A Longitudinal Study of Growth, Sex Steroids, and IGF-1 in Boys With Physiological Gynecomastia

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Context: Physiological gynecomastia is common and affects a large proportion of otherwise healthy adolescent boys. It is thought to be caused by an imbalance between estrogen and testosterone, although this is rarely evident in analyses of serum.

Objective: This study aimed to describe the frequency of physiological gynecomastia and to determine possible etiological factors (eg, auxology and serum hormone levels) in a longitudinal setup.

Design, Settings, and Participants: A prospective cohort study of 106 healthy Danish boys (5.8–16.4 years) participated in the longitudinal part of the COPENHAGEN Puberty Study. The boys were examined every 6 months during an 8-year follow-up. Median number of examinations was 10 (2–15).

Main outcome measurements: Blood samples were analyzed for FSH, LH, testosterone, estradiol, SHBG, inhibin B, anti-Müllerian hormone, IGF-1, and IGF binding protein-3 by immunoassays. Auxological parameters, pubertal development, and the presence of gynecomastia were evaluated at each visit.

Results: Fifty-two of 106 boys (49%) developed gynecomastia, of which 10 (19%) presented with intermittent gynecomastia. Boys with physiological gynecomastia reached peak height velocity at a significantly younger age than boys who did not develop gynecomastia (13.5 versus 13.9 years, $P = .027$), and they had significantly higher serum levels of IGF-1 ($P = .000$), estradiol ($P = .013$), free testosterone ($P < .001$), and FSH ($P = .030$) during pubertal transition. However, no differences in serum LH or in the estradiol to testosterone ratio were found.

Conclusions: Gynecomastia is frequent in pubertal boys. Increased IGF-1 levels and pubertal growth appear to be associated, whereas changes in estrogen to testosterone ratio seem negligible. **(*J Clin Endocrinol Metab* 100: 3752–3759, 2015)**

Physiological gynecomastia is a common, usually self-limiting, condition affecting 20–70% of pubertal boys (1–3). It is defined as the development of glandular tissue recognizable by palpation at the level of the nipple in pubertal boys, without any underlying endocrinopathy. The etiology is generally thought to be an imbalance in the estradiol to testosterone ratio (E2/T) that favors estrogen. This is because gynecomastia is observed in disorders

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Abbreviations: AMH, anti-Müllerian hormone; CV, coefficient of variation; E2/T, estradiol to testosterone ratio; ER, estrogen receptor; IGFBP, IGF binding protein; IGF-1R, IGF-1 receptor; LOD, limit of detection; PHV, peak height velocity.

known to influence this E2/T balance, such as Klinefelter syndrome (47, XXY), partial androgen insensitivity syndrome (4) and aromatase excess syndrome (5). Furthermore, breast development is a well-known side effect during GH therapy (6). However, sex steroid imbalances can rarely be detected in serum samples from boys with physiological gynecomastia (3, 7–11). It is therefore believed that other factors, such as other circulating hormones like GH, local imbalance in sex steroids, or endocrine disruptors may play a role in the pathogenesis (12), although controversy exists (13). We have previously shown in a cross-sectional study that serum IGF-1 concentrations were significantly higher in boys with physiological gynecomastia compared to boys without gynecomastia. However, the cross-sectional nature of the study prevented us from identifying boys that had gynecomastia before the examination or who develop it at a later time (14).

In a recent study Limony et al found the age at peak height velocity (PHV) to coincide with the development of physiological gynecomastia (15).

Large-scale animal studies have been used to elucidate hormonal effects on the growth of breast tissue and confirm the presence of estrogen receptors (ERs), IGF-1 receptors (IGF-1R), and progesterone receptors (16, 17), but the literature on receptors in the male breast, especially physiological gynecomastia, is sparse and somewhat contradicting (18, 19).

In this longitudinal study of 106 healthy Danish boys, we describe the frequency and natural course of physiological gynecomastia and evaluate auxological and hormonal factors.

Materials and Methods

Study subjects

In this longitudinal study, as part of the COPENHAGEN Puberty Study (20, 21) (ClinicalTrials.gov ID:NCT01411527), we examined 106 (5.8–16.4 years of age) healthy Danish boys every 6 months over the course of 8 years (2006–2014). A total of 968 examinations were carried out, and the median age at first examination was 9.2 years (range, 5.84–14.95). The median (range) number of examinations was 10 (2–15). All participants were recruited from two schools in the Copenhagen area. The schools were in the upper 20% of Danish schools with respect to higher parental income and socioeconomic status in a national investigation from 2011 (http://www.kora.dk/media/277013/Bilag_2_til_rapport_om_folkeskolens_faglige_kvalitet.pdf).

All boys, except two, were healthy. The two boys (one with and one without gynecomastia) had asthma and were treated with inhalation steroid, but were included in the study.

Other aspects of the COPENHAGEN Puberty Study have previously been published (14, 20, 21).

Clinical examination

Height was measured to the nearest 0.1 cm using a stadiometer (Holtain Ltd.). Weight was measured to the nearest 0.1 kg using a digital electronic scale (SECA). The children were weighed without shoes, wearing light clothing. Body mass index was calculated as weight (kilograms) divided by height (meters) squared (kg/m^2). The thickness of four skinfolds (biceps, triceps, subscapular, and suprailiac crest) was measured on the left side of the body using a skinfold caliper calibrated to 0.2 mm (Harpenden, British Indicators Ltd). The measurement of skinfolds is described in further detail in a previous publication (14). The sum of all skin folds (biceps, triceps, suprailiac crest, and subscapular) and the sum of scapular and the triceps skin folds were calculated. Furthermore, total body fat percentage was calculated according to Slaughter (22).

Pubic hair and genital stages (PH1–6 and G1–5) were assessed by clinical examination according to Marshall and Tanner (23). Five boys did not want their pubertal development assessed at debut of gynecomastia. Using Prader's orchidometer (24), testicular volume was measured to the nearest bead (mL) by palpation. The volume of the largest testis was used in case of discrepancy between the size of the left and right testes.

Gynecomastia was evaluated by inspection and palpation as described by Braunstein (25).

The examiners did not distinguish between uni- and bilateral gynecomastia.

Blood sampling procedure

Blood samples were drawn from an antecubital vein between 08:30 and 13:00 hours. Blood samples were clotted and centrifuged, and serum was stored at −20°C until hormone analyses were performed no later than 5 years after collection.

Serum hormone analyses

Serum concentrations of FSH, LH, and SHBG were measured by time-resolved immunofluorometric assays (Delfia, Perkin Elmer). Intra- and interassay coefficients of variation for FSH were 2.1% and 2.7% and with a limit of detection (LOD) of 0.05 IU/L; for LH, intra- and interassay coefficients of variation (CVs) were 3.0% and 1.94% with LOD 0.05 IU/L. Intra- and interassay CVs for SHBG were 5.1% and 7.5%, with an LOD of 0.2 nmol/L.

Serum T was measured by RIA using a Coat-A-Count RIA kit (Siemens), with an LOD of 0.23 nmol/L, and intra- and interassay CVs of 17% and 12.8%, respectively. E2 was measured by RIA (Pantex) with intra- and interassay CVs of 7.5% and 14.9%, respectively, and an LOD of 18 pmol/L.

Between 1990 and 2010, serum inhibin B was measured using one of two double antibody enzyme immunometric assays (Inhibin B DSL or Oxford Bio-Innovation Inhibin B), both with an LOD of 20 pg/mL and intra- and interassay CVs less than 16%. From 2010, inhibin B was measured using the Beckman Coulter Inhibin B genII assay, with an LOD of 3 pg/mL and intra- and interassay CVs less than 11%. The old and new inhibin B methods were compared and showed similar results for boys; therefore, no correction factor was needed. Serum anti-Müllerian hormone (AMH) was measured by a sensitive immunoassay (Immunotech) with a detection limit of 2 pmol/L. In our hands, the intraassay CVs were less than 7.8% and 11.6%.

Serum IGF-1 and IGF binding protein (IGFBP)-3 were determined by immunoassay (IMMULITE 2000, Siemens Healthcare Diagnostics) with detection limits of 20 ng/mL and 100 ng/mL,

respectively. IGF-1 intra- and interassay CVs were 2.1% and 10.1%; for IGFBP-3, CVs were 4.25% and 9.23%, respectively. Free -T was calculated according to Vermeulens with albumin fixed at 43 g/L (26).

Histological evaluation

Breast tissue was obtained after corrective breast surgery from four different males with gynecomastia. Representative histological slides from a 25-year-old patient with persistent physiological gynecomastia without any identifiable endocrinopathy were used. Histological sections were made using formalin-fixed, paraffin-embedded breast tissue, cut and mounted on glass slides, and stained with hematoxylin and eosin. Immunohistochemical analyses were made using the following antibodies (clone, product number, dilution): ER-α (SP1, 790–4325, ready-to-use ; Ventana Medical Systems Inc.), ER-β (polyclonal, PU385-UP, 1:100; Biogenex), androgen receptors (AR441, M3562, 1:400) and Ki67 (MIB1, M724001, 1:100; DAKO), IGF-1R (polyclonal, ab39398, 1:150; Abcam), prolactin receptor (polyclonal, A0569, 1:800; DAKO), progesterone receptor (IE2, 790–2223, ready-to-use; Ventana Medical Systems Inc.), and CYP19A1 (aromatase) (H4, LS-B7706, 1:50; LSbio). The paraffin-embedded tissue blocks were cut in 4μm sections, mounted on coated glass slides. The immunohistochemical reactions were performed using the BenchMark ULTRA Platform (Ventana Medical Systems Inc) including blocking of endogenous peroxidase activity, antigen retrieval at pH 8.5, 32 minutes at 100°C (ER-β, pH 9, 20 minutes at 100°C), and finally counterstained with hematoxylin and eosin.

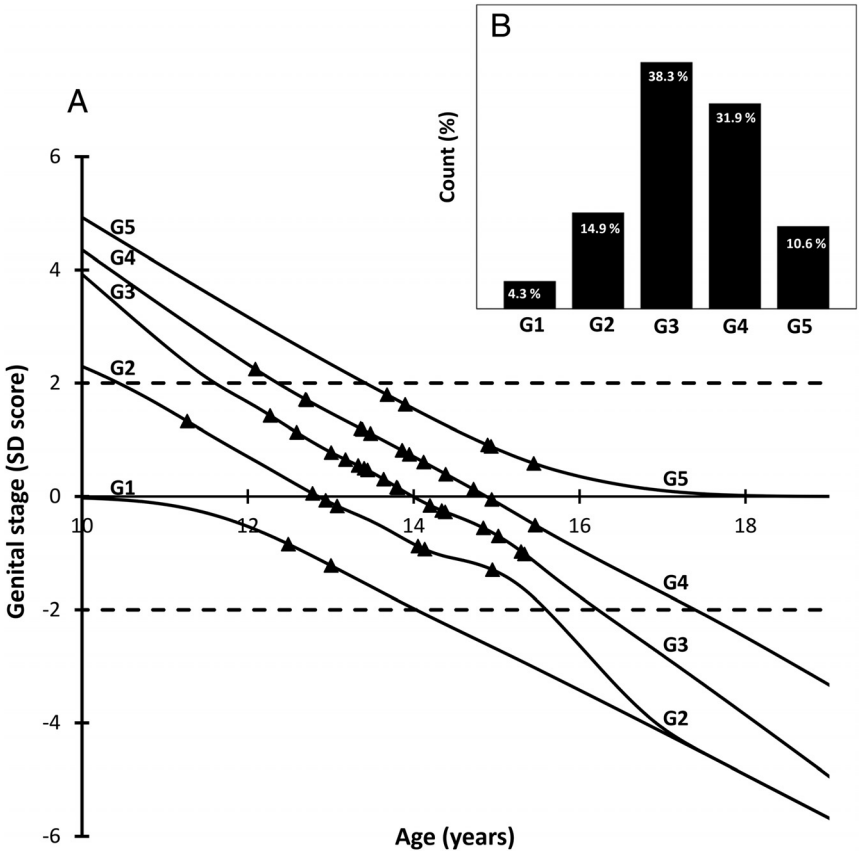

Figure 1. A, Puberty nomogram comparing age and genital stage at first examination with physiological gynecomastia (triangle) to a standard material based on healthy Danish boys (41). B, Distribution (%) of genital stages at first examination with presence physiological gynecomastia.

Statistical analyses

Data are presented as medians and range (min-max).

Duration of gynecomastia was calculated using Kaplan-Meier survival analysis and reported as median duration with 95% confidence interval.

The height of an individual was modeled by a population-wide growth curve plus an individual time-consistent height deviation. The model included a random warping function per individual that allowed temporal deformation of the population growth pattern, thus modeling a height development age for each subject. In the following, we refer to the predicted height development age as adjusted age. The models belong to the class of functional nonlinear mixed-effects models (27) and were fitted using maximum likelihood estimation. The population height curve was modeled by an increasing spline with 10 anchor points. The consistent height variations over time were modeled by a Gaussian Matérn process with smoothness 2. The time warping was done using an increasing spline with two anchor points driven by an underlying Brownian bridge.

Height velocity curves were calculated from the model by differentiation of the predicted height curves. Individual ages at PHV were found as the chronological ages that corresponded to the population PHV in adjusted age.

We tested if the boys without and the boys with gynecomastia could be accommodated by a single model, which would indicate similar growth pattern in the groups, and whether the gynecomastia group had different mean age at peak-velocity than the nongynecomastia group. These hypotheses were tested using the combined maximum log likelihood and the difference in mean predicted ages at PHV. *P* values were computed by means of a permutation test with 10 000 resamplings.

The remaining variables (weight, height, body mass index, body fat percentage, FSH, LH, T, E2, SHBG, inhibin B, AMH, IGF-1, IGFBP-3, free-T, E2/T, FSH/inhibin B ratios, genital and pubic stages, and testicular volume) were analyzed individually by a model similar to what was described previously, except that the mean function was modeled by cubic B-splines and that no temporal deformation of the curves was included. Instead, the curves were analyzed using both chronological and adjusted age predicted from the height measurements, thus also controlling for pubertal development. For each variable, we tested whether the measurements in the two groups could be accommodated by a single model. This was tested by means of the combined maximum log likelihood for the groups and *P* values were computed by permutation tests with 5000 resamplings for each variable. The test for difference between two groups using the maximum log likelihood difference tells us if there is some difference between the two groups. It does not, however, explicitly tell us what the difference is, nor differences at specific time points. It may be a difference in population mean pattern,

in the adjusted ages of the groups (if allowed), or in the variance parameters of the model.

Ethical considerations

The study was approved by the local ethical committee (KF 01 282214) and the Danish Data Protection Agency (2010–41–5042). All participants and their parents gave their informed consent before enrollment in the study.

Results

Fifty-two (49%) of the boys developed gynecomastia during the follow-up. Age at first examination with gynecomastia was 13.4 years (10.8–15.0 years; median [min-max]), gynecomastia occurred most frequently in pubertal stages G3 and G4 (Figure 1).

Fourteen of the 52 (27%) boys experienced intermittent gynecomastia. Six of these had two to three intermittent examinations without gynecomastia and lasting a median time of 2.1 years (range, 1.5–4.5 years). The remaining eight boys had one intermittent examination without gynecomastia.

Median time from G2 to development of gynecomastia was 1.9 years (<0.5–4.5 years) (n = 45). Median time from first examination with gynecomastia to PHV was 0.27 years (range, −2.7–2.21) (n = 52).

The median duration of gynecomastia was 1.9 years (range, 1.1–2.6 years). Nineteen of the boys still had breast development at the end of the study (median age at last exam 15.4 years; range, 12.0–16.3).

The two groups had significantly different growth patterns ($P = .017$), and the gynecomastia group experienced PHV at younger ages compared to the nongynecomastia group (13.5 versus 13.9 years, $P = .027$) (Figure 2).

Boys who developed gynecomastia were significantly more advanced in their pubic hair development compared to controls (for a given chronological age, $P < .001$; for a given adjusted age, $P = .001$), although not accompanied by advanced genital or testicular development.

Other anthropometric measurements (eg, weight, height, body mass index, body fat percent) did not differ between the two groups over the course of the follow-up.

When comparing hormone levels between the two groups using both chronological and adjusted age, we found significant higher IGF-1 ($P < .001$ and $P = .000$, respectively), FSH ($P = .015$ and $P = .030$, respectively), T ($P = .019$ and $P = .006$, respectively), free-T ($P < .001$ and $P < .001$, respectively), E2 ($P < .001$ and $P = .013$, respectively), and FSH/inhibin B ratio ($P = .007$ and $P = .01$, respectively), but lower AMH ($P = .000$ and $P = .003$, respectively) and SHBG ($P = .000$ and $P = .001$, respectively) in boys with physiological gynecomastia. No differences were found for, LH, E2/T ratio, or IGFBP-3. Free-T, E2, IGF-1 and IGFBP-3 are depicted longitudinally according to adjusted age in Figure 3.

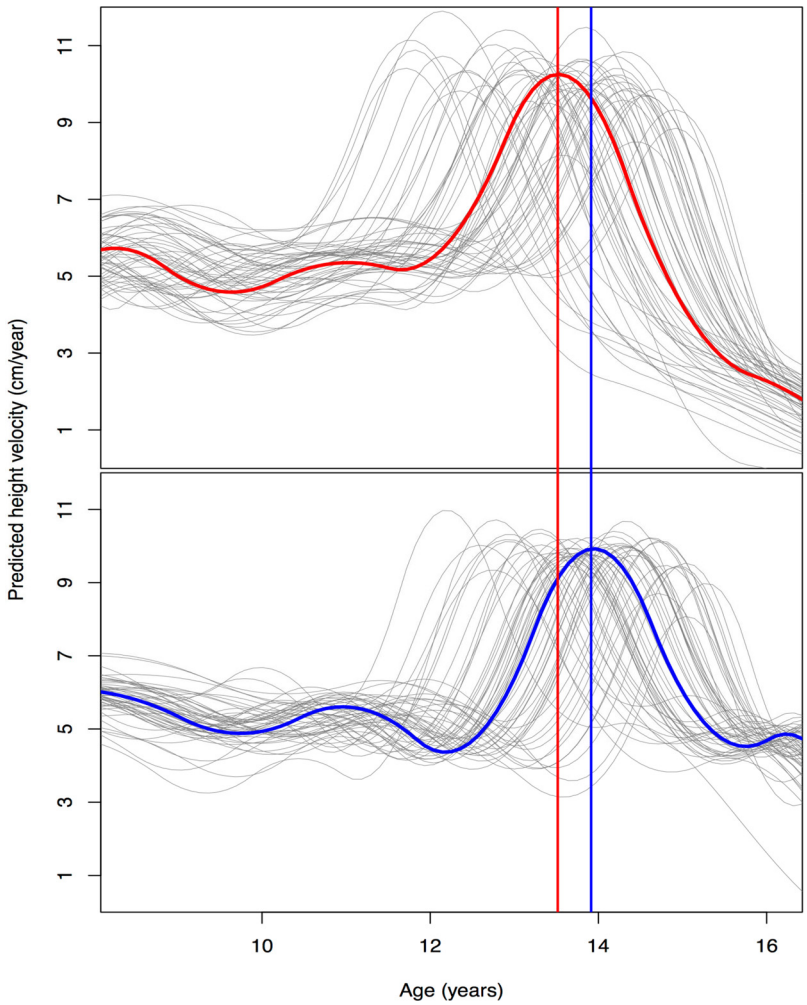

Figure 2. Modeling and comparison of predicted age at peak height velocity in boys with (red) and without (blue) gynecomastia.

Histological evaluation

The histological findings are in accordance with not-recently developed gynecomastia, with ductal hyperplasia surrounded by fibrous tissue and no presence of lobuli—as a sign of no current abnormal endo-

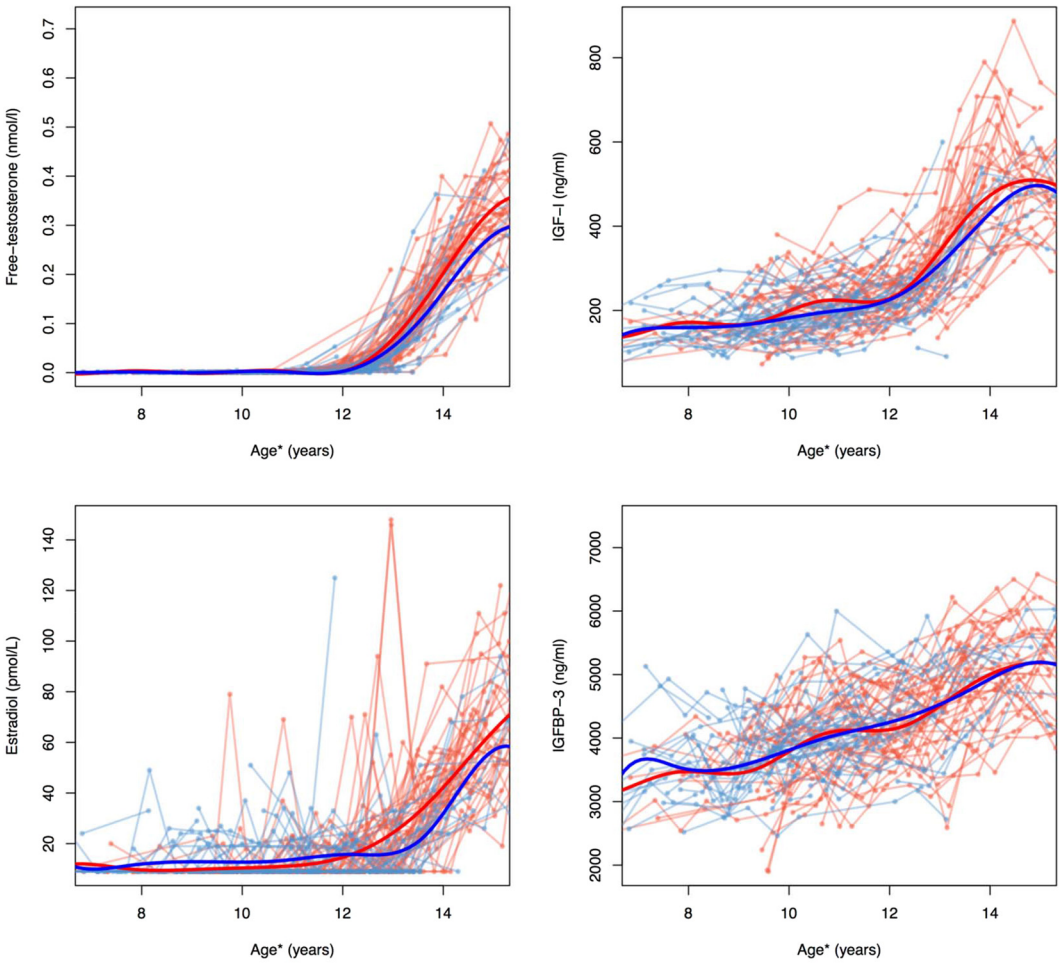

Figure 3. Serum free-T, E2, IGF-1, and IGFBP-3, according to adjusted age (age*) 7–15 years, in boys with gynecomastia (red, thin individual lines and mean) compared to controls (blue, thin individual lines and mean).

crine influence. The hematoxylin and eosin–stained slides showed fibrous tissue and ducts with ductal hyperplasia surrounded by various grades of fibrosis and no lobuli. Immunohistochemical staining show very low proliferation index (1–2%) based on Ki67 staining (not shown), which is consistent with "old" physiological gynecomastia (28). The ducts showed strong positive staining in ER-α, ER-β, androgen receptor, and IGF-1R, whereas CYP19A1 (aromatase) showed a weaker staining (Figure 4A-F). Progesterone receptors staining was strong, but prolactin receptor staining was negative (both not shown).

Discussion

In this prospective study, 49% of healthy boys developed physiological gynecomastia during pubertal transition. Boys who developed gynecomastia reached PHV earlier and had higher levels of IGF-1 as well as of FSH, free-T, and E2 compared to boys who did not develop gynecomastia.

In 27% of boys with physiological gynecomastia, we observed intermittent gynecomastia, defined as a period of time without gynecomastia after the initial appearance of breast development. This phenomenon has, to our knowledge, only been reported once in the literature by Bannayan et al in 1972, but they did not go into detail or provide further definition. In their study, 6% of patients had intermittent gynecomastia (29).

The prevalence of physiological gynecomastia in our cohort was high (49%), but in accordance to that found by Biro and colleagues (48.5%) (3). Both lower and higher prevalence has been reported: Nydick et al report a prevalence of 38.7% (2) and Lee et al (11) and Moore et al found that 69% and 56%, respectively, developed gynecomastia (8).

Most our boys developed gynecomastia in mid-puberty, G3 (38%) and G4 (32%)—a time when reproductive hormones and growth factors are increasing sharply. This is in accordance with previous reports (3, 8, 11, 15).

The duration of gynecomastia was 1.9 years. In the few longitudinal studies of physiological gynecomastia, the duration was reported to be between 1 and 2 years (2, 11), whereas one study reported gynecomastia lasting less than 6 months in 60% of the boys (3).

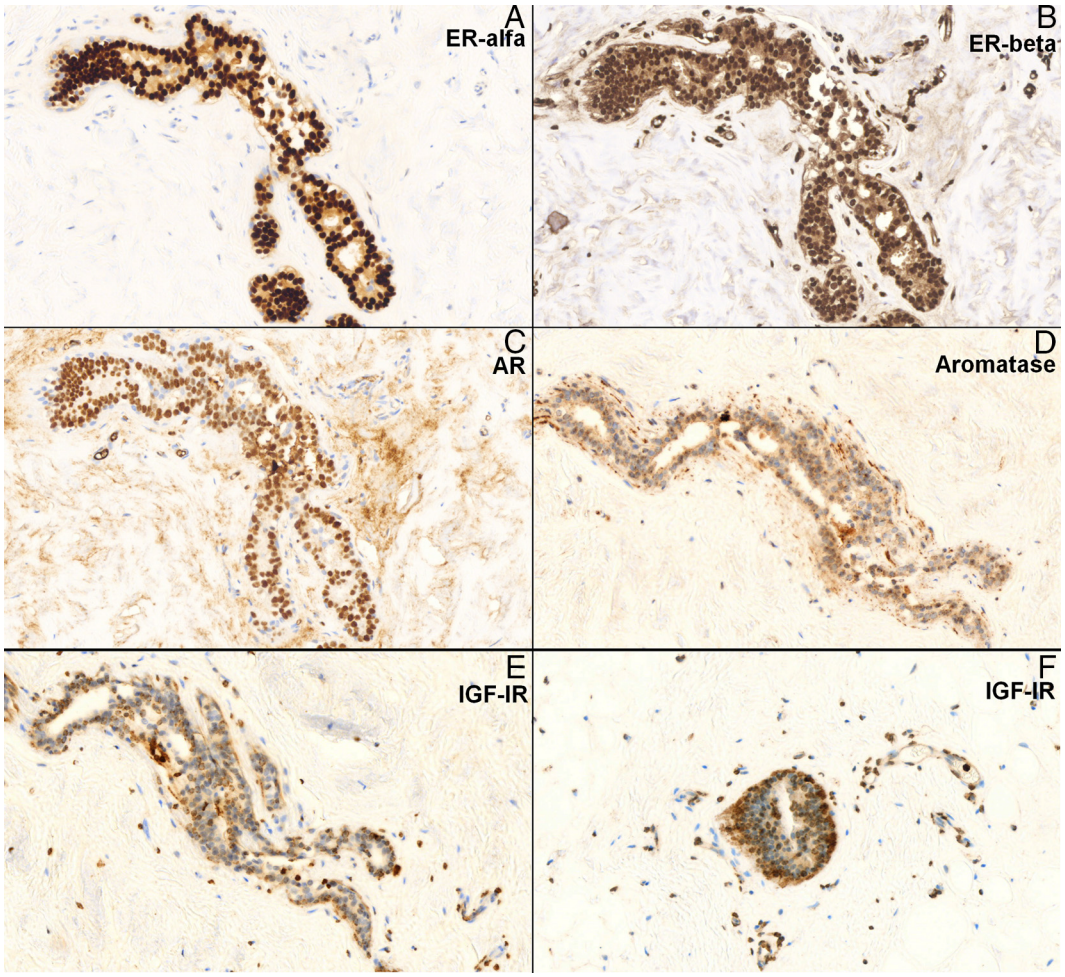

Figure 4. Illustrative immunohistochemical stainings of male breast tissue from a young man with persistent pubertal gynecomastia without any underlying endocrinopathy. Note ductal hyperplasia characterized by multilayered epithelium on a layer of myoepithelial cells, surrounded by a fibrous stroma. Immunohistochemical staining showing positive nuclear reaction in the epithelial cells (illustrated by dark brown color staining): A, ER-α; B, ER-β; C, androgen receptor; D, aromatase showing a cytoplasmic granular staining; E and F, IGF-1R showing a membranous as well as some cytoplasmic staining in most epithelial cells. All $\times$20 magnification.

We observed that 27% of boys with gynecomastia presented with intermittent gynecomastia. This could be argued to be due to interobserver variation; however, this is not likely because half of these boys had two to three consecutive examinations without breast development before recurrence of gynecomastia. Thus, we speculate that this intermittent gynecomastia is a physiological phenomenon.

We found that the boys with breast development reached PHV at a younger age than controls. The only other study evaluating PHV in relation to physiological gynecomastia found a positive correlation between age at PHV and the appearance of physiological gynecomastia (15).

The difference in growth was also reflected in higher serum IGF-1 levels in boys with physiological gynecomastia similar to what we observed in our cross-sectional data (14). Furthermore, IGF-1 was strongly correlated with height as also reported in a recent longitudinal study by Cole et al (30).

IGF-1, together with estrogen, is essential for the growth of breast tissue. It seems that the effect of GH on breast growth is mediated through IGF-1, circulating and even locally produced in the surrounding tissue (31, 32). The stimulatory effect of IGF-1 on breast formation was synergized by E2 (16, 33), which was also elevated in boys with gynecomastia in our study. However, serum T was similarly elevated leaving the E2/T ratio unaltered. We demonstrate the presence of IGF-1R, ER-α, ER-β, and androgen receptor (strong staining) in physiological gynecomastia by immunohistochemical staining. The presence of ER in gynecomastia has previously been shown (19, 34, 35), although contradicting studies exist (36).

Boys/men treated with GH risk developing breast tissue (6, 37), but to our knowledge the presence of IGF-1R in male breast tissue has not yet been described. We demon-

strate the presence IGF-1R in male breast tissue rendering the effect of IGF-1 in male breast formation plausible.

The low levels of AMH could suggest an impaired spermatogenesis or Sertoli cell function, as previously published in our cross-sectional data (14), although the interpretation of AMH levels in healthy boys is unclear (38). This raises the question if differences in the maturation of the Sertoli cells might influence the development of physiological gynecomastia.

Our study possesses both strengths and limitations. It is unique in its longitudinal design and modeling, evaluating etiological factors through pubertal transition, but we risk classifying very young and prepubertal boys that will eventually develop gynecomastia as controls. We do, however, believe that including all of these boys as controls will merely reduce the differences between the groups, and that thus leaving our reported significant differences larger.

Because the examinations throughout the study was carried out by three examiners in three consecutive periods, we cannot establish if there were any differences in detection of gynecomastia between examiners. We do note, however, that the incidence increased with age and genital development, as anticipated, and when looking at individual cases examined by several examiners, no suggestion of difference in detection of gynecomastia among the three examiners was found.

We did not note if the gynecomastia was uni- or bilateral; hence, we cannot establish if the breast development in some cases disappear on one side while appearing on the other. This might hypothetically influence the calculation of duration of gynecomastia.

As reported, T was measured with RIA, this has been shown to be less accurate in measuring T at low levels compared to liquid chromatography-mass spectrometry–based methods (39). Unfortunately, tandem liquid chromatography-mass spectrometry was not available on the present cohort. Because we have only measured T and E2, we cannot exclude the possibility that other androgens and estrogens might influence the development of physiological gynecomastia as well as the local conversion to estrogens by aromatization of T to E2 or desulfation of estrone sulfate to estrone might play a role (40). Increased ER sensitivity could be associated although this is difficult to demonstrate.

Because of the lack of available tissue from a boy with recent developed gynecomastia, our study of receptors in a 25-year-old man with persistent physiological gynecomastia might not reveal characteristics only present in the acute stage of physiological gynecomastia or in those speculated to be able to change over time (eg, aromatase activity, receptor expression).

Conclusion

In conclusion, our study confirmed that physiological gynecomastia is a frequent condition appearing in mid-puberty and is in most cases transient, lasting less than 1 year. We describe the finding of the physiological phenomenon "intermittent gynecomastia" in 27% of the boys with breast development. Finally, we demonstrate elevated serum IGF-1 levels, high expression of IGF-1R in breast tissue, and a significant younger age at PHV.

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