Anti-Müllerian hormone and letrozole levels in boys with constitutional delay of growth and puberty treated with letrozole or testosterone

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STUDY QUESTION: Does treatment of constitutional delay of growth and puberty (CDGP) in boys with aromatase inhibitor letrozole (Lz) or conventional low-dose testosterone (T) have differing effects on developing seminiferous epithelium?

SUMMARY ANSWER: Anti-Müllerian hormone (AMH) declined similarly in both treatment groups, and the two Sertoli cell-derived markers (AMH and inhibin B (iB)) exhibited differing responses to changes in gonadotrophin milieu.

WHAT IS KNOWN ALREADY: Boys with CDGP may benefit from puberty-inducing medication. Peroral Lz activates gonadotrophin secretion, whereas intramuscular low-dose T may transiently suppress gonadotrophins and iB.

STUDY DESIGN, SIZE, DURATION: Sera of 28 boys with CDGP who participated in a randomised, controlled, open-label trial at four paediatric centres in Finland between August 2013 and January 2017 were analysed. The patients were randomly assigned to receive either Lz (2.5 mg/day) (n = 15) or T (1 mg/kg/month) (n = 13) for 6 months.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The 28 patients were at least 14 years of age, showed first signs of puberty, wanted medical attention for CDGP and were evaluated at 0, 3, 6 and 12 months of visits. AMH levels were measured with an electrochemiluminescence immunoassay and Lz levels with liquid chromatography coupled with tandem mass spectrometry.

MAIN RESULTS AND THE ROLE OF CHANCE: AMH levels decreased in both treatment groups during the 12-month follow-up (P < 0.0001). Between 0 and 3 months, the changes in gonadotrophin levels (increase in the Lz group, decrease in the T group) correlated strongly with the changes in levels of iB (FSH vs iB, r = 0.55, P = 0.002; LH vs iB, r = 0.72, P < 0.0001), but not with the changes in AMH (P = NS). At 12 months, AMH levels did not differ between the groups (P = NS). Serum Lz levels (range, 124–1262 nmol/L) were largely explained by the Lz dose per weight (at 3 months r = 0.62, P = 0.01; at 6 months r = 0.52, P = 0.05). Lz levels did not associate with changes in indices of hypothalamic-pituitary-gonadal axis activity or Sertoli cell markers (in all, P = NS).

LIMITATIONS, REASONS FOR CAUTION: The original trial was not blinded for practical reasons and included a limited number of participants.

WIDER IMPLICATIONS OF THE FINDINGS: In early puberty, treatment-induced gonadotrophin stimulus was unable to counteract the androgen-mediated decrease in AMH, while changes in iB levels were associated with changes in gonadotrophin levels. AMH decreased similarly in both groups during the treatment, reassuring safety of developing seminiferous epithelium in both treatment approaches. Since a fixed dose of Lz induced variable serum Lz levels with a desired puberty-promoting effect in all boys, more research is needed to aim at a minimal efficient dose per weight.
The desired treatment effects (i.e. HPG axis activation) with a smaller dose as well. To elucidate this question, we report the circulating Lz levels in CDGP patients (Varimo et al., 2019) and correlate them to clinical and biochemical markers of puberty with special attention to the serum AMH and iB levels.

**Materials and Methods**

Thirty boys with CDGP were recruited to a randomised controlled trial, which compared 6-month aromatase inhibitor Lz treatment (2.5 mg/day, Letrozol Accord 2.5 mg; Accord Healthcare B.V., Utrecht, Netherlands) to low-dose intramuscular T treatment (1 mg/kg/month, Sustanon 250; Aspen Nordic, Ballerup, Denmark) (Varimo et al., 2019). The study protocol is illustrated in Supplementary Figure S1 and the distributions of hormonal and clinical markers of puberty during the study period in Supplementary Table S1. In brief, the boys were carefully examined, and those with other causes than CDGP for delayed puberty were excluded. The inclusion criteria were testicular volume between 2.5 and 4 ml and serum T < 5 nmol/L or serum T ≥ 1 nmol/L, if the mean testicular volume was < 2.5 ml, or Tanner genital stage 2 and serum T < 3 nmol/L (Varimo et al., 2019). At the start of the trial, the boys were above 14 years of age (mean 14.7 years 95% CI 14.4 to 14.9 years) and had a mean testicular volume of 3.1 ml (95% CI 2.8–3.5 ml) and a mean serum T concentration of 2.1 nmol/L (95% CI 1.6–2.5 nmol/L). During the study period, all boys progressed in puberty. At the study visits at 0, 3, 6 and 12 months, morning blood samples were drawn and sera stored at −80°C in 15 boys treated with Lz and 13 boys treated with T.

Two participants in the T group were excluded: one because of a protocol deviation, and the other due to missing samples. Data were thus available for 15 boys treated with Lz and 13 boys treated with T.

The levels of gonadotrophins, T, estradiol and iB were determined with routine laboratory techniques (immuno)electrochemiluminesometric, liquid chromatography/mass spectrometric and enzyme-linked immunosorbent assays), as described before (Varimo et al., 2019). GnRH-stimulation test was performed by injecting GnRH analogue (3.5 μg/kg, Relefact® LH-RH 0.1 mg; Aventis Pharma, Frankfurt, Germany) intravenously as a single bolus, and serum gonadotrophin concentrations were measured for up to 90 min.

Lz concentrations were determined at 3 and 6 months of the study by using a Shimadzu Nexera Liquid Chromatography System (Shimadzu Corporation, Kyoto, Japan) coupled to an API 3000 tandem mass
In order to evaluate the connection between Sertoli cell–secreted peptides with gonadotrophins, we calculated Pearson correlations between changes (0–3 months) in AMH and iB levels and changes (0–3 months) in FSH and LH levels. To further evaluate the change in AMH, we constructed a linear regression model explaining change in AMH (0–3 months) with the corresponding changes in T and FSH levels.

At 3 and 6 months, correlations between serum Lz concentrations and Lz doses by weight (mg/kg) were assessed with Pearson correlation. Correlations between Lz concentrations at 3 and 6 months and changes in clinical and hormonal markers of puberty between baseline and 3 or 6 months (serum T, estradiol, iB, LH, FSH and AMH concentrations; T/estradiol ratio and testis volume) were assessed with Spearman rank correlation. The correlation of number of patient-reported side effects and Lz concentrations at 3 and 6 months was calculated with Spearman rank correlation test. All P values were two-sided, and P values <0.05 were selected to indicate statistical significance.

Results

We investigated circulating AMH levels in boys with CDGP who had been treated either with traditional low-dose T or peroral aromatase inhibitor Lz. Correlations between serum AMH levels and clinical and hormonal markers of puberty at the start of the study are shown in Table I. In particular, AMH levels correlated negatively with FSH and GnRH-stimulated FSH and LH levels and positively with iB levels (Table I). We next investigated longitudinal changes in AMH levels between the treatment groups during the 12-month study period. Overall, the decrease in AMH level over time was significant (P < 0.0001). There were no clear differences in AMH levels between the study groups during the period of 6 months of medical intervention and 6 months of follow-up (P = NS) (Fig. 1). Between baseline and 12 months, AMH declined from 42.2 μg/L (95% CI 26.1–58.1) to 15.1 μg/L (95% CI 8.9–21.2) (P = 0.003) in the Lz group and from 35.8 μg/L (95% CI 20.9–50.8) to 13.8 μg/L (95% CI 7.5–20.2) in the T group (P = 0.002). The change was similar in both treatment groups (P = NS).

When the analyses were restricted to the first 6 months (i.e. during treatment), the change in AMH differed in the two treatment groups (P = 0.05): serum AMH decreased from 42.2 μg/L (95% CI 26.1–58.2) to 24.9 μg/L (95% CI 13.0–36.7) (P = 0.001) in the Lz group and from 35.8 μg/L (95% CI 20.9–50.8) to 29.2 μg/L (95% CI 14.6–43.4) (P = 0.051) in the T group. Individual AMH levels of the boys in the two treatment groups are shown in Fig. 2. When the baseline AMH levels were used to adjust the decrease in AMH from baseline to 6 months, the difference was, however, no longer statistically significant (P = NS).

Since it was evident that AMH exhibited a parallel change in both treatment groups (Fig. 1), and that low-dose T suppressed gonadotrophins and iB at 3 months of the study (Varimo et al., 2019), we were keen to investigate the relationships between gonadotrophins and the serum Sertoli cell markers. The results of a regression model between baseline AMH and T and FSH levels are shown in Table I. In particular, AMH levels correlated negatively with FSH and GnRH-stimulated FSH and LH levels and positively with iB levels (Table I). We next investigated longitudinal changes in AMH levels between the treatment groups during the 12-month study period. Overall, the decrease in AMH level over time was significant (P < 0.0001). There were no clear differences in AMH levels between the study groups during the period of 6 months of medical intervention and 6 months of follow-up (P = NS) (Fig. 1). Between baseline and 12 months, AMH declined from 42.2 μg/L (95% CI 26.1–58.1) to 15.1 μg/L (95% CI 8.9–21.2) (P = 0.003) in the Lz group and from 35.8 μg/L (95% CI 20.9–50.8) to 13.8 μg/L (95% CI 7.5–20.2) in the T group (P = 0.002). The change was similar in both treatment groups (P = NS).

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Table I: Correlations between baseline serum AMH level and baseline clinical and hormonal markers of puberty.

| AMH (μg/L) | Testosterone (nmol/L) | Estradiol (pmol/L) | Inhibin B (ng/L) | LH (IU/L) | FSH (IU/L) | LH * (IU/L) | FSH * (IU/L) | Testicular volume (ml) |
|-----------|-----------------------|-------------------|-----------------|-----------|------------|-------------|--------------|-----------------------|
| Spearman correlation | −0.36 | −0.35 | 0.39 | −0.24 | −0.49 | −0.39 | −0.53 | −0.26 |
| P value | 0.06 | 0.07 | 0.04 | 0.23 | 0.009 | 0.04 | 0.005 | 0.18 |

AMH, anti-Müllerian hormone
*Maximal stimulated value in GnRH test
Lz-levels, largely explained by Lz dose per weight (mg/kg), were not associated to HPG-axis activity or patient-reported side effects.

Medications that are used to manipulate puberty should be well-investigated and safe without long-term adverse effects. This has not always been the case. For example, high-dose estrogen treatment, used to prevent tall stature in girls, was found to be associated with increased risk of infertility (Venn et al., 2004; Hendriks et al., 2012; Benyi et al., 2014) and high-dose T for the same indication in boys warrants careful consideration due to short-term side effects and subtle long-term increase in FSH levels, decrease in endogenous T levels and testis volume compared to untreated men (Hannema and Sävendahl, 2016; Albuquerque et al., 2017).

Puberty, the second postnatal activation period of the HPG axis, is associated in boys with Sertoli cell proliferation, which is essential for future fertility (Johnson et al., 1984; Cortes et al., 1987; Orth et al., 1988). Since men with a history of delayed puberty exhibit decreased sperm counts (Jensen et al., 2016), we deduced that it would be important to investigate circulating markers of seminiferous epithelium function in boys with delayed puberty during treatment with conventional low-dose T or a potent third-generation aromatase inhibitor Lz.

During the course of treatment, serum AMH level decreased in both groups, suggesting that both exogenous low-dose T and an increase in endogenous T levels are sufficient to down-regulate AMH secretion in early puberty. As AMH expression is considered to reflect maturation status of Sertoli cells (Sansone et al., 2019), our finding argues against unduly rapid maturation of Sertoli cell population in Lz-treated boys exposed to elevated gonadotrophin levels. Considering that Sertoli cell maturation is intimately related to their replicative capacity, our finding indirectly argues against untoward effect of Lz on future sperm producing capacity. Definitive data to support this view are scarce, as only one report exists on post-treatment sperm counts after aromatase inhibitor treatment during adolescence. In that study, limited number of growth hormone (GH)–deficient adolescents previously treated with anastrozole, another third-generation aromatase inhibitor, had similar sperm parameters as other GH-deficient and GH-sufficient adolescent controls (Mauras et al., 2005).

Despite the previously shown difference in gonadotrophin concentrations in the Lz- and T-treated groups (Varimo et al., 2019), we detected no evident difference between the groups in the AMH decline. These findings are in agreement with earlier reports showing a strong suppressing effect of T on AMH (Young et al., 1999; Young et al., 2003; Young et al., 2005). It is important to note that, in boys (Kohva et al., 2018) and men (Young et al., 2005) with gonadotrophin deficiency, recombinant FSH increases AMH, whereas in healthy boys, this effect appears to be overcome by the pubertal increase in intratesticular T levels (Rey, 1998; Grinspon et al., 2013). Indeed, the expression of androgen receptors in Sertoli cells increases significantly at the age of 4 to 8 years (Chemes et al., 2008), and the pubertal decrease in AMH has been shown to be an early, androgen-dependent event (Hero et al., 2012). Interestingly, the current study and our previous work (Hero et al., 2012) both suggest that the pubertal decline in AMH occurs already as a consequence of low androgen levels, and that even very high intratesticular T levels achieved by Lz do not significantly accelerate this process. We have previously shown that, in boys with idiopathic short stature treated with Lz (Hero et al., 2005), AMH decline in early puberty did not differ from placebo group suggesting that Lz-induced

Discussion

In this study, we showed that (i) AMH decreased similarly in both treatment groups, although Lz induced gonadotrophin secretion while low-dose T suppressed it; (ii) treatment-induced changes in circulating gonadotrophin levels were not associated with the changes in serum AMH, while they correlated with changes in iB; and that (iii) varying
Figure 2 Individual AMH levels in CDGP boys during 6-month treatment. Boys treated with testosterone [T] in blue (n = 13) and letrozole [Lz] in red (n = 15).

Figure 3 Correlation between gonadotrophins and Sertoli cell–secreted peptides. Correlations between changes in FSH and LH and changes in AMH (in blue) and inhibin B (in red) from baseline to 3-month-measurements in 28 boys with CDGP treated either with testosterone or letrozole.

strong gonadotrophin stimulus was unable to counteract the androgen-mediated decrease in AMH (Hero et al., 2012), and our current data on CDGP boys support this.

At the same time, the relationship between the treatment-induced changes in gonadotrophin and iB levels appeared quite the opposite as we were able to detect a positive correlation between them. Indeed, these results support the idea proposed by Grinspon et al. that serum iB level has two components in it: the gonadotrophin-dependent and the Sertoli cell mass-related components (Grinspon et al., 2013). Furthermore, a dual model has been proposed, in which immature Sertoli cells express both alpha- and beta-B inhibin subunits, whereas during puberty, the expression of beta-B-subunit is shifted from Sertoli cell to pachytene spermatocytes, early spermatid stages and to lesser extend Leydig cells (Andersson et al., 1998). This dependency of circulating iB on germ cells may explain why low-mid range iB levels associate with sperm counts and help to identify impaired spermatogenesis in men (Jørgensen et al., 2010). In contrast, AMH level does not appear as a useful hormonal marker of spermatogenesis (Aksglæde et al., 2018).
Given the reported lowered sperm counts in men with a history of delayed puberty (Jensen et al., 2016), it is tempting to hypothesise that Lz-induced activation of the HPG axis in early puberty will improve Sertoli cell proliferation and future spermatogenesis. Future studies are required to test this hypothesis.

To the best of our knowledge, this is the first study to report serum Lz concentrations in paediatric patients. The main determinant of circulating Lz levels was expectedly Lz dose per weight. Since the response to Lz treatment was heterogeneous, we could not identify an optimal HPG axis–inducing level of serum Lz. It is important to note that the current work was not powered to detect associations between Lz levels and HPG axis activity. On the other hand, it seems rational to aim at a minimal efficient dose per weight without losing the desired puberty-promoting effect. It is apparent that the current Lz dose was sufficient in all treated boys to induce the desired effect. Overall, the serum levels of Lz obtained with daily 2.5-mg dosing were similar to those observed in postmenopausal women (ranges 124–1262 nmol/L and 88–1227 nmol/L, when 1 ng/ml equals 3.51 nmol/L, respectively) (Desta et al., 2011). In postmenopausal women treated for breast cancer with a standard Lz dose of 2.5 mg per day, the variation in plasma concentrations of Lz is largely explained by BMI, age and CYP2A6 genotype, and the therapeutic range of Lz is considered wide. Serum Lz level or CYP2A6 genotype have not been associated with arthralgia, a commonly reported adverse effect of Lz (Desta et al., 2011; Tani et al., 2011; Borrie et al., 2018), and in the current study, Lz was well-tolerated and high Lz levels were not associated with the patient-reported side effects.

In conclusion, we investigated serum AMH levels in boys with CDGP who had been treated with low-dose T or peroral aromatase inhibitor Lz. Reassuringly, AMH decreased similarly in both groups during the treatment. The treatment-induced changes in AMH levels were gonadotrophin-independent, while the change in iB correlated with the change in gonadotrophins. Circulating Lz levels were highly variable and the levels were not associated with the HPG axis activity in CDGP boys in early puberty. Thus, future studies are required to investigate if a Lz dose lower than 2.5 mg/day is sufficient for the stimulation of the HPG-axis in boys with CDGP.

**Supplementary data**

Supplementary data are available at *Human Reproduction* online.

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**Authors’ roles**

T.V., P.J.M., M.H. and T.R. designed the study concept. T.V., H.H., S.T., R.V., J.T., P.J.M., S.T., R.V., J.T., K.V., J.V. and J.T.B. acquired the data. E.K. and T.V. analysed the data and interpreted it together with P.J.M., J.T.B., M.H. and T.R. All authors contributed in drafting and revising the article and approved the final version to be published.

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**Conflict of interest**

The authors have nothing to disclose.

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