Serum levels of insulin-like factor 3, anti-Müllerian hormone, inhibin B, and testosterone during pubertal transition in healthy boys: a longitudinal pilot study

Marie Lindhardt Johansen^{*}, Ravinder Anand-Ivell^{1,*}, Annette Mouritsen, Casper P Hagen, Mikkel G Mieritz, Tue Søbørg, Trine Holm Johannsen, Katharina M Main, Anna-Maria Andersson, Richard Ivell² and Anders Juul

Department of Growth and Reproduction, GR 5064, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark, ¹School of Bioscience, University of Nottingham, Nottingham, UK and ²Leibniz Institute for Farm Animal Biology, 18196 Dummerstorf, Germany

Correspondence should be addressed to A Juul; Email: anders.juul@regionh.dk

^{*}(M L Johansen and R Anand-Ivell contributed equally to this work)

Abstract

Insulin-like factor 3 (INSL3) is a promising marker of Leydig cell function with potentially high clinical relevance. Limited data of INSL3 levels in relation to other reproductive hormones in healthy pubertal boys exist. In this study, we aimed to evaluate longitudinal serum changes in INSL3 compared with LH, FSH, testosterone, inhibin B, and anti-Müllerian hormone (AMH) during puberty in healthy boys. Ten boys were included from the longitudinal part of the COPENHAGEN Puberty Study. Pubertal evaluation, including testicular volume, was performed and blood samples were drawn every 6 months for 5 years. Serum concentrations of testosterone were determined by a newly developed LC-MS/MS method, and serum concentrations of INSL3, AMH, inhibin B, FSH, and LH respectively were determined by validated immunoassays. The results showed that serum INSL3 levels increased progressively with increasing age, pubertal onset, and testicular volume. In six of the ten boys, LH increased before the first observed increase in INSL3. In the remaining four boys, the increase in LH and INSL3 was observed at the same examination. The increases in serum concentrations of LH, testosterone, and INSL3 were not parallel or in ordered succession and varied interindividually. We demonstrated that INSL3 concentrations were tightly associated with pubertal onset and increasing testicular volume. However, the pubertal increases in LH, INSL3, and testosterone concentrations were not entirely parallel, suggesting that INSL3 and testosterone may be regulated differently. Thus, we speculate that INSL3 provides additional information on Leydig cell differentiation and function during puberty compared with traditional markers of testicular function.

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Introduction

Insulin-like factor 3 (INSL3) is a peptide hormone secreted by Leydig cells in the testis and by theca cells

in the ovary. INSL3 exerts its effect through the G-protein-coupled relaxin family peptide receptor 2 (RXFP2) (Huang *et al.* 2012), which in transfected cell systems activates the stimulatory G-protein leading to an increase in cAMP (Kumagai *et al.* 2002, Heng *et al.* 2008). In rodent gametes, RXFP2 may also couple to the inhibitory G-protein (Kawamura *et al.* 2004, Hutson 2013). The expression of *INSL3* is believed to be regulated by luteinizing hormone (LH; Foresta *et al.* 2004, Bay *et al.* 2006, Ferlin *et al.* 2006a, Cabrol *et al.* 2011, Roth *et al.* 2013) in a chronic, cell differentiation-related manner rather than the acute regulation that LH exerts on testosterone (Bay *et al.* 2005, Anand-Ivell *et al.* 2006a, Bay & Andersson 2011). Both gene expression and protein secretion studies in rodents show that INSL3 reflects a relatively mature Leydig cell phenotype (Ivell *et al.* 2013).

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INSL3 is, along with testosterone, responsible for the hormonal control of the descent of the testes in the male fetus (for reviews see Bay *et al.* (2011) and Hutson (2013)). The trans-abdominal phase of testicular descent is completely dependent on INSL3 due to its trophic effect on the gubernaculum (Zimmermann *et al.* 1999, Kubota *et al.* 2002). This hypertrophy of the ligament along with the growth of the fetus causes the testes to be placed at the superior end of the future inguinal canals. Functional mutations of either the *INSL3* or the *RXFP2* gene causes cryptorchidism in rodents as well as in humans (Nef & Parada 1999, Zimmermann *et al.* 1999, Tomboc *et al.* 2000, Ferlin *et al.* 2003, 2006b, Foresta & Ferlin 2004). The later phase(s) of testicular descent are driven by androgens, although the exact mechanisms are unknown (Hutson 2013).

The physiological role of INSL3 in adult life remains unclear. A role in the maintenance of spermatogenesis by ensuring germ cell survival has, however, been proposed (Kawamura *et al.* 2004), although studies using conditional knockout mice suggest that this is not an essential function (Huang *et al.* 2012). Non-gonadal roles of INSL3 are suggested by the presence of RXFP2 receptors in many tissues other than testis, including epididymis (Anand-Ivell *et al.* 2006b), brain, bone marrow, muscle, kidney, thyroid, and peripheral blood cells (Hsu *et al.* 2002).

Several studies have described serum levels of INSL3 from fetal life to adulthood in humans. In two human studies, INSL3 was detectable in second-trimester amniotic fluid (at the time of the trans-abdominal phase of testicular descent) of male fetuses (Anand-Ivell *et al.* 2008, Bay *et al.* 2008), whereas INSL3 was undetectable in the amniotic fluid of female fetuses (Bay *et al.* 2008). Serum concentrations of INSL3 increase during the postnatal activation of the pituitary–gonadal axis at around 2–3 months of age (a process termed mini-puberty) in healthy boys (Bay *et al.* 2007), as well as in boys with Klinefelter syndrome (Cabrol *et al.* 2011). In healthy adult men, INSL3 levels appear to be relatively stable (Foresta *et al.* 2004, Bay *et al.* 2005) until they decline from aged mid-thirties onwards, probably reflecting a decline in Leydig cell functional capacity (Anand-Ivell *et al.* 2006a). In adults with Klinefelter syndrome, INSL3 levels are low (Foresta *et al.* 2004, Bay *et al.* 2005). Furthermore, serum concentrations of INSL3 do not appear to respond acutely to gonadotropin stimulation in reproductively healthy men once a certain baseline level has been reached (Bay *et al.* 2005, 2006).

To our knowledge, serum concentrations of INSL3 throughout puberty have been investigated in only two previous studies: one cross-sectional study of 75 healthy boys aged 9–17 years (Ferlin *et al.* 2006a) and one longitudinal study of 14 boys with idiopathic short stature (Wikstrom *et al.* 2006). To further elucidate INSL3 levels throughout pubertal transition, we present unique longitudinal data from a pilot study on serum INSL3

concentrations in ten healthy boys before and during puberty in comparison with other reproductive hormones.

Materials and methods

Subjects

This is a secondary analysis of the longitudinal part of the COPENHAGEN Puberty Study (Aksglaede *et al.* 2009, Sorensen *et al.* 2010, Hagen *et al.* 2012, Mouritsen *et al.* 2013), which includes a total of 208 children. Ten boys were selected from the 208 participants based on three criteria: i) Caucasian origin, ii) having at least seven clinical examinations (simultaneous blood sampling was not a prerequisite), three before and four after the onset of puberty, and iii) male gender. Blood sampling and pubertal staging were carried out every 6 months between 2006 and 2011. The researcher selecting the ten boys was blinded to all hormone values. Part of the data (regarding LH, anti-Müllerian Hormone (AMH), and testosterone) has previously been published (Aksglaede *et al.* 2010, Mouritsen *et al.* 2013, 2014).

Clinical examination

Testicular volume was determined by Prader's orchidometer to the nearest milliliter (Prader 1966). Both right and left testicular volumes were recorded, and the larger volume was used as the determinant. Pubertal onset was defined as testicular volume >3 ml at all subsequent examinations. Pubertal staging was according to Tanner (G1–G5).

Statistical analyses

Changes in INSL3 concentrations between one examination before and one following pubertal onset were evaluated using Wilcoxon's signed-rank test.

Hormone assays

Blood was drawn from an antecubital vein in the morning between 0800 and 1000 h. Immediately after blood sampling, the samples were centrifuged and aliquoted into cryotubes, which were stored at -20°C until hormone analyses were performed. We analyzed all blood samples for all reproductive hormones, bar INSL3, in the same laboratory blinded for the technician for pubertal staging. Serum concentrations of follicle-stimulating hormone (FSH) and LH were determined using the time-resolved immunofluorometric assays (Delfia; PerkinElmer, Boston, MA, USA). The limits of detection (LODs) were 0.06 and 0.05 IU/l respectively with intra- and inter-assay coefficients of variation (CV) of <5% respectively in both assays. Serum concentrations of inhibin B were measured using the Beckman Coulter GenII assay with an LOD of 3 pg/ml, and inter-assay CV were <11%. Serum concentrations of AMH were measured using the Immunotech Beckman Coulter enzyme immunometric assay with an LOD of 2 pmol/l, and inter-assay CV were <14%. The AMH samples were diluted (1:10) if the boy was below the age of 13 years. If the concentration in a boy older than 13 was above 150 pmol/l,

the sample was diluted and then reanalyzed: 54 of 93 samples were diluted in this study. Serum concentrations of testosterone were quantified using an in-house TurboFlow-LC-MS/MS method as previously described (Soeborg *et al.* 2013), and serum concentrations of INSL3 were measured using a well-established TRFIA (Anand-Ivell *et al.* 2006a), recently modified for higher sensitivity (Anand-Ivell *et al.* 2013). The LOD was 10 pg/ml, with intra- and inter-assay CV across the range of measurement of <3 and <10% respectively.

Ethical considerations

The Copenhagen Puberty Study (ClinicalTrials.gov ID: NCT01411527) was approved by the Local Ethics Committee (numbers KF 01 282214 and V200.1996/90) and the Danish Data Protection Agency (2010-41-5042). All participants and parents gave informed consent.

Results

Serum concentrations of INSL3 increased with age with large interindividual variability (Fig. 1A) and with pubertal onset (Fig. 1B). The increase in INSL3 concentrations between one examination before and one following pubertal onset was significant ($P=0.009$). Furthermore, INSL3 concentrations increased with genital stages (median INSL3 (ng/ml) (range)): G1 (0.145 (0.036–0.272)), G2 (0.375 (0.118–0.693)), G3 (0.844 (0.367–1.370)), G4 (1.080 (0.896–2.135)), and G5 (1.579 (0.999–1.905)). Testicular volume appeared to increase with both age and puberty (Fig. 1C and D).

Figure 2A, B, C, D, E, F, G, H, I and J illustrates the longitudinal hormone concentrations (INSL3, LH, testosterone, AMH, inhibin B, and FSH) along with testicular volumes for each individual boy. In six of the

ten boys (Fig. 2A, D, E, G, I and J), serum concentrations of LH were the first to increase, at around the time of pubertal onset, followed by an increase in INSL3 concentrations observed at the next examination. In the remaining four boys (Fig. 2B, C, F and H), the increase in LH and INSL3 was observed at the same examination. The increases in serum concentrations of LH, testosterone, and INSL3 was not parallel or in ordered succession and varied interindividually (Fig. 2). All the boys showed a decrease in AMH and increase in inhibin B and FSH following puberty.

Most of the boys went through pubertal transition with hormonal onset (increasing LH and in some cases also testosterone and/or INSL3 concentrations) before clinical pubertal onset. Boy I appeared to progress through puberty slowly, with a clinical onset occurring before significant increases in reproductive hormones. Furthermore, we also noted an intra-individual variability, with testicular volumes decreasing between some clinical examinations (boys A, B, C, D, G and H).

Discussion

This pilot study presents longitudinal measurements of serum INSL3, LH, FSH, testosterone, inhibin B, and AMH in ten healthy boys before and during pubertal transition. Our data provide unique details on the associations between gonadotropins and Leydig and Sertoli cell markers in each boy. Our study suggests that INSL3 may provide additional information on Leydig cell function in pubertal boys compared with the information provided by the measurement of serum testosterone alone.

The purpose of this small pilot study was to look at the intra-individual progress of the reproductive hormones and their interrelationship longitudinally during puberty.

Figure 1 Serum concentrations of insulin-like factor 3 (INSL3) according to age (A) and time to pubertal onset (B). Testicular volume according to age (C) and time to pubertal onset (D) (defined as testicular volume above 3 ml). Each color and corresponding letter (A, B, C, D, E, F, G, H, I and J) represents the same boy in (A), (B), (C) and (D).

Figure 2 Reproductive hormones according to pubertal onset. Each horizontal pair of graphs represents one boy. Left column represents the pituitary–Leydig cell axis (luteinizing hormone (LH), testosterone, and insulin-like factor 3 (INSL3)); right column represents the pituitary–Sertoli cell axis (follicle-stimulating hormone (FSH), inhibin B, and anti-Müllerian hormone (AMH)). Numbers along the INSL3 curves represent individual testicular volumes (ml) at the clinical examination. Letters (A, B, C, D, E, F, G, H, I and J) correspond to letters (and thus the same boy) in Fig. 1.

Thus a major limitation of this study is its small size, which does not allow conclusions to be drawn about interindividual hormone levels, i.e. our data are not as suitable as a general population-based reference.

Our data provide novel and detailed information on the increases in serum concentrations of LH, testosterone, and INSL3 seen throughout puberty in ten healthy boys. There was no discernible pattern in the succession of increase in gonadotropins vs sex steroids and INSL3 around pubertal onset. This is slightly contradictory to cross-sectional data previously published, which suggested that the pubertal increase in INSL3 anticipates the increase in testosterone (Ferlin *et al.* 2006a). Differences in study design, i.e. cross-sectional vs longitudinal, may explain this divergence, as individual ‘increases’ cannot be deduced from such cross-sectional studies, but require a longitudinal design as in our present study. Furthermore, we found that in all boys, serum concentrations of LH increased either before or at the same examination, as does INSL3. As blood sampling was performed every 6 months, we may in some cases have missed the LH surge. Our findings are indicative of INSL3 being chronically regulated by LH

throughout childhood and adolescence, which supports the conclusions of previous studies. Several studies have found positive correlations, some significant, between LH and INSL3 concentrations (Foresta *et al.* 2004, Bay *et al.* 2005, Ferlin *et al.* 2006a, Cabrol *et al.* 2011). Furthermore, INSL3 concentrations in serum increase upon long-term stimulation (weeks or months) with human chorionic gonadotropin in gonadotropin-suppressed men, and decrease in response to gonadotropin suppression, further supporting the chronic regulation of INSL3 by LH (Bay *et al.* 2006, Roth *et al.* 2013).

The fact that the pubertal increases in INSL3 and testosterone seen in this study were non-parallel could indicate that although both hormones may be regulated by LH, the regulation may differ. It is believed that LH has a non-acute, long-term effect on Leydig cells, promoting their differentiation to a more mature phenotype (Ivell *et al.* 2013), and thus a chronic effect on the production of INSL3, as opposed to the acute regulation of testosterone (Bay *et al.* 2005). Together with the observed constitutive expression in cultured Leydig cells (Sadeghian *et al.* 2005), and the high within-individual consistency (<10% variation) of INSL3 measurements

in men over periods of several months (R Anand-Ivell and R Ivell, unpublished data), this implies that INSL3 is not subjected to the acute variations known for testosterone (Ivell & Anand-Ivell 2009, Bay *et al.* 2011). If that is the case, it is unlike testosterone (and inhibin B), for which substantial diurnal patterns have been described (Resko & Eik-nes 1966, Veldhuis *et al.* 1987, Carlsen *et al.* 1999, Ankarberg-Lindgren & Norjavaara 2004). Thus, INSL3 may be a valuable supplement to testosterone as a biomarker of Leydig cell function. The value of serum INSL3 is especially obvious in the case of pubertal induction with mild androgen supplementation in a boy with delayed puberty, as a serum testosterone measurement would not distinguish between endogenous and exogenous testosterone.

The increase in INSL3 concentrations seen between 3 months before and 3 months following clinical pubertal onset was significant. We furthermore observed an increase in INSL3 concentrations between all genital stages from G1 to G5. These findings could signify that measurements of serum INSL3 can be used as a predictor of puberty onset and progression, although the limited size of our study needs to be kept in mind. Significant increases in serum levels of LH, testosterone, and INSL3 between Tanner stages G1 and G2 have also been reported in a former study (Wikstrom *et al.* 2006). Previously, early morning testosterone has been shown to be a fairly accurate predictor of imminent puberty (Wu *et al.* 1993). Another study suggested that significant increases in the concentrations of LH in morning urine occur 1–2 years before clinical onset of puberty (Demir *et al.* 1996). A recent study found that urinary concentrations of LH and FSH at the age of 9 were able to predict Tanner stages at the age of 12 (Koenis *et al.* 2013). A study similar to these focusing on the applicability of INSL3 as a forecaster is, however, necessary to investigate the potential significance of INSL3.

While this pilot study provides detailed information on the pituitary–Leydig cell axis and on Leydig cell maturation, we also present similar, detailed information on the pituitary–Sertoli cell axis (FSH, inhibin B, and AMH) and Sertoli cell maturation. We observed the expected increase in FSH concentrations, followed by a decrease in the serum concentration of AMH and an increase in the serum concentration of inhibin B around the onset of clinical puberty, as previously published (Aksglaede *et al.* 2010). This underlines that the ten boys in fact are healthy and seem to progress through puberty as expected.

To determine testicular volume and thereby clinical pubertal onset in this study, Prader's orchidometer was used. Intra-individual variability in testicular volumes was observed. Boy I, for example, seemed to progress through puberty slowly, which may be explained by an inaccuracy in the assessment of testicular volumes. The boy may in fact have had his pubertal onset at the time of pituitary–gonadal activation, as would be expected.

Although an actual decrease in testicular volume between examinations cannot be ruled out, it is more likely that the observed variability is due to observer variations when using the orchidometer. The inaccuracy of Prader's orchidometer is well described in the literature, although varying degrees of inter- and intra-observer biases have been found (Rivkees *et al.* 1987, Behre *et al.* 1989, Tatsunami *et al.* 2006), and some authors did not find large variations (Karaman *et al.* 2005). Ultrasonography is reported as a far more accurate and reproducible way of measuring testicular volume (Rivkees *et al.* 1987, Behre *et al.* 1989, Sakamoto *et al.* 2007, Lin *et al.* 2009), but is limited by its impracticality. Our study took place at two schools rendering it difficult and impractical to determine testicular volumes by ultrasound.

In conclusion, this small but unique study showed longitudinal data on serum concentrations of INSL3, LH, FSH, AMH, testosterone, and inhibin B during pubertal transition in ten healthy boys. INSL3 levels were tightly and significantly linked to pubertal onset, the LH surge, and also to increasing testicular volume. The increase in serum concentrations of LH, INSL3, and testosterone during pubertal transition was not parallel, thus suggesting different regulating mechanisms and indicating a potential clinical value of INSL3 as a biomarker of Leydig cell function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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