Insulin-like factor 3 levels in amniotic fluid of human male fetuses

Ravinder Anand-Ivell1, Richard Ivell1,4, Deborah Driscoll2 and Jeanne Manson3

1School of Molecular and Biomedical Science, University of Adelaide, Adelaide, 5005 SA, Australia; 2Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA, USA; 3Division of Human Genetics and Molecular Biology, Department of Pediatrics, University of Pennsylvania, Philadelphia, PA, USA

BACKGROUND: Rodent studies suggest that the peptide hormone insulin-like factor 3 (Insl3) made by the fetal testis is responsible for the first transabdominal phase of testicular descent, and may be affected by xenobiotics to disrupt male reproductive tract development. To date, there is very little information on the production of INSL3 by the human fetus during gestation. The objective of the present study was to determine the concentrations and time course during pregnancy of INSL3 and testosterone production in human fetuses and their associations with maternal characteristics, pregnancy complications and outcome. METHODS: This is a retrospective cohort study in which women who contributed amniotic fluid specimens to a bank from 2003–2006 were followed to determine their pregnancy complications and pregnancy outcome. Amniotic fluid specimens were collected from the Reproductive Genetics Laboratory of the Hospital of the University of Pennsylvania subsequent to routine amniocentesis. INSL3 and total testosterone levels were measured in amniotic fluid (from n = 50 female, n = 237 male fetuses) by validated immunoassays and correlated with maternal characteristics, pregnancy complications and outcome. RESULTS: INSL3 was only detectable in amniotic fluid from male fetuses, and highest levels occurred from weeks 15–17 of gestation. INSL3 concentration was positively associated with increased birth weight, the occurrence of pre-eclampsia and advanced maternal age, but not with testosterone levels. CONCLUSIONS: INSL3 concentration in human amniotic fluid is potentially predictive of fetal sex and pre-eclampsia, and presumably reflects the functioning of the fetal Leydig cell population.

Keywords: insulin-like factor 3; amniotic fluid; sex determination; pre-eclampsia

Introduction

Insulin-like factor 3 (INSL3) is a member of the insulin/relaxin family of peptide hormones. However, unlike insulin, INSL3 signals through a G-protein coupled receptor, RXFP2 (previously known as LGR8 or Great) (Bathgate et al., 2006), distantly related to the receptors for LH and FSH. In the adult testis, the INSL3/RXFP2 system appears to represent a paracrine hormone-receptor system that conveys information about Leydig cell status to germ cells, and is possibly part of an autocrine feedback loop (Anand-Ivell et al., 2006a; Feng et al., 2007). Mature Leydig cells are the principal source of INSL3, and adult men have circulating concentrations between 0.5 and 2 ng/ml (Foresta et al., 2004; Bay et al., 2005; Anand-Ivell et al., 2006b). In the adult male, this peptide hormone is believed to have a protective, anti-apoptotic effect on male germ cell survival, and production levels are maintained consistently and constitutively (Kawamura et al., 2004; Anand-Ivell et al., 2006b). In the fetal rodent, Insl3 expression is associated with the transabdominal phase of testicular descent; ablation of the Insl3 gene or that of its receptor Rxfp2 leads primarily to cryptorchidism (Nef and Parada, 1999; Feng et al., 2004). The fetal expression of Insl3 does not require gonadotropins as hpg-mutant mice (hypogonadal mice), which lack GnRH and a functional hypothalamic–pituitary–gonadal axis, express the Insl3 gene (Balvers et al., 1998). Accordingly, both hpg mice and LH receptor knockout mice have normal transabdominal testicular descent, but the inguino-scrotal phase, which depends upon androgens, is blocked (Yuan et al., 2006). INSL3 gene expression is altered in relation to the differentiation status of Leydig cells; it is most highly expressed in the normal adult testes, and is reduced in prepubertal and aging rats, sexually inactive seasonal breeders and in dedifferentiated Leydig cell tumors (Ivell and Bathgate, 2002). The proximate intracellular pathways activated by INSL3 have not been identified, and the precise role of this peptide hormone remains unknown.

Exposure of pregnant rodents to estrogens (diethylstilbestrol) or phthalate esters results in a down-regulation of Insl3...
expression in the fetal Leydig cells and an inhibition of the transabdominal descent of the testes (Emmen et al., 2000; Wilson et al., 2004; McKinnell et al., 2005; Borch et al., 2006). The molecular mechanism of such xenobiotic action in vivo is largely unknown. In a population of persistently cryptorchid boys, the INSL3 concentration in cord blood appears to be reduced, and the LH/INSL3 ratio in serum was elevated at 3 months of age compared with healthy male infants (Bay et al., 2007). These findings suggest that INSL3 may serve as a marker of testicular function in infants, particularly during the transient activation of the hypothalamic–pituitary axis in the first 3 months of age. The onset of puberty is associated with a marked increase in peripheral INSL3 concentration, with adult levels being achieved at an age of 13–14 years (Wikstrom et al., 2006). During pubertal development, there is a strong positive association between INSL3 concentration and serum LH and testosterone, as well as testicular development, reflecting the increasing differentiation of the Leydig cells.

Mutations in the INSL3 gene or of its receptor, RXFP2, are exceedingly rare in the etiology of human cryptorchidism (Feng et al., 2004). This would suggest that defective differentiation of the fetal Leydig cell is largely responsible for cryptorchidism, rather than any direct influence on INSL3 production itself (Toppari et al., 2007). Measurement of INSL3 production in the human fetus could nonetheless be predictive of the potential for cryptorchidism as well as for exposure to endocrine disrupting chemicals that produce cryptorchidism in animal models. To date, however, there is no information available on INSL3 expression in the human fetus during this period of sexual differentiation. In the present study, we have utilized amniotic fluid samples from an amniocyte bank to characterize human fetal production of INSL3 and testosterone in mid-pregnancy. Amniocentesis is performed primarily from 14–18 weeks of gestation, which is within the critical period for sexual differentiation of the CNS and the reproductive tract (Knickmeyer and Baron-Cohen, 2006). Of all available sources, including maternal serum and cord blood, amniotic fluid has been identified as the best matrix to investigate the influence of any agent or condition that affects fetal androgen levels (van de Beek et al., 2004). Our results indicate that INSL3 can be measured in amniotic fluid of male fetuses only, and that there is no correlation with levels of amniotic testosterone. INSL3 levels were also found to be elevated in association with increased birth weight, the occurrence of pre-eclampsia and advanced maternal age.

Materials and Methods

Subjects

The amniotic fluid samples utilized in the present study were derived from a bank of amniocytes and amniotic fluid samples collected from women undergoing amniocentesis for routine indications. Subjects were drawn from five local hospitals or satellite clinics that are part of the University of Pennsylvania Health System, and consent was obtained from subjects at the time of amniocentesis to contribute samples to the bank. Once medically indicated tests were complete, back-up cultures and remaining supernatants from the amniocentesis specimens were collected from the Reproductive Genetics Laboratory at the Department of Obstetrics and Gynecology, Hospital of the University of Pennsylvania. Reproductive genetics chart reviews were carried out on consented subjects, and women were followed forward to obtain information on complications during pregnancy and delivery, and the outcome of their pregnancy.

Questionnaires were mailed out to each subject at the expected delivery date to obtain information on pregnancy outcome; subjects not returning questionnaires were called by a genetic counselor and information obtained via telephone interview. This study was approved by the Institutional Review Boards of the University of Pennsylvania as well as The Children’s Hospital of Philadelphia.

Database elements for subjects in the amniocyte bank comprised the following items:

(i) Patient enrollment: race, ethnicity, age, last menstrual period (LMP) date, personal identifiers
(ii) Reproductive genetics intake: reproductive history, indications for the procedure
(iii) Reproductive genetics chart review: first, second trimester screening tests, genetic findings from chorionic villus sampling, amniocentesis and peripheral blood analysis, as well as findings from Level I and II ultrasound
(iv) Pregnancy outcome: complications during pregnancy and delivery, live birth, fetal death, miscarriage, elective termination and any abnormality detected in these outcomes.

Hormone analyses

In the present study, a total of 250 amniotic fluid samples from male fetuses and 50 from female fetuses were randomly selected from pregnancies with a live birth outcome. Twin pregnancies and those with known chromosomal abnormalities were excluded. Total testosterone levels were measured in amniotic fluid using a semi-competitive time-resolved fluorescence immunoassay (TRFIA) modified from a previous enzyme-linked immunosorbent assay format (Paust et al., 2002; Anand et al., 2003). Since amniotic fluid contains no sex hormone-binding globulin, samples were subjected to a simple cold ethanol extraction procedure using 100 μl samples of amniotic fluid, as previously described (Paust et al., 2002; Anand et al., 2003). The range of detection for this assay was 0.06–14.58 ng/ml testosterone. Control experiments yielded 83.0 ± 13.2% recovery of testosterone standards spiked into amniotic fluid, representing low, medium and high values, similar to those obtained with an ether extraction procedure. The intra-assay and inter-assay coefficients of variation (CV) were <7 and <16%, respectively. Cross-reactivity of the assay was 42% for dihydrotestosterone, 1.6% for androstenedione, 1.7% for 17ß-dihydroandrosterone and <0.01% for a wide range of other steroids tested.

Human INSL3 levels in amniotic fluid were measured using a semi-competitive TRFIA, as previously described (Anand-Ivell et al., 2006b). Time-resolved fluorescence was measured by pulsing the sample for 1000/s with an excitation light of 340 nm and measuring the intensity of the emitted light at 615 nm. Intra-assay and inter-assay CVs were <9 and <11%, respectively, for all measured concentrations. Incubation parameters were adjusted to allow a lower limit of detection (LOD) of 20 pg/ml.

Statistical analysis

For samples with INSL3 levels below the LOD of 20 pg/ml, the LOD was divided by the square root of 2 to yield a value of 14.1 pg/ml. INSL3 and testosterone concentrations were log transformed prior to statistical analysis to better meet the assumptions of normal
distribution for the statistical tests employed. Gestational age at the
time of amniocentesis was calculated from the Level II ultrasound
date; this date was found to be more accurate and to represent the
typical periods during gestation when amniocentesis is conducted
than the LMP date. A partial correlation test was employed to
compute the linear relationship between INSL3 and testosterone,
while controlling for gestational age at amniocentesis. Univariate
linear regression analysis was first conducted to assess the relationship
between INSL3 or testosterone levels and potential covariates derived
from the amniocyte database. Multivariable linear regression analysis
was then conducted on those parameters which yielded a significant
association in univariate analysis (maternal age, length of gestation,
birth weight and complications during pregnancy) to determine if
they were independent predictors of INSL3 or testosterone levels.
These latter analyses were controlled for gestational age at the time
of amniocentesis.
A value of \( P < 0.05 \) was considered significant.

**Results**

INSL3 levels were not measurable in any of the 50 amniotic fluid
samples obtained from female fetuses. Of the 250 samples ana-
lyzed from male fetuses, karyotype abnormalities were found in
13, leaving a total of 237 samples for evaluation. Among the 237
samples, INSL3 levels were above the LOD (20 pg/ml) in 161,
or 68% of samples. Table I contains data on the percentage of
samples with non-detectable levels of INSL3 by gestational
age at amniocentesis, and Fig. 1 is a graph of all of the INSL3
data. From these results it appears that INSL3 was not detectable
at 22 weeks and later in gestation, which may reflect the actual
time course of INSL3 production by the fetal Leydig cells,
although the number of samples at 24–31 weeks is limited.
The cause of the relatively high percentage of samples with non-
detectable INSL3 levels at earlier times in gestation (15–21
weeks) is not readily apparent.

Testosterone levels (ng/ml) were measurable in all but 2 of
237 samples obtained from male fetuses and in all 50 samples
from female fetuses. The mean, SD and 95% confidence inter-
vals (CI) for these values in male fetuses were 0.52 ± 0.17 ng/
ml (0.50–0.54) and for female fetuses, 0.30 ± 0.09 ng/ml
(0.27–0.32), and results are plotted in Fig. 2. Testosterone
levels were significantly different between male and female
fetuses by analysis of variance (\( P < 0.001 \)). Levels in male
fetuses remained relatively stable from 15 weeks of gestation
through to 22 weeks. Testosterone concentrations for female
fetuses were also relatively stable through the sampling
period. This time course is quite different from that observed
for INSL3, where levels were highest at 15 weeks and then
sharply declined by 22 weeks. The relationship between
INSL3 and testosterone levels in male fetuses was evaluated
by computing a partial correlation coefficient while controlling
for gestational age at amniocentesis. There was no significant
correlation between these two hormones \( (P = 0.85) \) (data not
shown).

Attempts were made to determine the most appropriate para-
ter to use in normalizing INSL3 and testosterone levels. The

<table>
<thead>
<tr>
<th>Gestational age at amniocentesis (weeks)</th>
<th>No. samples analyzed</th>
<th>No. samples with non-detectable INSL3</th>
<th>% Non-detectable</th>
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<td>13</td>
<td>1</td>
<td>0</td>
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</tr>
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<td>3</td>
<td>2</td>
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<td>31</td>
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<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>237</td>
<td>76</td>
<td>32</td>
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</table>

![Figure 1: Insulin-like factor 3 (INSL3) concentrations in male fetuses by gestational age at amniocentesis. Samples at 13 and 24–31 weeks are represented by single data-points only. Means ± SEM](image1.png)

![Figure 2: Testosterone concentrations in male (squares) and female (circles) fetuses by gestational age at amniocentesis. Samples at 13 (0.43 ng/ml), 30 (0.68 ng/ml) and 31 (0.76 ng/ml) weeks are represented by single data-points for male fetuses only and are excluded from this figure. Means ± SEM](image2.png)
influence of gestational age at amniocentesis determined from the Level II ultrasound date, amniotic fluid alpha fetoprotein levels (AFAFP), and fetal weight determined from the Level II ultrasound were examined as covariates in linear regression analysis with the log of INSL3 levels as the dependent variable. Gestational age at amniocentesis was the most significant predictor of INSL3 levels ($P < 0.001$) while AFAFP levels were marginally associated ($P = 0.07$) and fetal weight was not significantly associated with INSL3 ($P = 0.90$). A similar approach was taken with amniotic testosterone levels. Gestational age at amniocentesis was significantly associated ($P = 0.03$) with testosterone levels, but AFAFP and fetal weight were not ($P = 0.51$ and 0.89, respectively). Consequently, in all subsequent analyses, gestational age at amniocentesis was used as a covariate in examining the relationship between INSL3 and testosterone and other indicators of pregnancy complication and adverse outcome.

In unadjusted linear regression analysis, a variety of parameters were explored for their relationship to the log of the INSL3 level (dependent variable). There was no significant relationship between maternal race and ethnicity, whereas there was a significant positive relationship with maternal age at amniocentesis ($P = 0.001$) (Fig. 3A). INSL3 levels were significantly higher across all gestational ages in women over 38 years of age than those younger than 36 years of age ($P = 0.03$) and 34 years of age ($P = 0.01$). There were no significant relationships between any of the reproductive genetics intake data elements (reproductive history, indications for the procedure), or in the reproductive genetics chart review data elements (screening tests, ultrasound Level I and II findings; fetuses with any abnormal genetic findings were excluded). There were a number of significant relationships between INSL3 levels and the pregnancy outcome data elements. Birth weight had a significant positive relationship with INSL3 levels ($P = 0.008$) (Fig. 3B); infants with birth weights $>3487$ g had significantly higher levels of INSL3 across all gestational ages than those with birth weights $<3090$ g ($P = 0.02$). Length of gestation indicated a marginally significant negative relationship with INSL3 levels ($P = 0.10$). Among the pregnancy complications reported, there was a significantly positive relationship between pre-eclampsia and INSL3 levels; amniotic fluid samples from pregnancies with pre-eclampsia had significantly elevated levels of INSL3 from 15–18 weeks of gestation compared with samples from normotensive pregnancies ($P = 0.03$) (Figs 3C and 4). Attempts were made to ascertain whether the elevation in INSL3 levels was simply due to reduced amniotic fluid volume associated with pre-eclampsia. To address this question, the effects of pre-eclampsia on AFAFP levels was examined with the assumption that reduction in amniotic fluid levels per se should be reflected in AFAFP levels. There was no significant association between AFAFP levels and pre-eclampsia in logistic regression analysis (odds ratio $= 4.3$, 95% CI $0.70$, 27, $P = 0.12$), while controlling for gestational age at amniocentesis. Consequently, the elevation in INSL3 levels in pregnancies complicated by pre-eclampsia is not likely due simply to a reduction in amniotic fluid volume. There were no significant relationships between INSL3 levels and gestational diabetes, hypothyroidism, preterm delivery, preterm premature rupture of membranes and vaginal bleeding.

Multivariable linear regression analysis was then conducted to determine whether maternal age at amniocentesis, birth
The dependent variable was the log of INSL3.

Table II. Multivariable linear regression results for independent predictors of INSL3 levels.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Standardized β coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at amniocentesis (weeks)</td>
<td>−4.13</td>
<td>0.0001</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>0.28</td>
<td>0.001</td>
</tr>
<tr>
<td>Pre-eclampsia (% yes)</td>
<td>0.18</td>
<td>0.006</td>
</tr>
<tr>
<td>Maternal age at amniocentesis (years)</td>
<td>0.15</td>
<td>0.025</td>
</tr>
<tr>
<td>Length of gestation (weeks)</td>
<td>−0.13</td>
<td>0.13</td>
</tr>
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</table>

The dependent variable was the log of INSL3.

weight, length of gestation and pre-eclampsia were independent predictors of INSL3 levels while controlling for gestational age at amniocentesis. Results of these analyses are presented in Table II. All remained highly significant and independent predictors of INSL3 levels with the exception of length of gestation.

The same approach was taken to determine which amniocyte bank parameters were significantly associated with testosterone levels in unadjusted analyses. The only marginally significant relationship was between testosterone levels and hypothyroidism during pregnancy \((P = 0.07)\). When this parameter was examined in multivariable linear regression analysis controlled for gestational age at amniocentesis, results were also marginally significant \((P = 0.054)\). There were only three cases of maternal hypothyroidism in the bank, and it is not likely that this is a meaningful finding.

Discussion

This is the first report on the levels of INSL3 derived from the human fetus at mid-gestation. This is a time when sex differentiation occurs following the activation of the SRY and SOX9 genes (Kim and Capel, 2006). It is also the time when Insl3 is first expressed in the fetal rodent gonad, and a suppression of Insl3 production is one of the earliest events associated with the onset of cryptorchidism due to xenoestrogen or phthalate exposure in utero (Wilson et al., 2004; Carruthers and Foster, 2005; McKinnell et al., 2005; Borch et al., 2006; Nikolova and Vilain, 2006; Toppari et al., 2007).

There are currently no data available on INSL3 production in humans at any time prior to birth. Bay et al. (2007) have examined levels of INSL3 in cord blood at birth and in serum of persistently cryptorchid boys and found reductions of the already very low levels of INSL3 in cord blood and elevations in the LH/INSL3 ratio at 3 months of age, indicative of a mild disturbance of testicular development at birth. The present study extends these findings to mid-gestation and show that INSL3 levels are considerably elevated during the critical period of sex differentiation in the second trimester of the normal male fetus. Unfortunately, too few samples were available from our amnio bank with outcomes of cryptorchidism and hypospadias to gain any information on an association between amniotic INSL3 levels and the occurrence of these anomalies. However, there were two major findings obtained from the present study. First, INSL3 expression is only detectable in the amniotic fluid derived from male fetuses, and that maximum levels were found at 15–18 weeks of gestation, declining thereafter to undetectable levels by week 22. Weeks 15–18 represent the period of gender determination in the brain, and this period occurs shortly after the initial abdominal phase of testicular differentiation and abdominal migration (Hutson and Hasthorpe, 2005; Nikolova and Vilain, 2006). Our data confirm that INSL3 is likely to be a unique product of the male gonad, since INSL3 in amniotic fluid from female fetuses was consistently below the level of detection. Also Bay et al. (2007) were unable to measure INSL3 in the cord blood of girls at any age in their study. These findings also reinforce that there is probably no significant contribution to the fetus from the maternal system or placenta.

It remains to be determined why 26% of samples obtained from weeks 15–18 had undetectable levels of INSL3. It seems likely that intra-individual variability in the temporal pattern of INSL3 expression, which could not be estimated with only single samples of amniotic fluid from any one subject, is the cause of this problem. According to Hutson and Hasthorpe (2005), the period of the first transabdominal phase of testicular decent in humans, when INSL3 is most likely to be impacting, is at weeks 8–15. It is thus highly probable that amniotic INSL3 concentrations are higher in this earlier period, which for ethically obvious reasons is hard to sample, and that in the subsequent weeks 15–18 INSL3 concentrations are already declining or have already reached baseline. The INSL3 assay is robust to temperature changes, and samples that were repeatedly frozen and thawed, or left at room temperature or at 4°C for 24 h, retained nearly identical values (data not shown). This is an important consideration as samples collected in a clinical environment can be left at room temperature for several hours prior to refrigeration and freezing. Thus, sample degradation is an unlikely cause of the non-detectable levels.

Total testosterone was also measured in the same samples of amniotic fluid. Although measurement of testosterone in sera is
known to be problematic (Rosner et al., 2007), the values for amniotic fluid obtained in the present study are in good agreement with other published data. Amniotic testosterone levels for male fetuses were reported by van de Beek et al. (2004) to be $1.41 \pm 0.34 \text{ nmol/l}$ (equivalent to 0.41 ng/ml) and for female fetuses were $0.69 \pm 0.33 \text{ nmol/l}$ (equivalent to 0.20 ng/ml). The present mean values were $0.52 \pm 0.17$ and $0.30 \pm 0.09 \text{ ng/ml}$ for males and females, respectively, which are in the ranges reported in several other studies where testosterone was measured by immunoassay and by gas chromatography/mass spectrometry (males: range 0–0.5 ng/ml, females: range 0–0.27 ng/ml) (Wudy et al., 1999). The maximum sex difference in amniotic testosterone levels occurred between 15 and 18 weeks in the present study, as previously reported by other investigators evaluating levels of steroid hormones in amniotic fluid (Knickmeyer and Baron-Cohen, 2006). The lack of correlation between amniotic INSL3 and testosterone levels may reflect priming of testosterone production in the fetal Leydig cell by an initial surge in INSL3. The maximum sex difference in amniotic testosterone occurred at the same time interval when INSL3 values were the highest (weeks 15–18). However, a considerable portion of the amniotic testosterone concentration is likely to be contributed from the fetal adrenal (Krone et al., 2007), also in females, and thus will not necessarily reflect testicular function.

In addition to the principal findings mentioned above, INSL3 was found to be significantly associated with gestational age at amniocentesis, elevated birth weight, advanced maternal age and the occurrence of pre-eclampsia. Gestational age at amniocentesis was derived from Level II ultrasound measurements, and thus can be considered a more accurate measure of developmental age than obtained from LMP date calculations. The variable pattern of INSL3 levels in amniotic fluid with gestational age is hypothesized to represent the actual time course of production by the human fetus. The amniotic concentration of INSL3 may be determined by both the production of INSL3 and the volume of amniotic fluid. The evident decline in INSL3 levels with the progression of pregnancy also implies that immunoreactive INSL3 is removed from amniotic fluid by some degradative mechanism. This presumably involves fetal imbibition, since the amniotic fluid itself does not appear to possess the appropriate proteolytic activity, as witnessed by the lack of INSL3 loss following prolonged incubation at room temperature.

When only those amniotic fluid samples with measurable INSL3 values were examined, gestational age at amniocentesis and pre-eclampsia remained highly significant, whereas birth weight and maternal age at amniocentesis did not. Consequently, the associations with these latter variables must be interpreted with caution and additional verification is needed before their true associations with INSL3 concentrations can be ascertained.

The second major finding from this study is the elevation of INSL3 levels in amniotic fluids from asymptomatic women who subsequently developed pre-eclampsia. Hombach-Klonisch et al. (2001) showed that trophoblast cells are able to express INSL3 transcripts and protein in the human placenta. However, this INSL3 gene activity was unaffected by either the site of implantation or the invasive properties of the cytotrophoblast as demonstrated by samples from patients with tubal implantation in the first trimester or pre-eclampsia in the third trimester, respectively. In the present study, INSL3 was measured in amniotic fluid from women in mid-pregnancy who were asymptomatic for pre-eclampsia. Studies have found that decreased amniotic fluid levels occur in patients who have pre-eclampsia (Mercer et al., 1984). This is not the likely cause of elevated INSL3 levels in the present study; AFAFP levels were not increased in samples from women who subsequently developed pre-eclampsia, and at the time of amniocentesis, none of the women in the present study were exhibiting signs of pre-eclampsia. Although advanced maternal age is a known risk factor for pre-eclampsia (Duckitt and Harrington, 2005), results from the multivariable linear regression analysis conducted in this study indicate that both advanced maternal age and pre-eclampsia each independently predict INSL3 levels while controlling for a number of other covariates. A number of studies have shown that maternal serum levels of testosterone and of leptin are elevated in women with documented pre-eclampsia in the third trimester compared with matched normotensive controls (Acromite et al., 1999; Atamer et al., 2004; Salamalekis et al., 2006). While it is difficult to extrapolate between these published findings and those obtained in the present study, the possibility exists that an early step in the development of pre-eclampsia involves alterations in androgen production in the fetal compartment. This alteration may first be expressed by elevated INSL3 levels in amniotic fluid, as documented here, leading to elevated or prolonged fetal production of testosterone. Elevated fetal testosterone levels could lead to increased placental production of leptin, leading to increased birth weight, and to elevations of testosterone and leptin levels in the maternal circulation. In the present study, the relationship between pre-eclampsia and levels of amniotic testosterone were not statistically significant, but amniotic testosterone levels were more variable than INSL3, and a larger sample of pre-eclamptic subjects may be needed to see meaningful differences. Other studies have found that markers such as altered vascular endothelial growth factor, nitric oxide, inhibin and fibronectin levels are associated with pre-eclampsia (Baumann et al., 2007; Mutter and Karumanchi, 2008). These markers are generally expressed in symptomatic patients late in the third trimester, though some may become significant earlier, and were not measured in the present study. Further investigation of this finding that amniotic INSL3 is elevated in asymptomatic women who later develop pre-eclampsia is needed and should also include measurements of these well validated markers.

Acknowledgements

We should like to thank Ms Bettina Hafen for excellent technical assistance, and Dr Matthias Schumacher for expert advice in regard to assay validation.

Funding

This work was supported in part by NIH grants R21ES11675 and P30ES013508, by the Faculty of Sciences Small Grant
Scheme from the University of Adelaide, and by grant DP0773315 from the Australian Research Council.

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Submitted on August 2, 2007; resubmitted on December 8, 2007; accepted on January 26, 2008