Insulin-like factor 3 as a monitor of endocrine disruption

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Abstract

Insulin-like factor 3 (INSL3) is generated and secreted by differentiated interstitial Leydig cells of the testes in both fetal and adult males of all mammalian species so far analyzed. All evidence to date suggests that it is produced constitutively, independently of acute regulation by the hypothalamo-pituitary–gonadal (HPG) axis, in amounts which reflect the numbers and differentiation status of the Leydig cells. This Leydig cell functional capacity is otherwise monitored only by androgen output, which, however, is massively confounded by acute regulation from the HPG axis and other factors leading to substantial and irregular short-term variation. Leydig cells are a primary target of endocrine-disrupting agents in the context of the testicular dysgenesis syndrome in the fetal male, as well as in the adult. In the male fetus, INSL3 is responsible for the first phase of testicular descent, and hence is directly linked to the etiology of cryptorchidism. In this study, by measuring INSL3 production, for example, during fetal life via amniotic fluid, or as secretions from fetal testis explants, or in adult peripheral blood, we and others have shown that INSL3 represents a useful quantitative and sensitive endpoint for assessing the impact of endocrine-disrupting agents and their mechanisms of action.

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Introduction

Until relatively recently, the growing embryo in viviparous mammals was considered to be protected from external influences by a combination of the fetal membranes and the placenta, together with the catabolic capacity of the maternal physiology, where the liver and kidney act as first lines of defense against noxious environmental agents. Since the construction of the DOHaD hypothesis (Developmental Origins of Health and Disease), we now know that in spite of these maternal and fetal defense mechanisms the growing fetus is indeed vulnerable to a range of intrinsic (maternal nutrition and metabolism) and extrinsic agents, mostly man-made chemicals which appear to be able to interfere with the establishment of fetal organ systems and their endocrine or paracrine control mechanisms (Swanson et al. 2009, Barouki et al. 2012). The DOHaD concept is reinforced by our modern understanding of the important role played by epigenetics, not only in determining cell fates and lineage phenotypes in the embryo, but also by acting as a memory for extrinsic insults, which may impact on adult physiology long after the exposure has ceased (Sinclair et al. 2007).

One of the most vulnerable periods during the life of the fetus is in the transition from first to second trimester. This is the time when most organ systems are developing rapidly, both anatomically as well as in the establishment of their endocrine control systems. It is also a time when the fetal skin is still relatively permeable, such that amniotic fluid largely comprises fetal serum exudate (Underwood et al. 2005, Beall et al. 2007), and thus offers very little protection against exogenous chemical insult. Moreover, at this time the placenta is establishing its vascular bed and functional dimensions. Typical for this period is the differentiation of the male organs of reproduction, the testes and Wolfian derivatives.
Very shortly after SRY-induced sex determination in the male fetus, the somatic cells of the fetal testes proliferate and differentiate into Sertoli and steroidogenic Leydig cells, providing both a niche for the developing totipotent germ cell stem cells (gonocytes) as well as delivering the hormones essential for adequate development of the male genitalia and reproductive organs (androgens, AMH, and insulin-like factor 3 (INSL3)). In contrast, the ovary of the female fetus at this time is relatively less active as an endocrine organ, with a lack of hormones determining the female phenotype. Therefore, because the testes are very actively differentiating at this vulnerable time, and because they are both producing and are themselves regulated by a very active hormonal system in this period of early gestation, environmental endocrine disruption by extrinsic hormone-like factors has been most emphatically observed in regard to the male reproductive phenotype. Consequently, symptoms have been most emphatically observed in regard to the male genitalia and reproductive organs during pregnancy (Skakkebaek et al. 2001, Sharpe & Skakkebaek 2008). Nevertheless, other organ systems are also likely to be affected, though possibly less obviously.

Unfortunately, the transition from first to second trimester of pregnancy in humans is one of the most difficult physiological time points to access. The fetus is still very small and fragile, and maternal serum offers few clues as to how the pregnancy is progressing. Moreover, of the endogenous hormones being produced by the fetus in measurable amounts, few have the required specificity to be able to offer any conclusive diagnostic outcome. So, for example, androgens are generated by both adrenal glands and gonads at this time, and thus are expressed by both male and female fetuses in not very different amounts (Anand-Ivell et al. 2008, Scott et al. 2009). An exception is provided by the small peptide hormone INSL3, which is produced in large amounts by the fetal testis shortly after sex determination (Anand-Ivell et al. 2008). It is produced by no other fetal organ, consequently does not appear to be produced by the female fetus (Anand-Ivell et al. 2008, Bay et al. 2008), and is made in only very low amounts by the maternal ovary (Anand-Ivell et al. 2013). It thus has the potential to be a highly specific monitor for any events impacting on the normal differentiation and development of the fetal testis during the important window for endocrine disruption at the transition from first to second trimesters (in the human).

**INSL3 in the fetal testis**

The fetal testis is established relatively early on during organogenesis, with the prospective somatic Sertoli and Leydig cells in place soon after the arrival of the gonocytes from the hindgut and only shortly after the expression of the SRY gene by the Sertoli cells to signal sex determination. In humans, this occurs at around week 7 of pregnancy and in mice at around day 11 (Viger et al. 2005, Sekido & Lovell-Badge 2013). Latest evidence suggests that the fetal Leydig cells (at least in the mouse) may have a dual origin, in that they appear not only to differentiate from mesenchymal cells within the gonadal ridge but also migrate in from close to the mesonephros (De Falco et al. 2011). In either case they begin to express the transcription factor SF1 almost immediately after Sry expression in the Sertoli cells at around day e10.5 in the mouse (Sekido & Lovell-Badge 2008). INSL3 is a gene which in the adult testis is regulated via multiple SF1-responsive elements in its promoter, but is a marker for a relatively late stage of Leydig cell differentiation (Sadeghian et al. 2005). If its expression is similarly regulated in the fetus, then this would imply that INSL3 in fetal Leydig cells should become detectable soonest after about day e12.5 in the mouse, or by weeks 8–10 in the human. Indeed, the earliest evidence for Insl3 mRNA detection in the mouse is on day e12.5 (Sarraj et al. 2010) and, for the human, INSL3 peptide has been measured in the amniotic fluid at gestational week 11 (R Anand-Ivell, unpublished observations). There is less evidence available for other species, although sporadic measurements of INSL3 in the bovine (Anand-Ivell et al. 2011), porcine, or rat systems (Anand-Ivell, Vernunft, Barthol & Ivell, unpublished observations) confirm that high levels of INSL3 are detectable in fetal blood, or in fetal fluids, at the time of the first transabdominal phase of testicular descent (Fig. 1).

All evidence so far indicates that the Leydig cells of the testes are the only source of INSL3 in the fetus. Female fetuses consequently appear to express no or only very low levels of INSL3, the origin of which is obscure (Anand-Ivell, unpublished observations). Thus the detection of INSL3 in fetal blood or amniotic fluid during mid-gestation is a very reliable indication of male fetal gender (at least in monotocous species). Moreover, in some species, e.g. cows, where maternal circulating
concentrations of INSL3 are low, this fetal INSL3 can even be detected in maternal blood during mid-gestation (Anand-Ivell et al. 2011).

**Comparative biology of testicular descent**

Mammals are unusual in that with a few notable exceptions (e.g. elephants) the male gonad does not remain in a peri-renal position like the ovary, but undergoes a migration, usually during fetal or early postnatal life, to an extra-abdominal location, the scrotum. The reasons for this are unclear, though may relate to a need for sperm in mammals to be stored at cooler than core body temperature before ejaculation (Ivell 2007). This ‘testicular descent’ into the scrotum occurs in two phases, which may be continuous or discontinuous. The first ‘transabdominal phase’ begins in most species soon after sex determination (Fig. 1), and is achieved by INSL3 from the fetal Leydig cells acting on its specific receptor, a G-protein-coupled receptor called RXFP2, which is expressed at high concentration on the cells of the gubernacular ligament (gubernacular bulb or gubernaculum) attached to the ventral surface of the fetal testis and which is also linked to the abdominal wall of the inguinal region. INSL3 causes thickening of the gubernaculum, effectively retaining the testes in the inguinal region, while the rest of the body, including the kidneys, grows away antero-dorsally. Androgens, though probably not the peptide hormone AMH, may modulate this process (Emmen et al. 2000a). The second phase of testicular descent involves the formation of the scrotum, the inguinal canal linking this to the peritoneal cavity, and the progressive eversion of the testis together with the gubernaculum and epididymis through the canal into the scrotum (Hutson et al. 2013). This phase occurs usually in the perinatal period and is regulated largely by androgens (Hutson et al. 2013).

When the Insl3 gene or its receptor, RXFP2, is deleted in mice, the major phenotype observed is a failure of testicular descent (cryptorchidism) with the testes retained in a high abdominal position (Nef & Parada 1999, Zimmermann et al. 1999, Gorlov et al. 2002). This cryptorchidism is bilateral, affecting both testes. Occasional defects in INSL3 production have also been found associated with cryptorchidism in dogs and horses (Klonisch et al. 2003, Pathirana et al. 2012). However, in humans, although cryptorchidism is relatively common amongst newborn boys (ca. 1–7%), this is mostly transitory, unilateral, and only in rare cases have any defects in the INSL3 gene or its receptor been identified (Bogatcheova et al. 2007).

There are some important differences between species in the relative timing of the first transabdominal phase of testicular descent (Fig. 1). Whereas in humans and ungulates this process and fetal testicular differentiation occur relatively early in pregnancy, with the first phase usually complete by the end of the second trimester; in rodents, it occurs much later and is not complete until after birth. Furthermore, fetal Leydig cell differentiation in rodents appears to occur independently of the establishment of the hypothalamic–pituitary–gonadal (HPG) axis (O’Shaughnessy et al. 2006), unlike in humans and probably other species, which require a gonadotropin (e.g. hCG or fetal luteinizing hormone (LH)) to drive Leydig cell differentiation. In the fetal pig, we had shown previously that LHβ is first evident in the pituitary on gestational day 50 but not on day 40 (Ma et al. 1996), whereas INSL3 is already well detectable in fetal fluids on day 45 (Anand-Ivell, Vernunit & Ivell, unpublished observations).

Transabdominal testicular descent, particularly in those species where this occurs relatively early in gestation, such as the human, is a critical anatomical event. Because it is occurring at a time of rapid fetal growth and the development of other organ systems, and is directionally contrary to these, any disruption to the timing or dynamics of gubernacular development could easily lead to a discordant development of other organ and ligament systems, and vice versa (Hutson et al. 2013). It is probably for this reason that unilateral or inguinal cryptorchidism, rather than bilateral cryptorchidism, is relatively common, being caused by a relative disturbance in the growth trajectories of different ligament systems.

**Amniotic fluid as a surrogate for measuring fetal blood**

Mammals, birds, and reptiles (amniotes) develop two major fluid-filled compartments to surround and protect the growing fetus from which they originate. One of these, the allantois is best seen as an external extension of the mesonephros and hindgut, though in humans becomes rudimentary, in comparison with the amnion. The amnion develops rapidly from the first through third trimesters of pregnancy (Fig. 2) and, while early in gestation its fluid is contributed to mostly from the surrounding amniotic epithelium, later in pregnancy it becomes a reservoir for fetal urine, which, however, is turned over very rapidly through placental and cardiovascular function (Brace 1997, Underwood et al. 2005). Both allantois and amnion, as well as the fetal circulation, have differing composition of amino acids and electrolytes, together indicating that the fluid contents are all under strict and independent regulation, presumably as a function of their surrounding epithelial or endothelial layers (Goldstein et al. 1980, Brace 1997). Whereas, during the first and early-second trimester, amniotic fluid can also be regarded to a degree as an exudate of fetal serum, later in gestation, when the fetal skin has become keratinized, the composition of amniotic fluid changes and represents more that of fetal urine (Underwood et al. 2005).

Importantly, amniotic fluid is accessible by needle puncture (amniocentesis) already from the 10th week of gestation in humans, and thus represents one of the earliest points of access by which fetal health can be
Figure 2 Diagram showing the dynamic changes in amniotic fluid volume and fetal blood volume through gestation in human pregnancy. The calculated ratio of amniotic fluid (AF):blood volumes is indicated in green to estimate the possible dilution factor of INSL3 from fetal blood. The approximate time of maximum INSL3 production is shown by the purple cross-hatched rectangle. Based on Smith & Cameron (2002), Beall et al. (2007), and Anand-Ivell et al. (2008).

Amniocentesis is routinely offered at around weeks 12–18 of pregnancy for older women (>35 years) at higher risk of presenting a fetus with aneuploidy, or where there is a family genetic risk. This time point at the transition from the first to the second trimester coincides with the time when there should be maximal INSL3 expression by the fetal testis, correlating with the first transabdominal phase of testicular descent. It also represents a critical period of fetal organogenesis as well as placental development, which are both highly susceptible to extrinsic disrupting effects (DoHAD hypothesis). We know from numerous epidemiological and experimental studies that the male reproductive system is indeed very vulnerable to such extrinsic effects (Sharpe & Skakkebaek 2008). Moreover, this is a time period where, other than using ultrasound, no other methods are routinely available to monitor fetal blood or fetal–maternal interaction.

In a preliminary study (Anand-Ivell et al. 2008), we have been able to characterize INSL3 expression in human amniotic fluid collected at amniocentesis and show that maximal concentrations, which can only have been derived from male fetal testes, are indeed measurable in this medium (<0.5 ng/ml). Both maternal serum concentrations as well as amniotic fluid from female fetuses at this time are at or below the assay limit of detection. Amniotic INSL3 concentration from male fetuses follows a curvilinear relationship retaining a maximum between weeks 12 and 16, thereafter declining to below detection levels. It is not yet clear whether this decline represents a downregulation of INSL3 expression, or an increasing dilution effect, or decreasing fetal skin permeability, or catabolism. The only information available on INSL3 mRNA for the human fetus (O’Shaughnessy et al. 2007) suggests little change in expression at least between weeks 12 and 18. In our relatively small study, it could also be shown that amniotic INSL3 concentration was significantly increased for mothers who later became pre-eclamptic, or reduced for those who delivered small for gestational age babies (Anand-Ivell et al. 2008). Both of these symptoms are believed to have their origins in the establishment of a dysfunctional placenta in the first trimester. Thus, these observations confirm that measurement of INSL3 in amniotic fluid, because of its fetal specificity and its dynamic range over a short critical time window of organogenesis, can indeed act as a monitor for more general aspects of fetal health at this critical time point. Moreover, INSL3 is probably more effective as a parameter than, for example, steroid hormones, because the latter result from both fetal gonads as well as from the fetal adrenal glands, and probably also the placenta, besides possible maternal origins (Scott et al. 2009). Consequently, this lack of source specificity for steroids and hence increased variance in the system could mask any DoHAD-related effects.

Fetal INSL3 and endocrine disruption

The linking of cryptorchidism to environmental endocrine disruption, and its identification as a key symptom within the TDS already predicted that, in addition to reduced androgen production, INSL3 might also be involved. This was supported by clinical studies showing that the xenoestrogen diethylstilbestrol (DES) given to pregnant mothers in the 1940s–1970s led to a high incidence of cryptorchidism in the male children (Palmer et al. 2009), as well as the key experimental study showing that DES given to pregnant mice caused bilateral cryptorchidism and a downregulation of Insl3 gene expression in the fetal testis (Emmen et al. 2000b). Later studies on rats then showed that the cryptorchidism and other TDS-like effects induced in rats by administering phthalates during a critical window of testis differentiation also led to a marked downregulation of fetal Insl3 expression (McKinnell et al. 2005, Mahood et al. 2006). Combined with the knowledge from the mouse knockout studies, where either INSL3 or its receptor RXFP2 had been ablated leading to primary cryptorchidism (Nef & Parada 1999, Zimmermann et al. 1999, Huang et al. 2012), this placed INSL3 firmly within the signaling pathways, together with altered androgen production, linking endocrine disruptors to TDS-like defects – at least in rodents.
Firstly, in the rat, where most such environmental disruptor studies looking at TDS symptoms have been carried out, Insl3 expression by the fetal Leydig cells corresponds in time quite precisely with the so-called ‘window of sensitivity’ that was defined for phthalate exposure (Fig. 3; Carruthers & Foster 2005). Moreover, immunohistochemical analysis of day 17.5 fetal rat testes exposed or not exposed to dibutyl phthalate showed maximal expression of Insl3 in Leydig cell in the controls and almost complete ablation in the phthalate-exposed animals (McKinnell et al. 2005). In Fig. 3, we also show parallel INSL3 measurements from both fetal blood and from amniotic fluid. The ratio of these two values appears to correspond to the relative volumes of these two fluid compartments in this prenatal phase (Fig. 4).

But the question then arises: are the effects of phthalates or other xenobiotics on INSL3 expression direct effects or secondary effects? Part of the difficulty here is that, although some in vitro studies have attempted to show direct hormone action on the INSL3 gene promoter (Lague & Tremblay 2008, 2009), other studies cannot show this (e.g. Sadeghian et al. 2005). Secondly, concomitant with such endocrine disruptor exposures, there is usually also a disruption of steroid, particularly androgen production. Thus effects of endocrine-disrupting agents are more likely to be indirect via impacts on Leydig cell differentiation, than directly on single genes like that for INSL3. Nevertheless, recent studies using xenografts of human testis (Desdoits-Lethimonier et al. 2012) or rat fetal testis (Kristensen et al. 2012) have shown gene discriminating effects of phthalates or paracetamol, whereby steroidogenesis but not INSL3 are affected. However, other recent studies using human adult or fetal testis explants indeed have shown that paracetamol was able to impact negatively on INSL3 expression (Albert et al. 2013, Mazaud-Guittot et al. 2013). It is also important to note that such effects may also be species-specific; for example, xenoestrogens may impact on INSL3 production by human fetal Leydig cells but not by those from rodents (N’Tumba-Byn et al. 2012). In a new study of INSL3 expression in human amniotic fluids taken during amniocentesis, a negative correlation could be shown between INSL3 concentration and phthalate load (Anand-Ivell, Toft & Jensen, unpublished observations), thus providing indirect evidence for an impact of endocrine disruptors on INSL3 production in the normal population.

**INSL3 in the ‘mini-puberty’**

Most mammals indicate a continual somatic development from fetus through birth to puberty and adulthood. Using rodents as example, we see that the fetal Leydig cell population involutes after birth. With the subsequent establishment of the HPG axis, a new population of Leydig cells differentiate during puberty from resident peritubular Leydig stem cells within the testes (Mendis-Handagama & Ariyaratne 2001, Ge et al. 2006). These are the adult-type Leydig cells. In humans and higher primates, there is an unusual situation in that, following birth, there is a prolonged period of relative gonadal quiescence during which there is substantial

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**Figure 3** INSL3 concentration in rat fetal blood serum and amniotic fluid collected from male fetuses at various gestational days as indicated, and measured using the specific rat INSL3 TRFIA as described (Anand-Ivell et al. 2009). Unpublished data courtesy of Dr Julia Barthol (Wilmington DA) and Dr Ravinder Anand-Ivell (Germany).

**Figure 4** Diagram showing the dynamic changes in amniotic fluid volume and fetal blood volume through gestation in the rat. Blood volume is estimated as 10% of fetal body weight. The calculated ratio of amniotic fluid (AF):blood volumes is indicated in green to estimate the possible dilution factor of INSL3 from fetal blood. The approximate time of maximum INSL3 production is shown by the purple cross-hatched rectangle. Based on Tam & Chan (1977), Park & Shepard (1994), and Anand-Ivell & Barthol (unpublished observations) (see Fig. 3).
brain, skeletal, and behavioral development, with puberty and the activation of the HPG axis occurring only after several years. An exception to this is during the immediate postnatal period at ca. 3 months of age, when there appears to be a short and transient upregulation of androgen production by the infant testes associated with a brain masculinization window (Prince 2001). These androgens, in what is referred to as the ‘mini-puberty’, appear to be derived from a newly developed population of Leydig cells, which later regress. It is not known whether these neonatal Leydig cells represent a lineage of cells entirely discrete from either the fetal or adult-type populations, or whether they represent a reactivation of the involuted fetal Leydig cell population. Only one study has looked at INSL3 during the mini-puberty (Bay et al. 2007). Like testosterone, it is also elevated compared with later ages, showing that the Leydig cells at that time are indeed exhibiting a more mature phenotype. Importantly in this study a significant reduction in circulating INSL3 concentration was detected comparing both transiently and especially persistently cryptorchid (unilateral and bilateral) boys with normally descended controls (Bay et al. 2007). Whether this was due to an original disruption of INSL3 expression during fetal life, or whether this was a consequence of the cryptorchid phenotype and the maintenance of the postnatal testes at abdominal temperature could not be determined. Interestingly, in this study expressing Leydig cell functionality as a ratio of LH:INSL3 gave much more significant differences between cases and controls than the ratio of LH:testosterone (Bay et al. 2007). This reinforces the viewpoint expressed earlier that testosterone, compared with INSL3, exhibits high within- and between-individual variance and consequently is less useful as a parameter to monitor Leydig cell function.

**INSL3 in the adult male**

Puberty in the male mammal represents the phenotypic consequence of Leydig cell differentiation under the influence of the postnatal awakening HPG axis. Pulsatile GNRH leads to increased secretion of pulsatile LH from the anterior pituitary, specifically stimulating Leydig cells to differentiate from resident testicular stem cells and produce androgens as well as the peptide hormone INSL3. This is well documented for INSL3 at RNA and/or secreted peptide levels in rodents (Balvers et al. 1998, Sadeghian et al. 2005), ruminants (Kawate et al. 2011), and humans (Ferlin et al. 2006, Wikström et al. 2006, Johansen et al. 2013). Because of their relatively more homogeneous genetic nature, rodents exhibit a consistent age-dependent increase in circulating INSL3 up to a maximum at the time of first appearance of sperm in the epididymis (ca. day 42), thereafter declining somewhat to attain a lower steady state by about 6 months of age (Anand-Ivell et al. 2009). Because INSL3 is secreted constitutively rather than being acutely regulated like testosterone, this has been interpreted to indicate that during puberty initially the large amounts of LH produced first cause an excessive differentiation of Leydig cells, which later stabilize at a lower functional capacity because of androgen feedback to the pituitary (Anand-Ivell et al. 2009). A similar observation has recently been found also in the dog (Pathirana et al. 2012).

Less information is available for humans and other species. In the former, circulating INSL3 concentrations have only been measured in boys up to about age 15 years, where although they have attained Tanner stage 5, INSL3 concentration is evidently still increasing (Ferlin et al. 2006, Wikström et al. 2006, Johansen et al. 2013). INSL3 has also been measured in adult men (Bay et al. 2005, Anand-Ivell et al. 2006), where it shows a significant and steady decline with age from about 30 to 80 years, reflecting the gradual loss of Leydig cell functionality with age (Anand-Ivell et al. 2006). What is important to note from these studies is that already at age 30 years men indicate a very broad range of INSL3 expression, consistent with a range of Leydig cell functional capacity (numbers per testis×differentiation status), which is likely to persist into old age and may determine androgen-dependent health outcomes during aging. We have no notion as to the cause(s) for this broad range in Leydig cell functional capacity in young men, except that it is already evident during puberty and may have its origins in some fetal or postnatal developmental disruption.

In order to investigate such a possibility, studies were conducted on rats using two models of endocrine disruption (Heng et al. 2012, Ivell et al. 2013). In the first model, pregnant female rats were dosed with either dibutyl phthalate (7×500 mg/kg body weight (BW) between GD14.5 and PND6) or DES (2×125 μg/kg BW on GD14.5 and GD16.5), or appropriate vehicle, during late gestation and/or early lactation. Litters were then followed through puberty and male offspring were assessed for INSL3 secretion and expression of Leydig-cell specific genes at day 24 (during puberty) and at 3 months (adulthood). Although the adult offspring showed no obvious effects of the treatments, compared with controls, the rate of progression of puberty, in terms of peripheral INSL3 concentration and Leydig cell numbers, and the establishment of final Leydig cell functional capacity were indeed significantly disrupted (Ivell et al. 2013). In the second experiment, advantage was taken of the property of the alkylating agent, ethane dimethane sulfonate (EDS), in vivo to completely eliminate all precursor and mature Leydig cells from the adult rat testis. Following such treatment, new Leydig cells regenerate within the testes with a developmental trajectory similar to that during puberty. In this study, similar to the previous experiment, rats were treated with either dibutyl phthalate (500 mg/kg BW daily on days 5–7 or days 3–9, following EDS injection) for a short
period, or DES (125 μg/kg BW daily as for dibutyl phthalate), or vehicle following EDS injection, again measuring INSL3 secretion and specific Leydig cell gene expression during the subsequent differentiation of the Leydig cells (Heng et al. 2012). As before, at day 27 after EDS treatment, a time equivalent to mid-puberty, there is a marked increase in Leydig cell numbers and in the rate of INSL3 secretion (elevated for phthalate and/or DES compared with controls), as well as in specific gene expression, largely caused by a significant impact on the rate of Leydig cell proliferation and differentiation (Heng et al. 2012). Together, these results from rodents show that typical environmental-disrupting agents may impact not only during gestation but also on the adult phenotype during puberty or earlier. Importantly, they demonstrate that INSL3 measured at an appropriately dynamic time point during pubertal development can be very informative about effects of earlier environmental endocrine disruption. It should be noted, however, that the changes observed during Leydig cell differentiation in these studies appeared to be completely resolved in adulthood. This does not mean that these effects are without impact, as there is a disruption of endocrine status at an earlier time when other organ systems are still developing, and these might consequently be subtly and chronically altered, for example, bone metabolism (Ferlin et al. 2013). Although such studies use high doses of potential endocrine disrupting substances, unlikely to be found naturally, they nevertheless point to aspects of developmental physiology which might otherwise escape our attention.

Comparable studies in humans are obviously difficult. However, using human adult testis explants xenografted into immune-deficient mice, it has recently been shown that various analgesics in moderate doses are also able to influence Leydig cell function in terms of INSL3 as well as testosterone output (Albert et al. 2013), thus confirming also for the human that the adult testis is susceptible to endocrine disruption which can be monitored in terms of INSL3 and/or androgen production.

### Conclusions

INSL3 is a small peptide hormone which is constitutively produced by fetal and adult Leydig cells, and by no other tissue in appreciable amounts. Importantly, it is produced by the fetal testis during the important developmental window at the transition from first to second trimester, a time window when we know that male reproductive development is very vulnerable to a range of endocrine disrupting insults. By measuring INSL3 in amniotic fluid at this time via routine amniocentesis, or by making use of alternative surrogates, such as cord or neonatal blood, we may be able to obtain significant insights into potential exposure of the fetus and consequences for infant and adult health.

### Ethical statement

All of the experimentation involving animals or humans, with which the authors have been involved and which is reported here, conformed to NIH guidelines, and had been approved by the appropriate local Animal and Human Ethics Committees.

### Declaration of interest

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

### Discussion from meeting

**Luiz França** (Belo Horizonte, Brazil): In addition to its role in testicular descent and apoptosis, there is evidence emerging that INSL3 influences spermatogonial stem cells and spermatogenesis. We have preliminary evidence from zebrafish.

**R Ivell** (Dummerstorf, Germany): There is a possible role for INSL3 involvement in the testicular niche, but there are as yet no solid data.

**Niels E Skakkebæk** (Copenhagen, Denmark): Can you speculate about the wide range of values of INSL3 found in normal men in control populations. Is it better to have higher values or lower values, and what is the ideal value? Most data suggest that testicular failure and hypogonadism are associated with low INSL3, but your data seems compatible with higher INSL3 following fetal exposure to endocrine disrupters.

**R Ivell**: More studies are required to be conclusive. Our data show an acceleration of Leydig cell growth and development during rat puberty or following treatment with ethanedimethanesulphonate (EDS). However, there are no long-term consequences in the animals followed into adulthood. Our Australian study showed an association between INSL3 and the metabolic syndrome, but not with BMI. There is a link to bone metabolism whereby low INSL3 is associated with osteoporosis. Smoking reduces INSL3 but increases testosterone. Overall it appears as if a high level of INSL3 is beneficial, but we have no longitudinal data.

**Bernard Jégou** (Rennes, France): We have recently validated your hypothesis that some compounds might alter INSL3 production. In our model, we exposed adult human testes to paracetamol, aspirin, and indomethacin and found inhibition of INSL3 (Albert et al., Human Reproduction, 2013). Mazz et al. (Personal communication) showed the same effect in human fetal testes exposed to paracetamol.

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