Novel Insights from Clinical Practice

HORMONE RESEARCH IN PÆDIATRICS

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Impact of Estrogen Replacement throughout Childhood on Growth, Pituitary-Gonadal Axis and Bone in a 46,XX Patient with CYP19A1 Deficiency

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Established Facts

- Girls with aromatase deficiency are suffering from a 46,XX DSD, show large ovarian cysts already in infancy, do not go normally through puberty, present with inadequate skeletal maturation and do not reach normal bone mass.
- Thus, treatment with estrogens is essential to reach normal bone mass and to allow normal growth and pubertal development.

Novel Insights

- Estradiol is essential already in early childhood for normal growth, bone maturation and achievement of normal bone mass. The doses of 17β-estradiol needed during that age period range between 50 and 100 μg.
- However, higher doses of 17β-estradiol are needed for a successful negative feedback to act on the pituitary-gonadal axis than for the normalization of longitudinal growth in late prepuberty and puberty.

Key Words
Aromatase · Bone densitometry in children · Estradiol treatment · Estrogen · Growth/auxology · Gonadotrophic axis · Multicystic ovaries

Abstract
Background: The adequate replacement dose of estrogens during infancy and childhood is still not known in girls. Aromatase deficiency offers an excellent model to study how much estrogens are needed during infancy, childhood and adulthood. ***Objectives:*** We studied the impact of oral 17β-estradiol treatment, on longitudinal growth, bone age maturation, pituitary gonadotropin feedback, multicystic ovaries and bone mass in the long-term follow-up of a girl compound heterozygote for two point mutations of the *CYP19A1* gene. ***Results:*** Low doses of 17β-estradiol were needed to achieve normal height velocity and adequate bone age maturation from early childhood on. Serum estradiol levels needed for breast development and for the appearance of an endometrial reflex were not sufficient to achieve physiological gonadotropin levels. Without 17β-estradiol treat-

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ment the ovaries of the patient showed a multicystic appearance, which reversed on 17β-estradiol replacement. Bone mass was within normal ranges during the whole follow-up period. ***Conclusion:*** In summary, we have shown that estradiol is needed not only in puberty but also in childhood for normal growth, bone maturation and achievement of normal bone mass. Particularly, this observation underscores the importance of early low-dose estrogen replacement also in other estrogen-deficient conditions as for instance in Turner's syndrome.

Introduction

The microsomal enzyme aromatase (P450C19) is encoded on the *CYP19* gene, which is located on chromosome 15q21.1 [1] and catalyzes the conversion of C19 to C18 steroids. Aromatase is the key enzyme in estrogen synthesis in all vertebrates [1]. The coding region of the *CYP19* gene spans 9 exons corresponding to approximately 35 kb beginning with exon II. In addition, there are a number of alternatives to exon I that are spliced in a tissue-specific fashion [1]. Furthermore, there are several promoters upstream from the coding region [2]. Aromatase is expressed in a variety of different tissues like the placenta, ovary, brain, osteoblasts, vascular endothelium, breast and adipose tissue [3].

During pregnancy placental aromatase has two main functions: on the one hand aromatization of fetal androgens is essential for production of estrogens during gestation, on the other hand placental aromatase plays a critical role in protecting the fetus from excessive androgen exposure in utero. After cleavage in the placental unit of the sulfate moiety of DHEAS from the fetal and maternal adrenal gland, DHEA is converted to androstenedione and eventually to testosterone. Placental aromatase converts androstenedione to estrone and testosterone to estradiol. 16-OH-DHEAS from the fetus is converted by placental sulfatase and 3β-HSD to 16-OH-DHEA and 16-OH-androstenedione and, finally, by placental aromatase to estriol [4].

The first case of aromatase deficiency was reported by Shozu and co-workers in 1991 [5]. To date 12 cases in infants (11 girls and 1 boy) [5–16] and 7 cases in adult men [8, 17–22] have been described.

Although we concluded previously that affected females should be treated with low-dose 17β-estradiol (E_2) in amounts sufficient to result in physiological prepubertal estradiol concentrations using an ultrasensitive estrogen assay and that later estradiol replacement needs to be adjusted throughout childhood and puberty to ensure normal skeletal maturation, adequate adolescent growth spurt, normal accretion of bone mineral density, and – at the appropriate age – female secondary sexual maturation, published data remain scarce [9]. Therefore, we studied the impact of low-dose, oral E_2 treatment assessed and adjusted by the use of an ultrasensitive estrogen assay from infancy to final height in a girl with aromatase deficiency harboring a CYP19A1 point mutation G>A at the splicing site between exon and intron 3 and a base pair deletion C (P408; CCC, exon 9) yielding a compound heterozygote state [9]. In the long-term follow-up, the role of E_2 in pituitary feedback regulation was explored accordingly. In addition, the role of E_2 supplementation in achieving normal bone mass was assessed longitudinally.

Patient and Methods

Case

The medical history, clinical findings and results of the genetic analysis of this girl were previously reported [9]. The patient was treated with oral E_2 as follows: at the age of 3.5–3.67 years: 0.4 mg/day; 4.67–6 years: 0.1 mg/day; 7–9.5 years: 0.05 mg/day; 9.5–10.5 years: 0.1 mg/day; 10.5–12 years: 0.2 mg/day; 12–13 years: 0.4 mg/day; 13–13.5 years: 0.8 mg/day; 13.5–14 years: 1.4 mg/day; after 14 years: 2.0 mg/day (fig. 1a). Since the lowest commercially available dose for estradiol pills in Switzerland, like in most European countries, is 1 mg, our Hospital Pharmacy prepared appropriate confections as low as 0.05 mg. From the age of 13.5 years on gestagen (dydrogesteron 20 mg/day from day 15–25 of each cycle) was added. Under this treatment our patient had regular vaginal bleedings. At six monthly intervals an ultrasonographic examination of uterus and ovaries as well as GnRH testing [23] was performed.

Hormonal Measurements

Plasma LH and FSH were measured by a fluorometric enzyme immunoassay using Stratus (DADE International, Miami, Fla., USA). During infancy and prepuberty E_2 was assessed with an ultrasensitive bioassay as described previously [24, 25]. Thereafter, a high-sensitive serum E_2 kit (DSL's Ultrasensitive E_2 RIA, DSL-4800) was used. The intra- and interassay coefficients of variation (CVs) were 8.5 and 7.2%, respectively, at 5.0 pg/ml (18.3 pmol/l). At 30 pg/ml (110 pmol/l), the intra- and interassay CVs were 6.1 and 9.2%, respectively. The lower detection limit was 2.0 pg/ml (7.3 pmol/l). Free testosterone, DHEA and DHEA-S were determined by commercially available RIA kits (Diagnostic Systems Laboratories, Diagnostic Products Corp., Los Angeles, Calif., USA). The conversion factors are as follows: E_2, serum: pmol/l = 3.67 × pg/ml; DHEA-S, serum: μmol/l = 0.0027 × pg/ml; DHEA, serum: nmol/l = 3.467 × μg/l; free testosterone, serum: pmol/l = 3.467 × pg/ml; Δ4-androstenedione, serum: pmol/l = 0.028 × ng/dl; LH, plasma: IU/l: 1.0 × mIU/ml; and FSH, plasma: IU/l = 1.0 × mIU/ml.

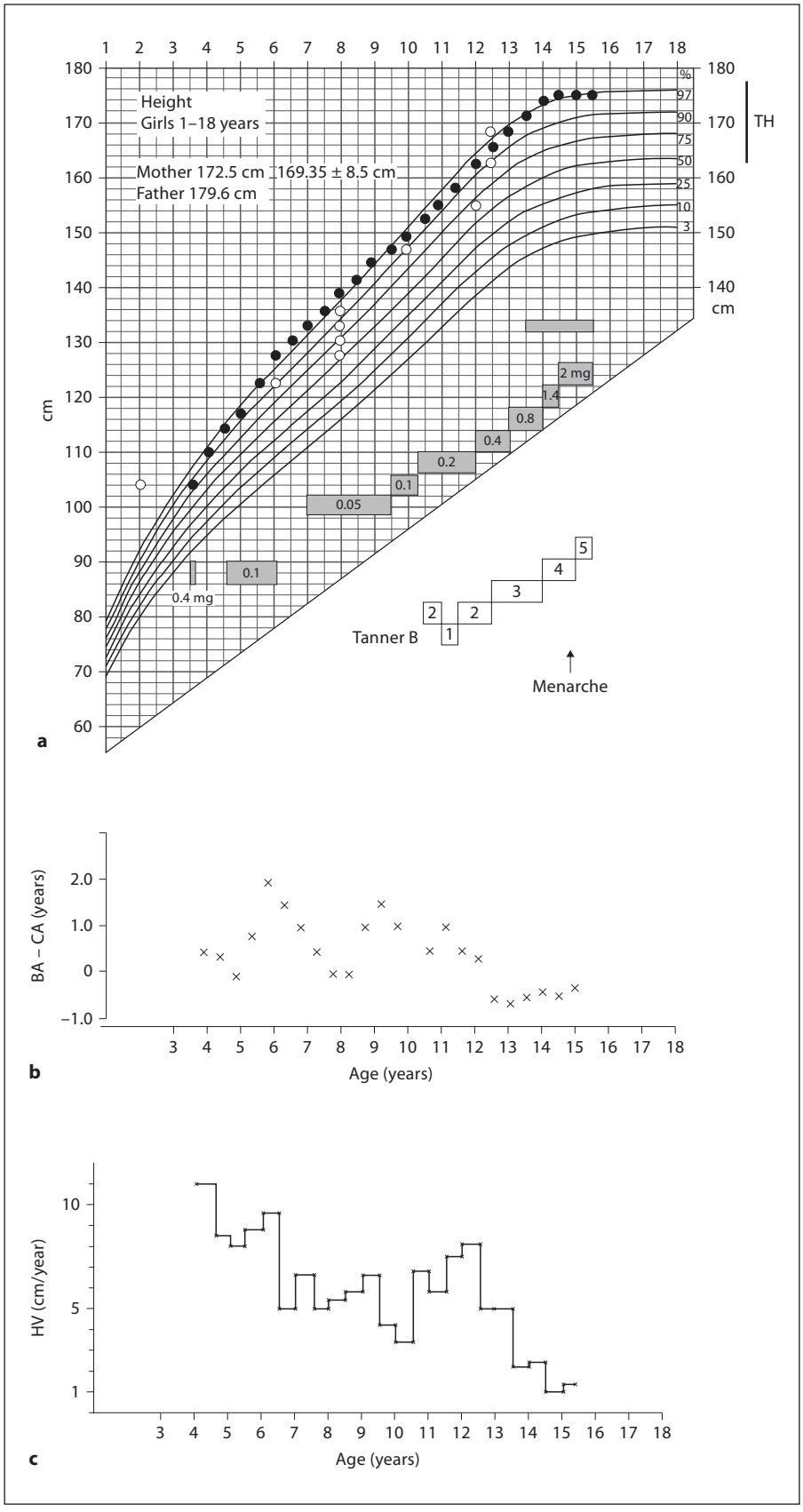
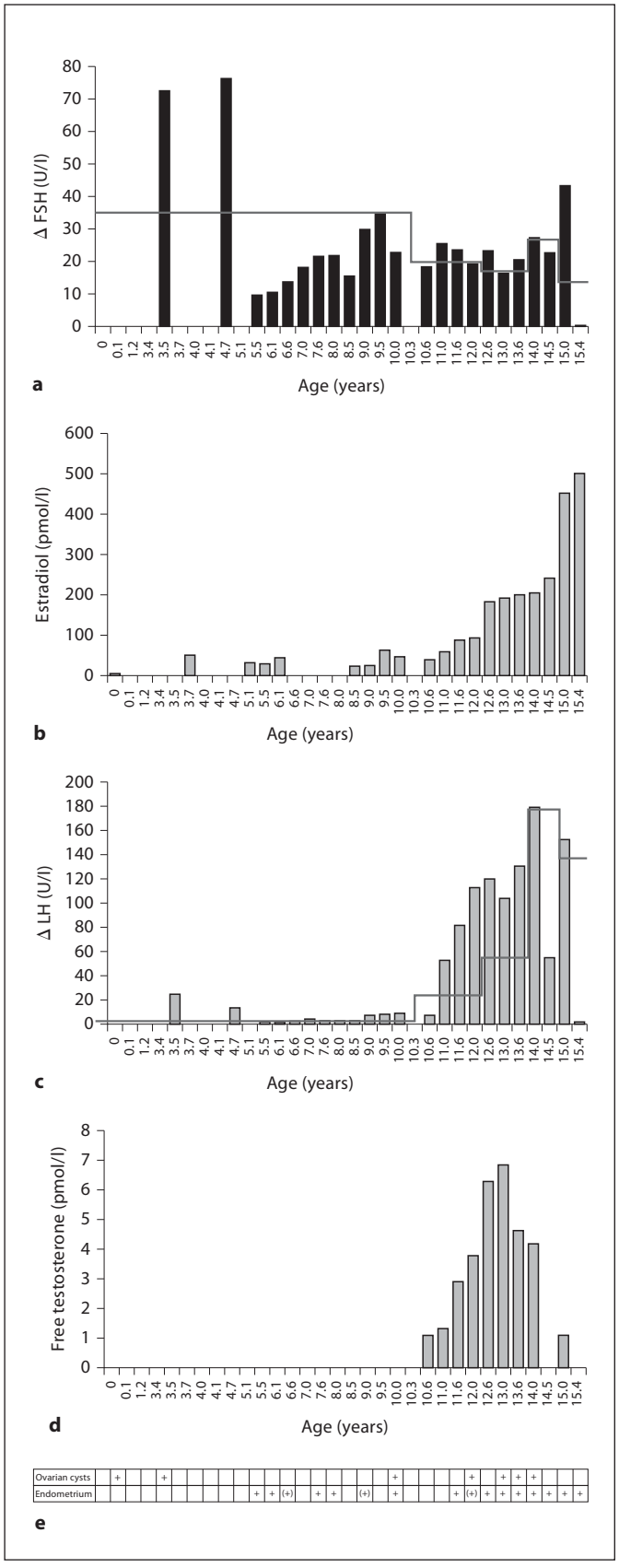


Fig. 1. Auxology of the patient up to near-final height. **a** Growth chart with schematic treatment schedule and Tanner stages for breast development. Closed circles indicate height for age, open circles indicate the corresponding bone age. For better readability not all bone age measurements are shown. The grey bars below indicate the daily oral dose of 17β-estradiol in mg. The hatched bar indicates added cyclic gestagen treatment. The arrow indicates the age of the first vaginal bleeding. Target height (TH) is shown as a black bar on right side of the graph. **b** Difference between bone age and chronological age (BA – CA) at every given time point. **c** Height velocity (HV).



Dual Energy X-Ray Absorptiometry (DEXA)

Whole body and lumbar spine bone mineral density (BMD) as well as whole body bone mineral content were measured using DEXA (Hologic QDR 4500, Hologic, Waltham, Mass., USA) using published reference values [26] three times between three and four years of age as published earlier [9] and then in yearly intervals starting with 7 years.

Results

Auxology

The auxological follow-up from 3.5 to 15 years of age is depicted in figure 1a–c. The main finding is that low doses of E_2 (0.05–0.1 mg/day) are needed for normal growth and bone age maturation (expressed as [bone age] – [chronological age]) already in early childhood. In our patient withdrawal of E_2 resulted in arrest of bone age maturation and decrease of height velocity as seen between 3.67 and 4.67 years as well as 6.0 and 7.0 years (fig. 1b, c). However, in late prepuberty and puberty the patient showed a discordant picture between an already decreasing bone age maturation indicating relative estrogen deficiency, and a rising height velocity indicating sufficient serum estradiol levels. Overall, under the aforementioned treatment the patient attained normal near-adult height (174.5 cm; +2.4 SDS) within the upper target range (169.6 ± 8.5 cm).

Pubertal Development

The main finding is that pubertal E_2 levels in serum needed for adequate breast development as well as for the appearance of an endometrial reflex are not sufficient to suppress the pituitary-ovarian axis via feedback mechanism at the level of the pituitary gland. Evidence for this is that the patient shows follicular cysts particularly in Tanner stages 3 and 4 indicating inadequately elevated gonadotropins. Furthermore, this effect seems to be age dependent since it is not seen to that extent in infancy, childhood and early puberty (fig. 2a–e). Importantly, however, adequate E_2 replacement leads to the disappear-

Fig. 2. Effect of treatment with 17β-estradiol on FSH, LH and free testosterone. **a** FSH amplitude (maximal FSH – basal FSH) after GnRH stimulation. The grey line shows the 97.5th centile for age. **b** Serum estradiol measured by an ultrasensitive assay. **c** LH amplitude (maximal LH – basal LH) after GnRH stimulation. The grey line shows the 97.5th centile for age. **d** Basal serum free testosterone. **e** Schematic representation of the presence of ovarian cysts and an endometrium reflex in the pelvic ultrasound.

ance of follicular cysts in the presence of normal FSH amplitude/peak value following GnRH stimulation although basal FSH still remained elevated.

Pituitary-Ovarian Axis

Basal FSH is mostly above the 97.5th centile for age and sex throughout the fifteen years of follow-up. In contrast, FSH amplitude (peak FSH – basal FSH after GnRH) was obviously increased between 3.67 and 4.5 years while not being on E_2 replacement therapy. Furthermore, this became clear at the early pubertal stage between the chronological age of 11–12.5 years as well as in late puberty at the age of 15 years indicating insufficient serum estradiol levels for normalization of pituitary-ovarian axis during pubertal development, which did not hold true for the effect on growth (fig. 1a, 2a, b). Finally, during puberty there is a discordance between progressing bone age maturation (age 11–15 years) and increasing height velocity (age 11–12.5 years) indicating sufficient serum E_2 levels on the one side and unsuppressed FSH on the other side suggesting a different threshold for growth and gonadotropin secretion.

Basal LH and LH amplitude are normal during prepuberty and lie above the 97.5th centile for age and sex in early-mid-puberty (Tanner stages 2 and 3) (fig. 2c).

Increased free testosterone mirrors elevated basal LH during early and mid-puberty (fig. 2d).

Bone Mass

Whole body mineral content (WB-BMC), lumbar spine bone mineral density (LWS-BMD) as well as femoral neck bone mineral density are within normal ranges. We observed a physiological rise in WB-BMC during the pubertal growth spurt (fig. 3a–c).

Discussion

We report on the long-term follow-up and impact of low-dose estrogen (E_2) replacement controlled and adjusted with the help of an ultrasensitive estrogen assay in a girl with aromatase deficiency on longitudinal growth, bone age maturation, regulation of pituitary gonadotropin feedback, multicystic ovaries and bone mass.

Firstly, we found that minimal doses of E_2 (50–100 μg/day) are required for normal longitudinal growth and bone age maturation even in early childhood. Evidence that E_2 is needed for normal longitudinal growth and bone age maturation is that when off treatment the patient showed increasing bone age delay and decreasing height velocity. This finding fits in with other reports, which also showed a delay in bone age maturation in untreated aromatase deficiency patients over a much shorter observation period [12] as well as the advance in bone age during treatment with estradiol [8]. Interestingly, a recent study in girls with Turner's syndrome using comparable equivalent doses of ethinylestradiol (EE) from 5 years of age onward in addition to growth hormone treatment showed an improved growth if compared to girls treated with growth hormone only [27].

Secondly, our patient achieved normal near-adult height and showed normal pubertal development. Multiple ovarian cysts were present particularly in puberty indicating insufficiently suppressed gonadotropin feedback drive probably due to unphysiologically low estradiol levels. Follicular cysts have already been described [7, 9, 12] in ovaries of girls with aromatase deficiency as well as in ovaries of aromatase knock-out mice [28].

Ovarian cysts are also present in girls with P450 oxidoreductase deficiency (PORD) [29–32]. P450 oxidoreductase (POR) is an electron donor to all microsomal P450 cytochrome enzymes and for that reason acts as an essential cofactor of P450 aromatase, 17α-hydroxylase and 21-hydroxylase [33]. Therefore, girls with PORD show congenital adrenal hyperplasia combined with sex steroid deficiency. In a recent study [29] five out of five girls with PORD showed ovarian cysts, variable hypogonadism and elevated gonadotropins. In a larger Japanese study involving 18 girls and adult women with PORD, 8 presented with ovarian cysts and in one case the cyst diminished in size after treatment with estradiol [30].

Thirdly, we could show that higher E_2 doses were needed for successful negative feedback to the pituitary-ovarian axis than for normalization of growth in late prepuberty and puberty. Evidence that higher E_2 doses are needed for suppression of pituitary-ovarian axis than for normalization of growth is that particularly in Tanner stages 3 and 4 the ovaries showed a multicystic appearance while the patient showed normal growth and bone age maturation. During the critical time window between the age of 12 and 14 years basal FSH, LH as well as testosterone values were persistently elevated with serum estradiol levels around 200 pmol/l in spite of the fact that oral E_2 doses were increased from 0.4 mg/day to 1.4 mg/day during that time interval. We think that this observation is in line with the current pathogenetic concept of the development of multicystic ovaries in which FSH seems to play a critical role. So, mutations in the FSH β subunit as well as mutations in the FSH receptor result in a phenotype of small ovaries containing only primordial fol-

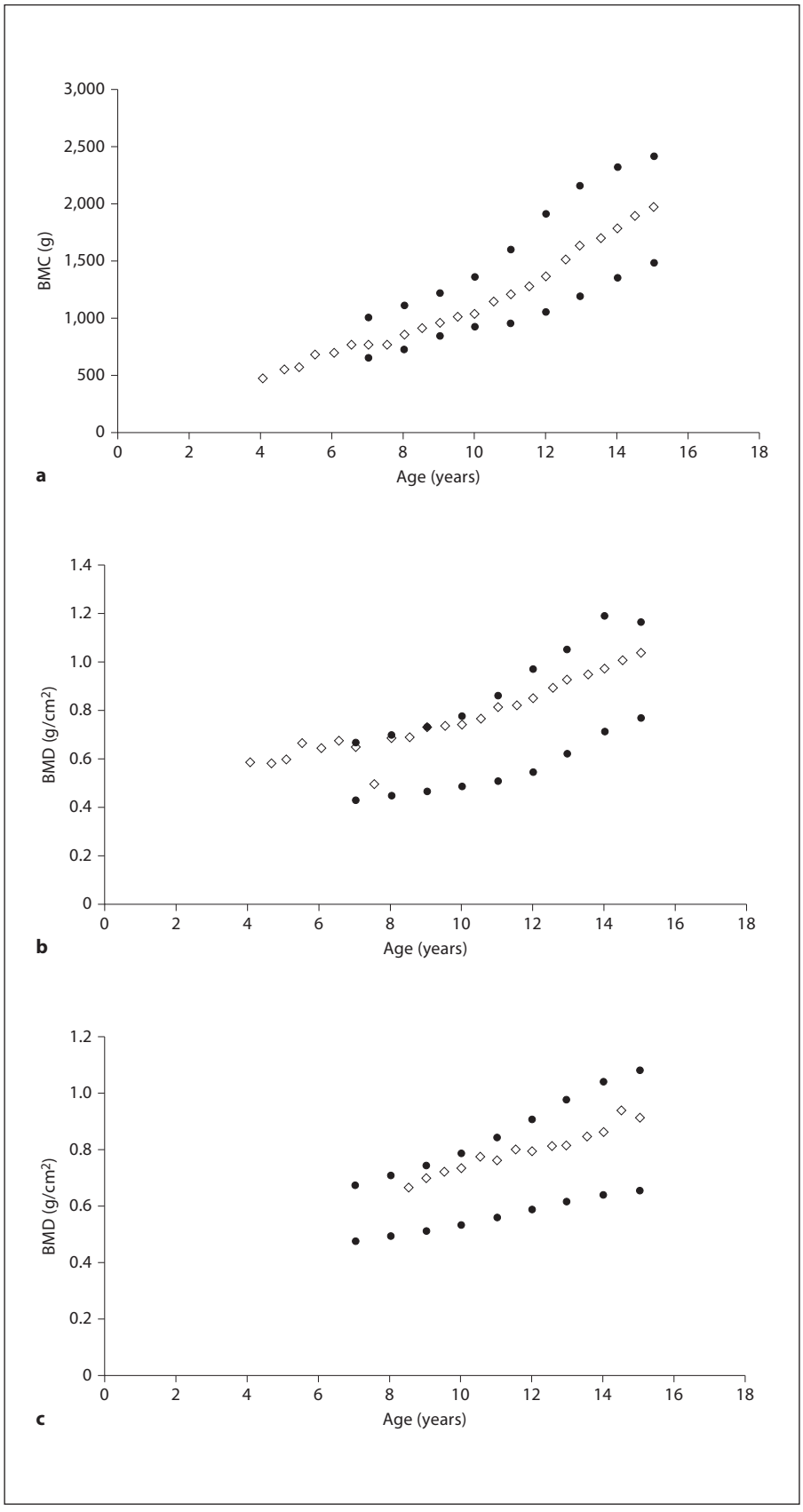


Fig. 3. Bone mineral content (BMC) and bone mineral density (BMD). The results of the patient's measurements are shown as open diamonds, the filled circles at the top and at the bottom indicate the 97.5th and the 2.5th centiles, respectively. **a** Whole body BMC. **b** BMD at the femoral neck. **c** BMD at the lumbar spine.

licles in mice [34] as well as in humans [35]. On the other hand, women with LH receptor mutations show normal follicular growth up to the antral stage [35] but neither dominant follicles nor cysts. Targeted overexpression of the LH receptor in mice results in a phenotype similar to PCOS [35], whereas activating mutations of the LH receptor in humans appears to have no phenotype [35]. Finally, high circulating androgens have been shown to increase the expression of FSH receptors on granulosa cells in rhesus monkeys resulting in an amplification of the FSH effect [36]. Putting these data together, it appears that LH is not critical for follicular development at least until the tertiary stage and that FSH action is essential for follicle growth although other hormones like androgens or paracrine factors seem to be necessary too.

An additional observation during GnRH testing was that the LH amplitude was normal in prepuberty except at three years of age and supraphysiologically elevated during puberty. The FSH amplitude showed a similar pattern although it rose already in late prepuberty and did not rise that much during puberty. This observation is in contrast to an earlier study which showed an elevated LH amplitude already in late prepuberty at 7.16 years [12].

Finally, we also show that our patient, who was adequately treated with E_2, developed normal bone mass during the whole observation period under treatment with increasing doses of E_2. Little is known about the bone phenotype of girls with aromatase deficiency. At the age of 3.5 years our patient showed low BMD and was subsequently treated with 0.4 mg of E_2 during 50 days. This treatment led to an increase of whole body BMD of 1% [9]. In contrast to our case, a girl compound heterozygote for two mutations on the *CYP19* gene, who was not treated with estrogens, showed a normal BMD at 6 years of age [12]. On the other hand, men with aromatase deficiency show a distinct bone phenotype characterized by high bone turnover and osteopenia [37]. This is very much in line with other conditions associated with estrogen deficiency like Turner's syndrome, which is also associated with low bone mass if not adequately treated with estrogens [38].

In summary, the long-term follow-up of our aromatase deficient patient on and off E_2 replacement illustrates that estradiol is needed not only in puberty but also in early childhood for normal growth, pituitary-gonadal development and bone maturation. This observation might establish a role for early low-dose estrogen treatment in other estrogen deficient states like in Turner's syndrome or PORD.

Disclosure Statement

All authors have nothing to disclose.

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