INSL3 Levels in Second Trimester Amniotic Fluid

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Abstract

Background: According to animal studies, the testicular Leydig cell hormone insulin-like factor 3 (Insl3) exerts a fundamental role in abdominal testis translocation, which occurs in the beginning of second trimester in humans. Despite this, human prenatal INSL3 production has been poorly investigated.

Methods: Amniotic fluid from 91 pregnant women undergoing amniocentesis was analysed for INSL3 and testosterone levels. Data were related to gestational age (15-25 weeks) at the time of amniocentesis and to sex (45 males and 48 females).

Results: INSL3 was present in amniotic fluid from all but one of the investigated male fetuses (range: <0.02-0.36 ng/ml, mean±sd: 0.12±0.07 ng/ml), whereas the hormone was undetectable in the female fetuses. Testosterone was significantly higher in male (range: 0.54-1.71 nmol/l, mean±sd: 1.04±0.30 nmol/l) as compared to in female amniotic fluid (range: 0.19-0.50 nmol/l, mean±sd: 0.34±0.06 nmol/l) ($p < 0.001$). In males, there was no correlation between INSL3 and testosterone. A statistically borderline negative association was found between INSL3 and gestational age ($p = 0.07$), whereas the corresponding association was not significant for testosterone ($p = 0.12$). In contrast, testosterone in females correlated positively with gestational age ($p = 0.02$).

Conclusion: INSL3 is clearly present in human male amniotic fluid in second trimester, where abdominal testis translocation takes place. In contrast, the hormone is undetectable in female amniotic fluid. Prenatal presence of INSL3 supports the hypothesis that this hormone is essential for testicular descent in humans.

Introduction

Abdominal testis translocation occurs in early second trimester in humans (1). According to animal studies, the Leydig cell hormone insulin-like factor 3 (Insl3) is essential for this first part of testicular descent (2). A single rodent study has also suggested that postnatal Insl3 expression is associated with germ cell survival (3).

Human INSL3 serum levels have been described from early infancy throughout childhood, puberty and adulthood (4-7). However, even though the most well characterised function of INSL3 is exerted *in utero*, human prenatal INSL3 levels have only been characterised in a single recent study (8). We have therefore taken the approach to analyse INSL3, along with testosterone (T), levels in amniotic fluid from pregnant women. Amniocentesis is normally performed in early second trimester, a time period that overlaps substantially with testicular development and testicular descent in the male fetus (9).

Insl3 mediates the outgrowth of the ligament gubernaculum, thereby facilitating the anchoring of the testes to the abdominal wall, close to the inguinal canal (10). Hence, *Insl3* knockout mice appear with testes located intraabdominally, due to an undifferentiated gubernaculum (2). Following the abdominal translocation of the testes, which may be completed already at gestational week 15 in humans, the subsequent transinguinal and inguinoscrotal migrations are responsible for the passage of the testes through the inguinal canal and further down to the bottom of the scrotum (1). These two processes have traditionally been regarded as a single inguinoscrotal phase mediated primarily by androgens. Prior to the transinguinal migration of the testes, a timely pause is present during which the part of the gubernaculum embedded in the abdominal wall undergoes extensive remodelling/enlargement, thereby dilating the inguinal canal, making room for the testes to pass through (1).

Incomplete descent of the testes, cryptorchidism, is one of the most common congenital malformations in infant boys (11), and a risk factor for reduced semen quality and development of testicular cancer in adult life (12). It has been hypothesized that these male reproductive health problems often share a common fetal origin and may be related to prenatal exposure to endocrine disrupting chemicals, e.g. phthalate esters (13). Interestingly, rodent studies have shown that gubernacular lesions and cryptorchidism, induced by prenatal exposure to phthalate esters, is associated with reduced *Insl3* expression (14).

Also adult female ovarian theca cells have been reported to produce INSL3 (15), although in minimal amounts as compared to male INSL3 production. The hormone is undetectable in amniotic fluid from human female fetuses (8).

In this paper we present INSL3 levels in human second trimester amniotic fluid, which provide information of the potential importance of this hormone in human testicular descent.

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Materials and methods

Subjects

Pregnant women undergoing amniocentesis at the University Hospitals of Copenhagen (Rigshospitalet and Hvidovre Hospital) in 2007 were invited to participate. In Denmark, pregnant women are offered a first trimester nuchal translucency scanning including biochemistry for detection of chromosomal abnormalities. In cases of increased risk, an amniocentesis is offered. Karyotyping on amniotic fluid cells were performed at Rigshospitalet. For the present study, only redundant supernatant not necessary for karyotyping was utilized. Available information included maternal age, gestational age at time of amniocentesis (based on ultrasonography) and genetic test outcome. A total of 91 women participated in our study; of these, three had a dichorionic twin pregnancy, giving a total of 94 fetuses (46 males, 48 females). A single male fetus had a trisomy 18 and was excluded from this study. Twin fetuses were included. All women had their amniocentesis performed in gestational week 15-25 (week 15-21 for male fetuses and 15-25 for female fetuses). Written informed consent was given by all participants. The study was approved by the local ethical committee (KF-01-259468, KF-11-2006-4093).

Hormone analysis

Amniotic fluid was stored at –20 C. INSL3 concentrations in amniotic fluid were determined using a human INSL3-specific fluorescence immunoassay (for details, see (4)). To adapt to amniotic fluid matrix, female amniotic fluid was used as new blank (female amniotic fluid has been reported not to contain detectable amounts of INSL3 (8) and the female amniotic fluid used as blank in the present study was undetectable when analysed under traditional assay conditions). Standards consisted of 100 µl blank spiked with defined concentrations of synthetic human INSL3 (0.02–1.60 ng/ml). The detection limit of this set up was 0.02 ng/ml. Intraassay coefficient of variance was 0.9-48.8% in the very low range (0.2-0.8 ng/ml) and 0.1-18.9% in the higher range (0.9-1.6 ng/ml). All samples were analysed in a single assay run.

Testosterone was measured by liquid-chromatography mass-spectrometry (LC-MS) (16) at Statens Serum Institute using an accredited method. The limit of detection was 0.01 nmol/l.

Statistical analyses

Data are presented as range and mean±sd. In male amniotic fluid, INSL3 and T levels adhered to normal distribution. INSL3 and T correlation was investigated by Pearson's Correlations. The influence of gestational age and maternal age on INSL3 and T was tested using linear regression. In female amniotic fluid, T levels adhered to normal distribution after Ln transformation. In females, the influence of gestational age and maternal age on Ln T was tested using linear regression. Gender differences in T levels were examined using t-test.

Results

Male amniotic fluid: All but one male amniotic fluid samples had measurable INSL3 levels (range: <0.02-0.36 ng/ml, mean±sd: 0.12±0.07 ng/ml) and all had measurable T levels (range: 0.54-1.71 nmol/l, mean±sd: 1.04±0.30 nmol/l). There was no correlation between INSL3 and T (p=0.98). In the linear regression analyses, there was a borderline negative association between INSL3 and gestational age (p=0.07), whereas the negative association was not significant between T and gestational age (p=0.12). Neither of the Leydig cell hormones was associated to maternal age at time of amniocentesis (p=0.11 for INSL3 and p=0.28 for T).

Female amniotic fluid: INSL3 was unmeasurable (<0.02 ng/ml), whereas T was measurable (range: 0.19-0.50 nmol/l, mean±sd: 0.34±0.06 nmol/l) in all female amniotic fluid samples. A significant positive association was found between Ln T and gestational age (p=0.02). There was no association between Ln T and maternal age (p=0.35). T levels were significantly higher in male compared to in female amniotic fluid (p<0.001).

Discussion

We report clearly measurable INSL3 levels in amniotic fluid from human male fetuses of gestational age 15-21 weeks. The observed borderline negative correlation between INSL3 and gestational age suggests that INSL3 concentrations in amniotic fluid may peak at or before gestational week 15. Thus, INSL3 is produced in human fetal Leydig cells during second trimester, at the time of gubernacular outgrowth and abdominal testis translocation. In contrast, all samples from human female fetuses were negative for INSL3, confirming that human fetal ovaries do not express INSL3 in amounts detectable in amniotic fluid. Notably, the concentration range of amniotic fluid INSL3 in male fetuses reported here (<0.02-0.36 ng/ml) is similar to the ranges found in male cord blood (<0.05-0.39 ng/ml) and serum from 3-month-old boys (<0.05-0.35 ng/ml) (7).

Our findings are in accordance to data presented in the recent study of INSL3 levels in human amniotic fluid (8), in that they also found INSL3 levels to decline with increasing gestational age in males. However, despite usage of the same antibodies and detection limits, Anand-Ivell et al reported INSL3 to be undetectable in 32% of male amniotic fluid samples (8), whereas only a single sample was undetectable in the present study. This discrepancy is most likely due to our adaptation of the assay to amniotic fluid rather than biological differences in the samples analysed.

Importantly, our data indicate high INSL3 production also after week 15, where the testes may already be anchored close to the inguinal canal (1). This renders it possible that INSL3 is also involved in the subsequent process of dilating the inguinal canal via gubernacular enlargement, thereby facilitating the transinguinal testis migration. This theory is further supported by the phenotype of transgenic female mice overexpressing Insl3. Such mice are characterised not only by ovary descent, but also development of the processus vaginalis (17). In this regard, analysis of INSL3 amniotic fluid levels throughout embryogenesis would have been optimal. However, of note is the presence of INSL3 in male cord blood (7), indicating a continued production of INSL3 throughout the remaining fetal development.

Cryptorchidism in humans is predominantly of the mild type, involving only the latter, androgen dependent, inguinoscrotal part of testicular descent (11). However, reduced INSL3 expression could theoretically cause a delay in the entire process of gubernacular outgrowth and testicular descent, thereby also affecting the final inguinoscrotal phase. Indeed, many boys born with cryptorchidism have spontaneous testis descent within the first 3 months of life (11), suggesting that process delay could be a contributing factor.

Maximal gender differences in amniotic T levels were reported to occur around gestational week 12-18 (18). In agreement with this, we found markedly higher T levels in male as compared to female amniotic fluid. Amniotic fluid T concentrations in the two sexes should become similar with increasing age, as testicular T biosynthesis in the male decreases and T synthesis from non-testicular sources increases in both male and female fetuses. Indeed, we found a significant positive correlation between amniotic T levels and gestational

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age in female and a tendency towards a negative corresponding association in male amniotic fluid, resulting in T levels approaching each other in the two sexes towards gestational week 20-25.

Numerous factors influence hormone levels in amniotic fluid, including fluid volume, hormone stability and fetal growth relative to growth of the hormone-producing organ. In a recent study of developmental changes in human fetal testes (9), the exponential increase in Leydig cell numbers registered in the first part of second trimester slowed down after gestational week 15. Gene expression related to INSL3 and T production was constant during second trimester. In comparison, Sertoli and germ cell numbers and testis volume in general increased exponentially through the entire second trimester (9). Thus, the negative association between gestational age and INSL3 (and T) levels in amniotic fluid reported here may reflect constant levels of expression per Leydig cell combined with a decreased Leydig cell/testis volume ratio. Furthermore, amniotic fluid volume increases approximately three fold between gestational weeks 15 and 22 (19). No exact data are available on the stability of INSL3 in amniotic fluid. However, amniotic fluid concentrations of the structurally related hormones insulin-like growth factors I and II represent very well fetal serum levels of the same hormones (20).

In conclusion, INSL3 was clearly present in human second trimester male amniotic fluid. In contrast, the hormone was absent from female amniotic fluid. Presence of INSL3 in male fetuses suggests that the hormone is involved in the abdominal testis translocation in humans. Continued production of INSL3 after gestational week 15 could suggest an involvement of the hormone in the modification of the inguinal canal through continued enlargement of the gubernaculum, thereby facilitating also the subsequent transinguinal testis migration.

References

1. **Amann RP, Veeramachaneni DN** 2007 Cryptorchidism in common eutherian mammals. Reproduction 133:541-561

2. **Nef S, Parada LF** 1999 Cryptorchidism in mice mutant for Insl3. Nat Genet 22:295-299

3. **Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, H Morita, Toppari J, Fu P, Wade JD, Bathgate RA, Hsueh AJ** 2004 Paracrine regulation of mammalian oocyte maturation and male germ cell survival. Proc Natl Acad Sci U.S.A 101:7323-7328

4. **Bay K, Hartung S, Ivell R, Schumacher M, Jurgensen D, Jorgensen N, Holm M, Skakkebaek NE, Andersson A-M** 2005 Insulin-like factor 3 serum levels in 135 normal men and 85 men with testicular disorders: relationship to the luteinizing hormone-testosterone axis. J Clin Endocrinol Metab 90:3410-3418

5. **Ferlin A, Garolla A, Rigon F, Rasi CL, Lenzi A, Foresta C** 2006 Changes in serum insulin-like factor 3 during normal male puberty. J Clin Endocrinol Metab 91:3426-3431

6. **Anand-Ivell R, Wohlgemuth J, Haren MT, Hope PJ, Hatzinikolas G, Wittert G, Ivell R** 2006 Peripheral INSL3 concentrations decline with age in a large population of Australian men. Int J Androl 29:618-626

7. **Bay K, Virtanen HE, Hartung S, Ivell R, Main KM, Skakkebaek NE, Andersson A-M, Toppari J** 2007 Insulin-like factor 3 levels in cord blood and serum from children: effects of age, postnatal hypothalamic-pituitary-gonadal axis activation, and cryptorchidism. J Clin Endocrinol Metab 92:4020-4027

8. **Anand-Ivell R, Ivell R, Driscoll D, Manson J** 2008 Insulin-like factor 3 Levels in Amniotic Fluid of Human Male Fetuses. Human Reproduction *in press*

9. **O'Shaughnessy PJ, Baker PJ, Monteiro A, Cassie S, Bhattacharya S, Fowler PA** 2007 Developmental Changes in Human Fetal Testicular Cell Numbers and mRNA Levels During the Second Trimester. J Clin Endocrinol Metab 92:4792-4801

10. **Kubota Y, Nef S, Farmer PJ, Temelcos C, Parada LF, Hutson JM** 2001 Leydig insulin-like hormone, gubernacular development and testicular descent. J Urol 165:1673-1675

11. **Boisen KA, Kaleva M, Main KM, Virtanen HE, Haavisto AM, Schmidt IM, Chellakooty M, Damgaard IN, Mau C, Reunanen M, Skakkebaek NE, Toppari J** 2004 Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries. Lancet 363:1264-1269

12. **Kanto S, Hiramatsu M, Suzuki K, Ishidoya S, Saito H, Yamada S, Satoh M, Saito S, Fukuzaki A, Arai Y** 2004 Risk factors in past histories and familial episodes related to development of testicular germ cell tumor. Int J Urol 11:640-646

13. **Skakkebaek NE, Rajpert-De Meyts E, Main KM** 2001 Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 16:972-978

14. **Wilson VS, Lambright C, Furr J, Ostby J, Wood C, Held G, Gray LE** 2004 Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. Toxicol Lett 146:207-215

15. **Balvers M, Spiess AN, Domagalski R, Hunt N, Kilic E, Mukhopadhyay AK, Hanks E, Charlton HM, Ivell R** 1998 Relaxin-like factor expression as a marker of differentiation in the mouse testis and ovary. Endocrinology 139:2960-2970

16. **Cawood ML, Field HP, Ford CG, Gillingwater S, Kicman A, Cowan D, Barth JH** 2005 Testosterone Measurement by Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry: Validation of a Method for Routine Clinical Practice. Clinical Chemistry 51:1472–1479

17. **Adham IM, Steding G, Thamm T, Bullesbach EE, Schwabe C, Paprotta I, Engel W** 2002 The overexpression of the insl3 in female mice causes descent of the ovaries. Mol Endocrinol 16:244-252

18. **Finegan JA, Bartleman B, Wong PY** 1989 A window for the study of prenatal sex hormone influences on postnatal development. J Genet Psychol 150:101-112

19. **Beall MH, van den Wijngaard JP, van Gemert MJ, Ross MG** 2007 Amniotic fluid water dynamics. Placenta 28:816-823

20. **Reece EA, Wiznitzer A, Le E, Homko CJ, Behrman H, Spencer EM** 1994 The relation between human fetal growth and fetal blood levels of insulin-like growth factors I and II, their binding proteins, and receptors. Obstet Gynecol 84:88-95
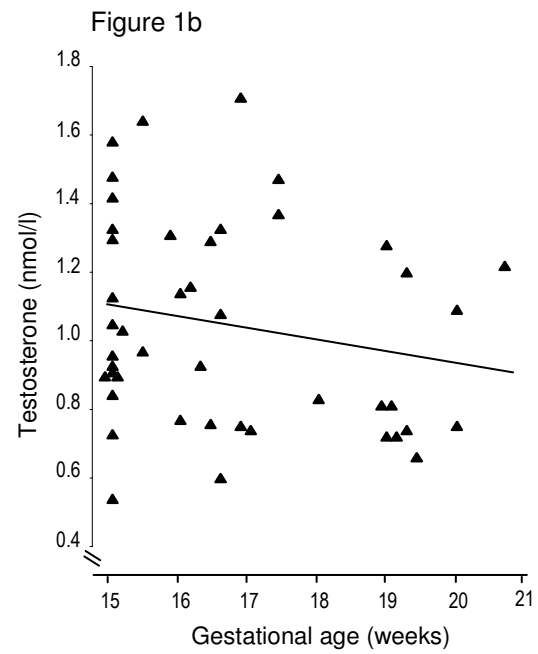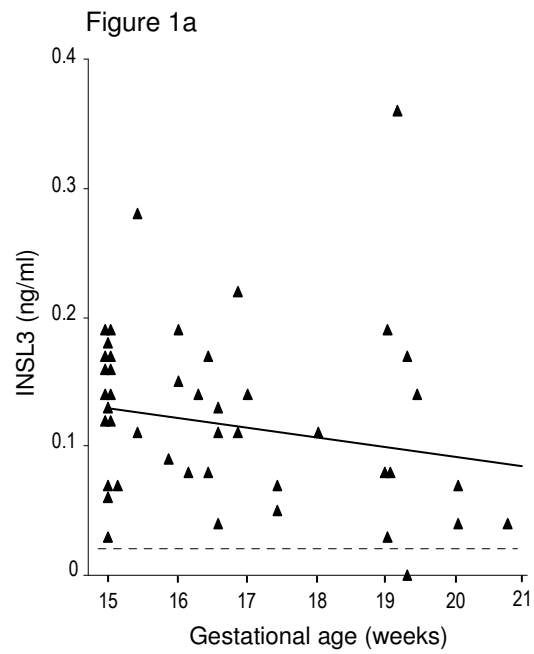
Figure 1

Scatter plots of the two Leydig cell hormones INSL3 (1a) and testosterone (1b) against gestational age at the time of amniocentesis in male amniotic fluid. Plotted are also lines of tendency. The dotted line in (a) indicate the detection limit for INSL3 (0.02 ng/ml).
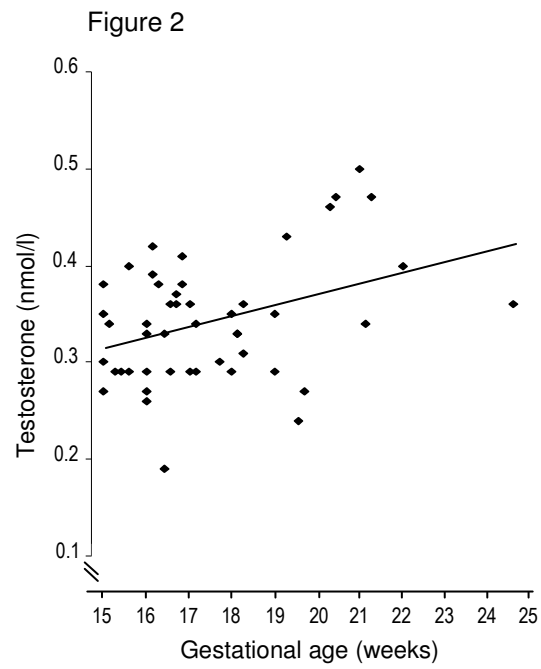
Figure 2

Figure 2

Scatter plot of testosterone levels against gestational age at the time of amniocentesis in female amniotic fluid. Plotted is also a line of tendency.