delemarre-van de Waal). As of January 2010, she works at the Juliana Children's Hospital in The Hague, the Netherlands (Dr. F. Brus), to fulfill the non-academic part of her training in pediatrics.

List of publications

List of publications

Van Gool SA, Wit JM, de Schutter T, de Clerck N, Postnov AA, Kremer Hovinga S, van Doorn J, Veiga SJ, Garcia-Segura LM, Karperien M. Impaired body weight and tail length gain after treatment with the aromatase inhibitor exemestane in male rats. Horm Res Paediatr 2010;73(5):376-85.

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List of abbreviations

List of abbreviations

AH	Adult height
AI	Aromatase inhibitor
ALS	Acid-labile subunit
ANOVA	Analysis of variance
AR(KO)	Androgen receptor (knockout mouse)
ArKO	Aromatase knockout mouse
BA	Bone age
BM(A)D	Bone mineral (apparent) density
BMI	Body mass index
BMP	Bone morphogenetic protein
CA	Chronological age
CAH	Congenital adrenal hyperplasia
CPP	Central precocious puberty
DHT	Dihydrotestosterone
DSD	Disorder of sex development
DXA	Dual energy x-ray absorptiometry
E	Exemestane
ECM	Extracellular matrix
ER(α/β/$\alpha\beta$ KO)	Estrogen receptor (alpha/beta/alpha+beta knockout mouse)
FH	Final height
FSS	Familial short stature
GF	Growth factor
(h)GH	(human) Growth hormone
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GHD	Growth hormone deficiency
GnRHa	Gonadotropin releasing hormone agonist
GWAS	Genome-wide association studies
H	Height
H&E	Haematoxylin and Eosin
IGF-I	Insulin-like growth factor I
IGF1R	Insulin-like growth factor receptor
IGFBP3	IGF binding protein 3

ISS	Idiopathic short stature
KEGG	Kyoto encyclopedia of genes and genomes
LL	Leg length
MAS	McCune-Albright syndrome
micro-CT	X-ray microtomography
(hf)MSC(s)	(human fetal) Mesenchymal stem cell(s)
NFSS	Non-familial short stature
NHANES	National health and nutrition examination survey
ORCHX	Orchidectomy
OVX	Ovariectomy
PAH	Predicted adult height
PBI	Pediatric bone index
PCA	Principal component analysis
PCO	Polycystic ovaries syndrome
qPCR	Real-time quantitative polymerase chain reaction
P/H	Proliferative zone/hypertrophic zone ratio
PICP	Procollagen type IC propeptide
PIIINP	Procollagen type III N propeptide
PI3K	Phosphoinositide-3-kinase
PLC	Placebo
PTHrP	Parathyroid hormone related protein
RCT	Randomized controlled clinical trial
SD(S)	Standard deviation (score)
SERM	Selective estrogen receptor modulator
SGA	Short for gestational age
SH	Sitting height
SNP	Single nucleotide polymorphism
TBW	Total body weight
TGF	Transforming growth factor
(c)TH	(conditional) Target height
Wnt	Wingless-type MMTV integration site family