

Executive Summary:

Reproductive health is based on normal endocrine-regulated development. It is also a sensitive indicator of overall health and disturbances are linked to cardiovascular and metabolic diseases, such as metabolic syndrome, obesity and diabetes. DEER (Developmental Effects of Environment on Reproductive Health) project tackled the problems of reproductive health by investigating:

- connections between normal and abnormal fetal and neonatal reproductive development and subsequent maturation of reproductive function at puberty and in adulthood
- systemic gene-environment interactions underlying reproductive disorders, taking into account genetic susceptibility and multiple exposures
- connection between perinatal development and metabolic disorders in later life (obesity).

DEER studies have added important epidemiological and mechanistic information for reproductive and developmental effects of environmental contaminants. The puberty studies revealed an advancement of the onset of puberty in Danish children, particularly in girls. Genetic predisposition affecting the timing was also emerging as well as associations to e.g. phthalate exposure that was linked to delayed pubarche (appearance of pubic hair) in girls. Programming of puberty was successfully analyzed in the rat model in which neonatal exposure to steroids, endocrine disruptors or nutritional stress caused organizational effects in the controlling network. These studies help to understand the normal regulation of puberty and its disorders. This forms the basis for prevention of puberty disorders.

DEER studies highlighted the importance of the so called masculinization programming window in the establishment of normal reproductive health during fetal development. Its pertinence for human health became also obvious, and this guides the programmes to prevent reproductive disorders, such as cryptorchidism, hypospadias, testicular cancer and impaired semen quality. Different in vitro models complemented in vivo studies in verifying mechanisms of endocrine disruption in this context. These will be useful tools for assessment of chemical safety. The data and techniques will provide tools for these assessments that are instrumental in the work of the European Chemical Agency to implement REACH.

Metabolomics was also used to describe on a global and untargeted way serum chemical phenotypes associated with semen quality and puberty timing. Novel systems biological approaches for analyzing exposure data, genetic analyses and available data banks and other data sources were introduced to studies of reproductive health. This methodological development provides powerful tools for future analyses of complex gene-environment networks to identify cause-effect relationships.

The wealth of new data generated in the DEER project will continue to produce new analytical results that will be published and publicly presented. The information will be disseminated to the policy makers and other stakeholders, as well as the public. The project will support improved risk assessment of endocrine disruptors.

Project Context and Objectives:

Many of the male reproductive health issues (low sperm count, testicular cancer, low production of male hormone), which a large portion of the male population in Western countries face today are thought to arise because of maldevelopment and malfunction of the fetal testis. These reproductive disorders are thus thought to comprise a testicular dysgenesis syndrome (TDS).

Several pieces of evidence suggest that common environmental chemicals, probably acting together in mixtures or in combination with other factors (genetic, lifestyle) could contribute causally to TDS. However, there are numerous obstacles to proving this scientifically, such as the long latency (up to 40 years) between cause (in fetal life) and health consequence, coupled with inherent difficulties in evaluating human fetal exposure to complex chemical mixtures; assessing interactions between chemical exposures and other factors adds greater complexity.

In order to circumvent the above-mentioned obstacles we took advantage in DEER of existing human birth cohorts/samples with their associated chemical exposure analyses. Established animal and in vitro models were used to improve our understanding of fetal testis development and function and its relationship to male reproductive development and disorders. The complexity of real-life chemical exposures was tackled using new bioinformatics approaches for assessing associations between real-life exposure scenarios and effects in humans.

The objectives of DEER were to approach the TDS problem by investigating

- connections between normal and abnormal fetal and neonatal reproductive development and subsequent maturation of reproductive function at puberty and in adulthood
- systemic gene-environment interactions underlying reproductive disorders, taking into account genetic susceptibility and multiple exposures
- connection between perinatal development and metabolic disorders in later life (obesity).

The scientific work planned in the DEER project was broken down into four themes:

Theme 1: Early exposures and adverse developmental effects

The overall aim of Theme 1 was to study early developmental effects of environmental factors. Human studies were complemented by experimental animal and in vitro studies, allowing us to investigate in detail the mechanisms behind observations made in the human population. This set-up, which provided new insights into mechanisms behind the effects of environmental factors, also allowed rapid implementation of this new understanding in further investigations of human material.

Based on the TDS hypothesis we aimed at generating new chemical exposure data to evaluate whether there is an association between chemical exposures and hypospadias. By using previously analyzed chemical data, this study also investigated whether there is any association between single or complex chemical exposures and congenital cryptorchidism. We also aimed at evaluating genetic factors

underlying cryptorchidism and TDS by traditional candidate gene analysis and by genome-wide association studies, integrated with systems biology approaches.

Here we also used a rat model of testicular dysgenesis syndrome (TDS) to identify mechanistic pathways in development of the male reproductive system, in particular of the testis, that are vulnerable to perturbation by endogenous and exogenous factors and that lead to male reproductive disorders (=TDS disorders) that are common/increasing in incidence in humans. The rat model involved exposure in pregnancy to high levels of the plasticizer dibutyl phthalate (DBP), to which there is ubiquitous human exposure. Within this overall context there were a number of specific objectives. First to evaluate the importance of the masculinisation programming window (MPW) and how androgen action in this time period relates to occurrence of dysgenesis per se (i.e. gross morphological abnormalities of testis set-up and structure), and how these relate to anogenital distance (AGD; as a read-out of androgen exposure in the MPW) and to occurrence of later male reproductive disorders. Second, to investigate the effects of fetal exposure to DBP on germ cell development, in particular on germ cell differentiation. Third, to establish if experimentally induced intra-uterine growth restriction (IUGR) affected events in the MPW and increased the occurrence of TDS disorders. Fourth to establish if impairment of fetal androgen exposure predisposed to the development of obesity and insulin resistance in adulthood. Fifth, although not an original objective, our emerging data identified a potentially important, novel mechanism via which DBP inhibits steroidogenesis by the fetal rat testis. In view of its importance, a considerable amount of time and effort were redirected to explore this mechanism (e.g. its species specificity, vulnerability to other exogenous factors).

To study the potential direct effects of two phthalates, di(2-ethylhexyl)phthalate (DEHP) and mono-(2-ethylhexyl) phthalate (MEHP), on the human and animal testis we decided to set up and use three different culture systems: a rat FETal Gonad Assay (FEGA), a human Cell Line Assay (CELIAS), and an adult human testicular organotypic culture (TEXAS: Testis EXplant Assay). One specific aim was to study the mechanisms of action of MEHP and DEHP on testicular steroidogenesis by using these different experimental approaches. As animal systems remain essential to decipher mechanism of action of EDs, it is also important to develop in vitro screening tests that can contribute to the replacing the use of animals.

Theme 2: *Early exposures and sexual maturation* (WP4 and 5)

Reproductive maturation and puberty onset are critically dependent on early developmental (organizing) events as well as on late (peri-pubertal) activational phenomena at the hypothalamic-pituitary (HP) unit, which also influences energy homeostasis. In addition, different forms of metabolic stress, acting at different developmental stages, can alter the timing of puberty and the function of the reproductive axis later in life. Assuming the concurrent mode of exposure to different environmental and metabolic insults along the life-span, we aimed to provide mechanistic data on the impact of single or combined chemical/hormonal and metabolic insults, as sequentially applied in preclinical models, on the hormonal profiles and central pathways responsible for the control of puberty and reproductive function, and how alterations of these could affect also the systems controlling puberty onset and reproductive function or vice versa. In this scenario, we aimed to implement a series of experimental studies using various rat models of early exposures to synthetic steroids, combined or not with specific metabolic insults, in order

to identify the consequences of such exposures on puberty onset and reproductive function, as well as their potential metabolic implications. In this general context, the Specific objectives included:

1. Characterization of target structures and expression profiles of genes linked to puberty onset at the HP unit after early and/or late exposures to synthetic steroids;
2. Identification of novel biomarkers/endpoints of endocrine disruption of puberty onset at the HP unit using exposure protocols used in point 1;
3. Assessment of the effects of early and late exposures to relevant EDCs upon puberty onset and HP unit function, using markers/end-points obtained in points 1-2;
4. Characterization of metabolic consequences (in terms of neuropeptide gene expression, food intake, body weight gain and hormonal profiles) of early and late exposures to compounds selected from points 1-3.

We also made use of two studies in humans: large and well-described Danish and Finnish longitudinal and cross sectional cohorts were used to explore the nature of puberty in Europe today. This enabled us to relate factors affecting pubertal onset to (prior) early perinatal events.

Theme 3: *Early exposures and late effects* (WP6 and 7)

Semen quality is disturbingly low in many Western countries. A trend for decreasing testosterone levels within the last decades has also been documented. As an endocrine organ, the testis is very susceptible to disruption by external factors, such as lifestyle or exposure to endocrine disrupting chemicals. This theme set out to examine whether exposure to certain endocrine disrupting chemicals (focus on the groups of perfluorinated compounds and phthalates) may interfere with human testicular function and reproductive health. Here, we also wanted to study the effect of consumption of beef on human semen quality. In the USA, cattle are often treated with various kinds of growth promoters. Thus, US citizens may be exposed to trace amounts of these growth promoters via meat consumption. Especially, in pregnant women eating beef, the growth promoters may disturb the delicate hormonal balance of the unborn child, resulting in long-term effects. To study the long-term reproductive health effects of early exposure, young men from Denmark and Finland were recruited from military drafting systems. Participation in this study required that the study subjects filled in questionnaire that included a detailed dietary questionnaire enabling disentanglement of the effect of in utero and current exposures. The subjects also underwent a physical examination and provided a semen, blood and urine sample. Additionally, the study included a questionnaire for the participants' mothers that included detailed information about diet, while pregnant with their son (including meat consumption), current diet, lifestyle, and other health information. For the US part of the study we investigated college students by using a protocol similar to that used to recruit Scandinavian populations of young men.

Theme 4: *Integrated systems biology studies* (WP8 and WP9)

The overall aim of the integrative systems biology studies was to create new cause-effect frameworks and knowledge networks to further refine research in the critical area of growth and reproduction. The

challenge was to establish associations of early developmental findings to adult reproductive health in order to facilitate prediction and prevention of male reproductive disorders. One aspect was to leverage the vast amount of data generated by the other partners of the consortium by applying data driven methods to produce cause-effects models, while at the same time pushing the frontier of integrative systems biology to encompass small molecule data. Such data integration in a relational infrastructure, linking the chemical exposure effect on a complete biological system, aimed to facilitate translational research for application in biology and medicine as well as for improving environmental risk assessment and risk management.

Project Results:

Theme 1

We investigated differences in combined chemical exposures between Denmark and Finland by using machine learning classifiers, which can take simultaneously all chemical concentrations into account. This analysis included breast milk levels of 121 endocrine disrupting chemicals, including dioxins, polychlorinated biphenyls (PCBs), pesticides, brominated flame retardants and phthalates. The previously analysed breast milk samples represented mothers of healthy boys. Distinct country-specific chemical signatures of EDCs were observed. The Danes presented higher concentrations of persistent chemicals (including dioxins, PCBs and some pesticides).

Differences in chemical exposures between healthy and cryptorchid boys

When using breast milk levels of the 121 above mentioned chemicals as a proxy for complex fetal exposure, a stronger correlation between chemical exposure and cryptorchidism was observed in the Danish than in the Finnish subjects. To further investigate the influence of correlating chemicals at a molecular level, a systems biology approach was used. In this analysis both PCB52 and PCB77 (which had a protective effect in the Danish subjects) were found to be connected to the gonadotropin releasing hormone pathway and to the arachidonic acid pathway. Of the chemicals associated positively with cryptorchidism, only two dioxin congeners had reported associations with genes other than cytochromes and the aryl hydrocarbon receptor. The genes are part of signalling pathways that affect cell proliferation, differentiation, motility and adenosine triphosphate (ATP) coupled biosynthesis.

We studied also a possible association between the levels of dioxins and PCBs in the placenta and cryptorchidism in the newborn. No evidence for an association was found.

Genetic studies

We have investigated whether 54 subjects with a history of cryptorchidism have mutations in genes underlying idiopathic hypogonadotropic hypogonadism. No mutations were found in the genes for FGFR1, PROK2, PROKR2, TAC3 or TACR3. Two subjects with a history of cryptorchidism had heterozygous mis-sense mutations in GNRHR. However, these mutations were present in control subjects with a similar frequency and were therefore unlikely to be important.

In the study including cases with symptoms of testicular dysgenesis syndrome (cryptorchidism, hypospadias, poor semen quality, testicular cancer), a genome-wide association study (GWAS) was combined with systems biology approaches. Markers located in the region of TGFBR3 (transforming growth factor β receptor III) and BMP7 (bone morphogenetic protein 7) showed associations with TDS phenotypes. The presence of TGFBR3 in peritubular and Leydig cells was confirmed in immunohistochemical examination of fetal and adult testes. These associations suggest that the transforming growth factor β signalling pathway has a role in the pathogenesis of TDS. The KITLG locus showed significant associations with testicular cancer, confirming previously published results.

In terms of the overall aims of studies using a rat model of TDS, the most important result to have emerged is the detailed evidence that morphological dysgenesis of the testis and impairment of fetal Leydig cell steroidogenesis, which leads to TDS disorders, both originate within the MPW. This is despite the fact that these two “endpoints” manifest at different ages, the impairment of fetal Leydig cell steroidogenesis manifesting within the MPW whereas the dysgenesis manifests after the MPW. The close inter-connection of these two different endpoints provides a strong endorsement of the original TDS hypothesis, proposed on the basis of occurrence and inter-connection of cryptorchidism, hypospadias, low sperm counts and testicular germ cell cancer (TGCC). However, perhaps the simplest but most important data to have emerged, is our demonstration that AGD provides a lifelong read-out of deficient androgen exposure just within the MPW and is closely correlated with occurrence of TDS disorders and ultimate size of all of the male reproductive organs (testes, penis, seminal vesicles, prostate) in adulthood. Our results show that AGD is a biomarker of both testicular dysgenesis and androgen action in the MPW and, as such, can be used in adulthood (or at earlier ages after birth) as a means of retrospectively divining the normality or otherwise of these “hidden” fetal events. In theory, AGD could be used to provide similar read-back in the human, and a number of publications have emerged in the past two years that have endorsed this use and shown similar inter-relationships between AGD, reproductive disorders and reproductive organ size in humans as has been shown in our rat studies. Our rat studies also imply that measurement of AGD at birth may be predictive of adult-onset reproductive disorders (e.g. low sperm production; small penis size), which again may have clinical implications.

The only component of human TDS that is not induced in our DBP-induced TDS rat model is TGCC and its precursor, carcinoma-in-situ (CIS) cells. This may be due to differences in the organisation and tempo of perinatal germ cell development, based on studies in our group. However, what we have shown in WP2 is that DBP exposure does significantly affect fetal germ cell development in several ways, and these are strictly age-dependent. The most vulnerable stage appears to be when the germ cells are undifferentiated and pluripotent, as DBP exposure induces significant loss of germ cells at this stage plus delaying their differentiation. Such effects may have some bearing on the origin of CIS cells, as this is thought to occur during the equivalent phase of development in the human fetal testis and involves failure of differentiation. Therefore, further study of the underlying mechanisms in the DBP model could be informative.

IUGR is a risk factor for TDS disorders in humans. Therefore we assessed if IUGR induced by maternal treatment with dexamethasone, on its own or together with DBP, was able to affect testis/reproductive tract development, AGD etc in rats. Our results showed that although dexamethasone induced IUGR, this was not sufficient to induce any TDS disorders although combination with DBP did significantly exacerbate the ability of DBP to induce such disorders. However, this effect was not ascribable to induction of IUGR but simply due to greater suppression of testosterone production by the fetal testis during the MPW. Our findings do not rule out that IUGR can induce TDS disorders, but our conclusion is that the growth restriction would probably have to occur during the MPW (1st trimester in humans), whereas the treatment-induced IUGR in our studies probably occurred after the MPW towards the end of gestation. Nevertheless, our studies of the mechanism via which DBP and dexamethasone induce suppression of testosterone production by the fetal testis show that both appear to target the same pathway (see below), which would neatly explain the additive effects of combined treatment. A novel finding to emerge from these studies was that suppression of testosterone production during the MPW, indicated by reduced AGD in adulthood, was associated with lower blood testosterone levels in adulthood with

evidence of compensated Leydig cell failure (i.e. elevated LH in the face of normal/low testosterone). This triggered new ideas and studies described at the end of this section on how fetal androgen exposure may programme adult Leydig cell function.

Preliminary studies had suggested that fetal exposure to DBP \pm dexamethasone might predispose to development of abdominal obesity and insulin resistance in adulthood, especially as this is associated in men with lower blood testosterone levels (as found in our exposed animals). However, detailed investigation of this possibility, including a glucose challenge test after overnight fasting, failed to provide strong evidence to support this. No further studies were therefore undertaken.

During our studies a paper was published showing that prepubertal knockout of a transcription factor, chicken ovalbumin upstream promoter transcription factor-II (COUP-TFII), resulted in failure of adult Leydig cells to develop and consequently near zero blood testosterone levels. This prompted us to investigate COUP-TFII expression in both postnatal and fetal testes in control and DBP-exposed rats and led to two major findings, only one of which has so far been worked up to publication. Our results showed that when fetal Leydig cells first differentiate in control animals they express COUP-TFII in their nucleus but during subsequent development, beginning in the MPW, the fetal Leydig cells progressively switch off COUP-TFII so that by e21.5 >85% of Leydig cells are negative for nuclear COUP-TFII expression. In DBP-exposed animals, this progressive switching off of COUP-TFII did not occur. Further studies showed that, in controls, normal switching off of COUP-TFII was associated with a 3-fold increase in cytoplasmic volume of each Leydig cell and a corresponding increase in testosterone production per Leydig cell, whereas these increases simply did not occur in DBP-exposed animals. In other words, DBP does not actually inhibit steroidogenesis by the fetal testis it prevents the normal age-dependent increase, a subtle difference, but with considerable implications. We propose that the DBP-induced maintenance of COUP-TFII expression in fetal Leydig cells competes with steroidogenic factor-1 (SF1) for binding to the promoter region of certain steroidogenic genes and thus inhibits expression of that gene and thus of steroidogenesis. This is based on our demonstration that Leydig cell genes with overlapping sites for COUP-TFII and SF1 in their promoter regions (StAR, Cyp11a1, Cyp17a1) are decreased in expression after DBP exposure whereas expression of a steroidogenic gene without overlapping sites (3 β -HSD) is unaffected. This interpretation fits with what has been described for COUP-TFII in other steroidogenic cells, namely that it is an active competitor with SF1. Further support for our interpretation came from our studies in which DBP treatment was not initiated until an age (e19.5) when COUP-TFII expression in fetal Leydig cells had switched off. We found that treatment for two days with DBP from e19.5 switched COUP-TFII expression in fetal Leydig cells back on (at e21.5) and this was associated with major suppression of steroidogenesis.

We went on to show that the effect of DBP on COUP-TFII expression in fetal Leydig cells was dose-dependent and that, in mice treated with the same regimen of DBP, COUP-TFII expression in fetal Leydig cells was not maintained and no impairment of steroidogenesis was found (as has been reported in the literature). Therefore, our results explain the dramatic difference in effect of DBP in fetal rats versus fetal mice. We also showed that other factors can also maintain/induce COUP-TFII expression in fetal Leydig cells of the rat, namely dexamethasone and diethylstilboestrol (DES), which may explain the ability of these treatments to inhibit fetal Leydig cell testosterone production. In this regard, DES also induced COUP-TFII expression in fetal Leydig cells in mice and reduced testosterone production. We also showed that COUP-TFII is expressed in some fetal Leydig cells in both humans and marmosets and

in the former there is a significant decline with fetal age in the % of fetal Leydig cells expressing COUP-TFII, as in the rat. Therefore, it is possible that certain factors might suppress fetal Leydig cell steroidogenesis in the human by maintaining/inducing COUP-TFII expression, although this remains to be shown.

In very recent studies we have identified that putative progenitor cells for adult Leydig cells are present in the fetal testis and express COUP-TFII strongly in their nuclei together with androgen receptor (AR), but do not express any established Leydig cell genes (e.g. LH receptor, SF1, steroidogenic genes). These cells give rise to the adult generation of Leydig cells from puberty onwards. Our preliminary findings show that decreased androgen exposure in fetal life (eg as a result of DBP exposure) leads to a substantial reduction in numbers of the putative adult Leydig progenitor cells in fetal and early postnatal life and to impaired function of adult Leydig cells. These studies are still ongoing.

The rat in vitro system (FEGA): Rat fetal testes at 14.5 days post-coitum were cultured in culture medium with or without DEHP or MEHP at different concentrations during 72hr. At the end of the culture, medium and tissues were stored at -80°C until use. It was found that DEHP produced a pro-androgenic effect after 3 days of culture, whereas MEHP displayed anti-androgenic effects on testis function. Histological observations and immunohistochemistry revealed that MEHP but not DEHP was able to increase the apoptosis index in a time- and dose-dependent manner. The pro-androgenic effect of DEHP could be link to the inability of the fetal rat testis to metabolize DEHP into MEHP (Chauvigne et al., 2009). The MEHP deleterious effects first occur on Leydig cell function by decreasing testosterone production, followed by a concomitant decrease in germ cell number and in Sertoli cell function as AMH production was also impacted (Chauvigne et al., 2009). Last, but not least, the FEGA allowed us to demonstrate that the metabolite of MEHP, 5OH-MEHP was also anti-androgenic in the rat fetal testis.

Investigations on the mechanism of action of MEHP on steroidogenesis using 5 different experimental approaches revealed a decrease in the production of androstenedione whereas the production of its precursor (17 β -OHprogesterone) was found to be increased, suggesting that MEHP blocks the activity of the C17,20 lyase enzyme converting 17 β -hydroxy-progesterone to androstenedione. This direct effect of MEHP on Leydig cell function was further established by microarray analysis and q-PCR showing that in addition to the cyp17a1 gene, several other genes involved in testicular descent, such as insulin-like factor 3(INSL3), or the alpha subunit of inhibin B, were also affected.

In addition to the work performed on phthalates described above, our studies have also revealed the ability of mild-analgesics to inhibit testosterone and prostagland production by the rat fetal testis. This is of major concern due to the fact that these pharmaceutical compounds are sold over the counter and are used widely by the general population, including by pregnant women.

The human cell line NCI-H295R: was used to decipher direct phthalate effects on steroidogenesis. DEHP, MEHP and ketoconazole inhibited testosterone production by NCI-H295R cells in a dose-dependent manner. DEHP inhibited testosterone with the same effects at 24 to 72 hr of exposure, whereas MEHP inhibited testosterone production at 24hr and 48hr but no effect was observed 72hr.

Biotransformation experiments allowed us to demonstrate that NCI-H295R cells were able to biotransform DEHP into MEHP and downstream products. The lower suppressive activity of DEHP

detected by 72 hr is likely to result from the metabolism of DEHP. The progressive loss of the suppressive action of MEHP reflects its biotransformation.

The global analysis of overall steroidogenic profiles in the presence of MEHP (steroidomics) confirmed the inhibition of testosterone and also showed inhibition of 17-hydroxy-pregnenolone and 17-hydroxy-progesterone levels, suggesting that MEHP could disturb testosterone production by targeting CYP17 hydroxylase activity.

Overall, we have observed an anti-androgenic effect of DEHP and MEHP in the human Cell Line Assay (CELIAS). The study of the production of testosterone precursors allowed us to identify potential targets of MEHP. CELIAS represents a good in vitro approach and a useful complementary tool to the organotypic culture system to screen the effects of potential endocrine disruptors on human steroidogenesis.

The potential effects of phthalates on human testicular function was also directly investigated using the Testis EXplant Assay (TEXAS). In this system, normal human testes obtained from prostate cancer patients, were cut into small pieces which were cultured in PET inserts in 1ml of medium \pm phthalates for 24 or 48 hours. We have demonstrated that phthalates have the ability to suppress human testicular steroidogenesis, notably androgen synthesis. DEHP and MEHP significantly inhibited testosterone production after 24hr of culture, whereas no effects were detected after 48hr without medium change. This decline of anti-androgenic effects after 48hr of culture most likely results from biotransformation of DEHP and MEHP. Indeed, we have demonstrated that as in the human cell line, the human testis was able to metabolize DEHP into MEHP and that the latter was further processed into 5OH-MEHP which was itself demonstrated by us to be anti-androgenic.

Phthalate anti-androgenicity did not affect the morphology of the testis as checked by histology examination, nor did it affect germ cell apoptosis. The study of both Δ^5 and Δ^4 steroidogenic pathways using the steroidomics approach, showed that all testosterone precursors were significantly inhibited by exposure of the human testis to MEHP.

In our experiments, ketoconazole known to exert potent inhibitory effects on testosterone production, showed a strong anti-androgenic effect. Ketoconazole also increased germ cell apoptosis, probably as the result of androgen deprivation.

In order to control the integrity of other testis function, we have investigated the effects of phthalates on insulin-like factor 3 (INSL3) production. INSL3 is a Leydig cell product responsible for the first phase of testicular descent during fetal life and is believed to be involved in germ cell survival and bone metabolism. Neither phthalates (nor ketoconazole) affected INSL3 production, showing that the phthalate effects on steroidogenesis was actually specific to the Leydig cells.

Furthermore, we report that neither DEHP nor MEHP had any effect on inhibin B, consistent with maintained Sertoli cell integrity. Accordingly, in humans, studies revealed that environmental exposure to DEHP was not associated with changes in inhibin B serum levels in healthy men, or in men recruited through a study on infertility.

Theme 2

The most salient results derived from the experimental studies on early exposures and sexual maturation are summarized below:

1. On the physiological front, experimental studies conducted under theme 2 have helped to establish that the hypothalamic Kiss1 system is sensitive to the early organizing effects of sex steroid-acting compounds and different metabolic manipulations, including over- or under-feeding. Our results have also documented the essential role of kisspeptin signaling in the timing of puberty and in generation of the pre-ovulatory surge, the hormonal trigger for ovulation. This physiological knowledge, obtained in suitable preclinical models, reinforces the view that early (chemical/hormonal/nutritional) disruption of the hypothalamic Kiss1 system is likely to have deleterious consequences in terms of pubertal maturation and fertility later in life.
2. In the same vein, studies conducted in theme 2 have been the first to document a relevant physiological role of the co-transmitter of kisspeptins, the neuropeptide neurokinin-B (NKB), in the control of puberty and adult gonadotropic function. In addition, our studies have demonstrated that development of NKB neurons in the hypothalamus, which is sexually dimorphic (females >> males), can be affected by early exposure to sex steroid-acting compounds, so that neonatal estrogenization results in lowering of the number of NKB-positive neurons. Furthermore, NKB expression is sensitive to conditions of metabolic stress. Altogether, these mechanistic data strongly suggest that the hypothalamic NKB system may also be a target for (chemical/hormonal/nutritional) disruption of pubertal maturation and reproductive function.
3. Sensitivity of the developing hypothalamic Kiss1 and NKB systems to the organizing effects of sex steroid-acting compounds was initially set in “proof-of-principle” studies, involving administration of a range of doses of synthetic estrogens, androgens and anti-androgens, during the neonatal critical period of brain sex differentiation. The initial observations in those “extreme” models have been later confirmed by exposure studies involving administration of environmentally-relevant doses of proven endocrine disruptors. Thus, via collaborative studies, we have documented the capacity of the xeno-estrogen, bisphenol A (BPA), to durably alter the patterns of expression of Kiss1 and NKB genes in key hypothalamic areas, such as in the arcuate nucleus and the rostral peri-ventricular hypothalamus. These changes were coupled to hormonal and phenotypic indices of pubertal disruption, thus suggesting their mechanistic relevance.
4. Changes in metabolic homeostasis and the feeding patterns during early developmental periods (such as early postnatal life in rat models) resulted in persistent alterations in the trends of body weight gain and the age of puberty; early exposures to androgen in the female and/or feeding on a high fat diet (HFD) enhanced the obesogenic action of postnatal overfeeding. In addition, obesogenic manipulations (neonatal overfeeding, neonatal androgenization, feeding on HFD) was associated with signs of metabolic disturbance and evidence for alterations in glucose homeostasis. As an example, female rats treated with the synthetic androgen, testosterone propionate, during the critical neonatal period, displayed a metabolic phenotype after the expected time of puberty, involving increased bodyweight and glucose intolerance, the magnitude of which was significantly augmented by the concurrence of other metabolic stressors, such as postnatal overfeeding or feeding on HFD from weaning onwards. Likewise, neonatal androgenization alters also the patterns of food intake (in basal and stimulated conditions), resulting in

enhanced feeding. In the same vein, male rats treated with the anti-androgen, flutamide, during the critical neonatal period, displayed variable but detectable alterations in the timing of puberty, as well as changes in bodyweight and LH levels, mainly when combined with obesogenic manipulations, such as postnatal over-feeding and/or feeding on a HFD after weaning. These results illustrate the potential synergistic interplay between sex steroid- and metabolic-insults as potential disruptors of the normal process of puberty. Again, this phenomenon would imply that, both in males and females, sensitivity to the disrupting effects of sex steroid-acting compounds could be modulated (and escalated) by the concurrent incidence of other stressors, such as metabolic insults.

5. Concerning pubertal maturation, our analyses in male and female rats unveiled changes in bodyweight gain and puberty onset due to postnatal and post-weaning over- and under-feeding, which were cumulative in nature. Obesogenic manipulations induced earlier puberty onset, but sensitivity to those manipulations was different between males (more sensitive to early postnatal overfeeding) and females (more sensitive to actual overfeeding during pubertal maturation). Differences were also detected between gestational and postnatal underfeeding. The above changes were associated with modifications in the circulating levels of key metabolic hormones, such as insulin and leptin, as well as in gonadotropin concentrations. As an illustrative example, in female rats, obesogenic exposures coupled to earlier puberty were associated with elevated insulin and leptin levels at the time of puberty. In addition, mechanistic analyses revealed that earlier puberty onset due to postnatal overfeeding was associated with increased expression of *Kiss1* mRNA expression in the hypothalamus and trends for higher numbers of kisspeptin fibres, whereas late puberty coupled to postnatal underfeeding was linked to lower *Kiss1* mRNA expression and decreased numbers of kisspeptin-positive neurons in the hypothalamic arcuate nucleus.

6. Recent recognition of a putative role of the RNA-binding protein, LIN28B, in defining the timing of menarche in humans, as evidenced by genome wide-association studies (GWAS), prompted us to conduct expression analyses of *Lin28b* mRNA (and the related *Lin28a*) in the hypothalamus of rat models, both during postnatal development and after early exposures to synthetic sex steroid compounds. In addition, given the fact that the major known role of LIN28b is to repress the synthesis of mature microRNAs of the let-7 family, complementary expression analyses of let7a and let-7b were also conducted in the above conditions. Our analyses have documented a significant drop in *Lin28* expression as a function of pubertal maturation, which was associated with a significant elevation of let-7A and let-7B miRNA levels. Such a reciprocal trend is in keeping with the proposed role of *Lin28* as a negative modulator of mature let-7 miRNA synthesis, suggesting a functional, dynamic interplay between these two sets of factors in the developing hypothalamus. Of note, early exposures to estradiol benzoate (males and females) and testosterone propionate (females) induced persistent reciprocal changes in the expression levels of *Lin28B* mRNA (increase) and *Let-7* miRNA (decrease) in the hypothalamus at the expected time of puberty. These changes seem to partially reverse the maturational switch in the balance between *Lin28B*/let-7 levels during pubertal maturation. Our data are compatible with a potential role of this novel miRNA regulatory system in the control of mammalian puberty, and suggest that it may be a target for the effects of sex steroid-acting compounds (either endogenous or synthetic) at critical periods of brain sex differentiation.

To determine whether there is a trend towards earlier pubertal timing in Europe, we systematically collected data from the same region at the beginning and end of a 15-year period, in 1991 and 2006.

In all, more than 2000 girls were studied. Pubertal timing, defined as onset of breast development occurred significantly earlier in girls investigated in 2006 as compared to girls investigated in 1991. Actually, breast development started on average a year earlier in 2006 as compared to in 1991, namely at the age of 9.9 years. In addition, duration of pubertal transition increased among girls born more recently. Alterations in reproductive hormones and BMI did not explain these marked changes, suggesting that other factors yet to be identified may be involved.

In boys, the examination of more than 1500 subjects showed that onset of puberty occurred significantly earlier in boys examined in 2006 as compared to in boys examined in 1991. Pubertal onset was defined as the age at attainment of testicular volume more than 3 ml. As compared to girls, the secular trend was less pronounced, in that boys born more recently had pubertal onset on average 3 months earlier (11.6 versus 11.9 years). Notably, in boys the decline was associated with the coincident increase in BMI.

Genetics and puberty

Pubertal timing is a strongly heritable trait (more than 60% of the variability in menarcheal age between subjects is genetically determined), but no single puberty gene has yet been identified. We have looked at genetic variation in different genes, for which there is some evidence of involvement in pubertal development. These include genes named KISS1, KISS1R, LIN28A and LIN28B. The variation in LIN28B, LIN28A, KISS1, and KISS1R was studied in girls with precocious puberty defined as pubertal onset before 8 years of age. We did not detect any rare variants for KISS1 or KISS1R in the subjects with precocious puberty. For LIN28B a mutation was found in one subject with precocious puberty. However, this variant was also detected in one of the 132 controls. No variation in LIN28A was found. In conclusion, we did not find any evidence that mutations in LIN28B or LIN28A would underlie precocious puberty. In addition, we confirmed that mutations in KISS1 and KISS1R are not a common cause for precocious puberty.

Also, genes encoding proteins important for gonadal function and sex steroid clearance were evaluated. These include, for example, genes involved in the function of growth. For example a certain and common mutation in the growth hormone receptor gene (called GHRd3) which occurs in 10% of Danish subjects, results in a growth hormone receptor which seems more responsive to GH activation. Also, a common polymorphism (9% of Danish boys) in the UGT2B17 gene which encodes an enzyme (glucuronidase) important for urinary excretion of androgens was evaluated. In boys, the GHRd3/d3 genotype was associated with smaller birth size and earlier age at pubertal onset compared with the GHRfl/fl genotype. Thus, this common polymorphism could play a role in prenatal growth and gonadal development in boys. The common mutation in the UGT2B17 gene strongly affected the urinary excretion pattern of androgen metabolites in pubertal boys but did not influence circulating androgen levels. Ongoing studies will evaluate whether or not it influences pubertal timing. Interestingly, these associations were not evident in girls.

Thus, the chosen genes have in various ways been coupled to the timing of pubertal development, which is the reason we included these in our analyses.

Non-genetic factors associated with pubertal timing

Early puberty is associated with marked changes in endogenous sex steroid levels, as well as increased risk of subsequent cardiovascular disease. At the very beginning of puberty, conventional methods cannot determine sex steroid levels in blood samples from young children, as the methods are too insensitive to detect the low levels. Consequently, we developed new sensitive techniques within DEER which enabled measurement of sex steroids in young children before and after onset of puberty. These results gave new insight into the importance of the different endogenous sex steroids for pubertal timing. Many sex steroids circulate in plasma bound to sex hormone-binding globulin (SHBG). Interestingly, we found that the individual SHBG level is a feature of early puberty as well as conditions associated with increased cardiovascular risk. Based on our results we found that measurement of SHBG is a valid pubertal marker which integrates the marked changes in glucose metabolism and body composition that occur during pubertal transition.

Exposure to endocrine disrupting chemicals and timing of puberty

We determined the urinary concentrations of 12 phthalate metabolites in urine samples from approx 500 healthy boys, and from more than 700 healthy girls. The vast majority of children had detectable and even high levels of phthalate metabolites in their urine sample. In boys we found that high phthalate levels were associated with fewer boys having detectable serum testosterone levels, but phthalate levels were not statistically significantly associated with the age at which clinical signs of puberty occurred. Furthermore, we could not confirm that phthalates were associated with pubertal gynaecomastia as suggested by others. In girls, we found no statistically significant associations with age at breast development, and urinary levels in girls with central precocious puberty were similar to that of control girls. Importantly, we found that girls with the highest phthalate exposure had significantly later pubic hair development. This is compatible with the anti-androgenic effects of phthalates which have been demonstrated in animal studies and with in vitro studies using adult human testes (theme 1).

Theme 3

Perfluorinated compounds

Perfluorinated compounds, among which PFOS and PFOA are man-made chemicals used in consumer and industrial products, for example, for impregnation of carpets, textiles, and paper. They are present in food packaging materials, such as paper and board, and have been shown to migrate into food. PFOS and PFOA are present ubiquitously in the environment, wildlife, and in the human body. They are persistent, extremely resilient to degradation in the environment and known to bio-accumulate in animals as well as in the human body, where the compounds have half-lives of years. The scientific field of perfluorinated compounds as endocrine disrupting chemicals is still new. Therefore, evidence for endocrine disrupting effects in animal studies and indications of a connection between exposure to PFOA and PFOS in humans are still limited. There are, however, some suggestions that the compounds can disturb testosterone function and thereby interfere with reproductive health.

We investigated the association between exposure to PFOA and PFOS and reproductive function in terms of semen quality and reproductive hormone levels in young Danish men. Our primary hypothesis was that

high concentrations of PFOA and PFOS would be associated with low testosterone levels and impaired semen quality.

We found, in a study of 105 healthy subjects, that men with high combined levels of PFOS and PFOA had a median of 6.2 million normal spermatozoa in their ejaculate in contrast to 15.5 million among men with low PFOS/PFOA. In addition, in a newer and larger study of 247 men, PFOS levels were inversely associated with testosterone levels and other hormonal markers of testicular function. Thus, we can conclude that high exposure to these perfluorinated compounds is associated with impaired testicular function. This is within a population of healthy men with “normal” exposure levels.

Phthalates

Phthalates are widely used for a multitude of purposes that include plasticizing, viscosity control, solvents and enteric coating of modified-release pharmaceuticals. Humans are continuously exposed, apparently both via the skin, through inhalation and via food. Phthalates are rapidly metabolized in humans, and the metabolites are excreted mainly in urine, with elimination half-lives of usually less than 24 hours. Urine is the preferred matrix in which to assess exposure, and consequently it is the primary and secondary oxidized metabolites that are measured. It is well established that some phthalates act as endocrine disruptors resulting in developmental abnormalities of the reproductive tract as well as inhibition of testicular testosterone production in animals exposed before and immediately after birth. Also in humans, various studies have documented associations between high phthalate exposure and impaired reproductive function.

In our phthalate studies, the focus was on investigating why some people may be more susceptible to the effects of phthalate exposure as compared to other people. In detail, we looked at how differences in phthalate metabolism affects the chemicals’ impact on testosterone levels, and how certain mutations affecting skin permeability may affect exposure to phthalates.

In one study, we found that the phthalate excretion pattern is negatively associated with reproductive hormone levels. The phthalates of interest are DEHP (di(2-ethylhexyl) phthalate) and DiNP (di-iso-nonyl phthalate). This study included 881 healthy young Danish men. Specifically, our findings suggest that if mostly the primary metabolites of phthalates are excreted (meaning that a person is not efficient at getting rid of the compound), it is more likely that phthalate exposure impairs testosterone levels and the communication between testicular testosterone production and the regulatory input from the brain. This is in comparison to individuals in whom it is primarily the secondary phthalate metabolites that are excreted (meaning that a person is good at getting the compounds inactivated and out of the system). Overall this means that the capability of the body to metabolize (and thereby change the toxicity of) phthalates plays a role in determining how harmful phthalate exposure is. One factor playing a major impact on the pattern of metabolism is genetics.

In another study we have investigated whether a certain gene mutation alters the degree to which people are exposed to phthalates. The mutation in question is found in a gene encoding the protein filaggrin, which is important for how strong the skin barrier is. Hence, people with a filaggrin mutation have more sensitive and permeable skin, which for example increases their risk of getting atopic dermatitis. The impaired skin barrier may also result in a higher permeability to various chemicals, such as phthalates

(which is also present in various skin lotions). Our hypothesis was therefore, that people with a filaggrin mutation would have higher phthalate levels in their urine.

The study showed that this was indeed the case. Adult Danes with a filaggrin mutation (on both alleles) had a significantly increased excretion of both short- and long-chained phthalates, up to 40% higher for the metabolite of the phthalate DnBP (di-n-butyl phthalate). We did not see associations between the filaggrin genotype and other reproductive hormones or semen quality.

The higher phthalate levels in filaggrin mutation carriers may reflect a higher level of exposure due to increased use of personal care products to alleviate dry skin. Alternatively, the filaggrin mutation carriers may be a group at risk for elevated exposure to any class of chemicals that is present in products that can come into contact with the skin and can permeate it. This cannot be resolved from our study. Anyway, our findings suggest that filaggrin mutants have higher internal exposure to phthalates and we speculate that this applies to other chemicals as well. It is important to treat and protect a defective skin barrier, but also to ensure that harmful chemicals are not overlooked as a possible side-effect of the treatment

Consumption of beef on human semen quality

New questionnaires aimed at describing meat consumption were developed and validated. The questionnaire for the young men included a detailed dietary questionnaire enabling disentanglement of the effect of in utero and current exposures. The questionnaire for the mothers of the young men included detailed information about diet, while pregnant with their son (including meat consumption), current diet, lifestyle, and other health information.

More than 200 American young men aged 18-21 years and living around Rochester NY participated in the project with their mothers. According to the questionnaire data, the mothers of the young men ate on average 2.5 meat meals per week while pregnant. No mothers reported eating more than 1 beef meal per day and only 8% reported eating 5-6 beef meals per week. Thus, meat consumption was markedly lower in the studied group as compared to the group investigated in the paper from 2007 by Swan et al. In the latter, the mean number of beef meals was 4.3 per week, and 13% of the women reported eating more than seven beef meals per week.

In the present study, there was no relation between mothers' beef consumption and semen quality of the sons (assessed as sperm counts, concentration, motility, and morphology). The lack of correlation may be explained by the more limited beef consumption when compared to the Swan study. Since it was in the group of "high beef consumers" that the association with sperm count was seen, the current study cannot address the hypothesis that high beef consumption during pregnancy is inversely associated with decreased semen quality in the sons. However, the current study does show that moderate beef consumption is not associated with a decreased semen quality.

There was no relationship between the men's own beef consumption, and any of the semen parameters that were assessed.

In addition to the association studies, serum samples from US citizens were analyzed for the growth promoters (or metabolites of) trenbolone acetate, melengestrol acetate and zeranol. In the first step, serum samples from citizens who reported having a high consumption of beef were analyzed. Specifically

developed analytical methods were used that permitted to reach a new standard in terms of sensitivity. Still, however, none of the analyzed growth promoters were detectable in the analyzed samples. This means that trace amounts of growth promoters in high beef consumers were below 1 pg/mL, which was the detection limit of the methods used.

However, we cannot conclude that high beef consumption (for example, up to 21 beef meals per week, consumption in the 1970s and 1980s reported by women from the Midwest in Swan et al 2007) is unrelated to growth promoters, since beef consumption in the current population was much lower.

Theme 4

One major challenge was to establish and maintain a relational database with the integration of chemicals and biological information from publicly available sources and integration of data resulting from the DEER consortium. For example, chemical exposure-outcome information is likely to be influenced by pathways or biological interactions between different exposures. Identification of such interactions is usually carried out for few chemicals and we decided to establish such links in a high-throughput fashion. First, we developed a chemogenomics pipeline, i.e. we collected small molecules with bioactivity against proteins. In a second step, with the assumption that molecular targets that interact (physically) each others (i.e. forming protein-protein interactions) are likely to be involved in the same cause-effect framework, we integrated high confidence human protein-protein interactions (PPIs) network for each of the bioactive targets. Such inference gives the possibility to enrich the biological entities that are involved, for example in reproduction disorders, and to explore new mechanism of action. Presently, the chemogenomics platform contains more than 700 000 compounds with biological activity for more than 30 000 proteins reaching more than 2 millions interactions. In addition, we gathered the chemical structure of all compounds. It is well accepted that chemicals sharing highly similar structures commonly share similar biological properties. Using this concept, two chemicals having positive biological activity for two different proteins, and sharing a similar structure can be suggested to have a similar effect on both proteins. This enables assessment of the chemical effect on biological systems. Such an approach is available through a publicly available web server, called ChemProt, designed during the DEER project. Using ChemProt users can obtain information at the cellular level, by linking a chemical that induces biological perturbations to diseases, phenotypes and specific tissues.

Using such database implementations and with the application of mathematical models, we intended to evaluate the influence of a panel of endocrine disruptors (EDCs) on congenital cryptorchidism at a systems level. Indeed, cryptorchidism, occurring in 2-9% of newborns, is one of the most common genital malformations among boys and is associated with decreased semen quality and a higher risk of testis cancer. In most cases no obvious genetic aberration can be revealed (apart of INSL3) and environmental factors (including chemicals) are suspected to have a major role in such male reproductive disorders. Recently, a study of possible association between in utero exposures to certain persistent organic pollutants (POPs) and cryptorchidism was performed in a joint longitudinal study between 1997-2001 at the National University Hospital, Rigshospitalet, Copenhagen, Denmark and the Turku University Hospital, Turku, Finland. A collection of breast milk samples from Finnish and Danish mothers of newborn boys analyzed for the presence of 130 EDCs with known endocrine disrupting effects in animals were considered for analysis. In DEER project, we explored the overall exposure of 130 EDCs in the

breast milk of pregnant women from Denmark and Finland in healthy boys. We depicted a higher exposure level for many persistent EDCs (essentially PCBs and PBDEs) in Denmark compared to Finland. To complement this study, we assessed also the potential relationship between healthy and cryptorchid boys to the distribution of the chemical levels measured in breast milk from both populations. Whereas in the Finnish cohort, no chemical exhibited a statistically significant difference between cases and controls, the concentration of 30 chemicals (including PBDEs, PCBs and dioxins) varied much more between cases and controls in the Danish cohort. In order to investigate the influence of these chemicals at a molecular level, we interrogated ChemProt. As an example, for PCB 52 and PCB 77, we extracted 12 and 31 known interacting proteins respectively. All proteins were integrated in a protein-protein interactions network, adding a total of 124 proteins. Biological enrichment information (pathways) was performed on this network of protein complexes. The outcome of the pathway analyses showed two pathways related to reproductive disorders, which were common to PCB52 and PCB77: (1) the gonadotropin releasing hormone pathway (GnRH) and (2) the arachidonic acid metabolism pathway. Such results support the hypothesis that the mixture of environmental chemicals may contribute to observed adverse trends in male reproductive health. These pathways can therefore be targeted in future studies.

Finally, we investigated the integration of the protein-protein interaction networks with genome-wide association study (GWAS) in order to evaluate genetic predisposition associated to testicular dysgenesis syndrome (TDS). Considering a cohort comprised of 927 individuals of Danish descent: 439 healthy young men and 488 cases (testicular germ cell cancer, cryptorchidism, hypospadias or infertility with low sperm concentration), a strong association has been found for nine markers with all phenotypes of TDS ($p < 10^{-6}$) notably the genes KITLG, TGFBR3, BMP7 and HOXDx in agreement with other GWAS studies. Combining these results with heterogeneous data types such as gene expression time-series studies of the developing fetal testis in mouse and human, protein-protein interactions data, targeted mice knockouts resulting in developmental defects of the testis, 14 top-ranked genes, many on which are active during early development, were selected. A replication of this study on 671 Nordic men allowed validating three genes KITLG, TGFBR3 and BMP7. Overall, integrative systems biology analysis suggests several disparate loci that are functionally linked to the TGF β superfamily-signaling pathway.

Regarding the metabolomic activities, a global methodological workflow based on liquid chromatography coupled to high resolution mass spectrometry was developed, allowing the generation of reproducible and informative metabolic chemical phenotypes from human serum samples. This methodology was then applied to around 100 serum samples from adults presenting various semen qualities. The existence of different serum biological signatures between men presenting poor versus high sperm concentrations was then demonstrated. The association between these metabolic profiles and the spermatozoa concentration variable was observed and confirmed on two independent sets of samples. The structures of a few metabolites of potential interest were elucidated and allowed to point out peptide marker candidates. However, the extremely low concentrations of the corresponding metabolites as well as the very discrete fold changes observed even statistically significant have limited this structural elucidation step and subsequent biological interpretation. Finally, this project has demonstrated that metabolomics is promising in clinical studies for descriptive purposes, by opening the door to wide and multi-endpoint chemical characterisation of biological samples. For explicative purposes, metabolomics is promising as well, given that the generated chemical phenotypes to be associated to the studied health outcomes may be an important source of renewed research from a mechanistic point of view. Nevertheless, an important

input is still required in terms of biological and mechanistic interpretation of the generated results. One main reason for this observation is the very high inter-individual variability commonly encountered in human that undoubtedly contributes to decrease the correlation between the abundances of metabolites and defined clinical outcomes. Indeed, in the frame of in vivo human studies conducted at a population scale, the observed changes in terms of chemical profiles may be the result of many different sources of variability (diet, diurnal variation, cultural influences, but also therapeutics, underlying pathologies, ethnic differences). This multifactorial determination of final chemical phenotypes from human biological material is now well recognized by the metabolomics community as a main difficulty and challenge for discovering diagnostic biomarkers. Thus, the simultaneous consideration of genetic, epigenetic, lifestyle and environmental factors today appears as the major issue of integrative biology.

Potential Impact:

Theme 1

The results from human studies on early developmental effects of environmental factors have been published in peer-reviewed scientific journals. This project has demonstrated that integration of GWAS with systems biology approaches can be useful for identification of new markers and candidate genes involved in TDS and thus in male reproductive health. The integrative assessment of complex chemical exposure patterns in relation to congenital cryptorchidism by use of machine learning classifiers and systems biology indicated that the use of machine learning classifiers together with systems biology seems useful for modelling complex human scenarios in relation to health outcomes.

This study has also generated new sets of human exposure data, which will be exploited when evaluating the associations between exposure to the analysed chemicals and development of hypospadias. The exposure data may also be used for temporal trend analyses of chemical levels.

The overall impact of results from the studies on the rat model of TDS has been to fundamentally advance understanding of TDS disorders, their origins, the importance of fetal androgens in normal development and in TDS, to identify a useable biomarker (AGD) that informs on this, and to identify a completely new mechanism important for regulating testosterone production by the fetal rodent testis, and potentially also by the human fetal testis. Unquestionably the most important impact has occurred via identification of the MPW and of AGD as a read-out of androgen exposure during the MPW. Not only does this provide the ability to “look back in time” in experimental rodent studies, it has opened up this possibility also for human studies, something not previously possible. Indeed, a number of publications in the past 3 years have used the information on AGD as a biomarker of fetal androgen exposure in the MPW, as the basis for studies in human males with cryptorchidism, hypospadias, low sperm counts or low testosterone levels, and shown similar relationships of these to AGD as in the WP2 rat studies. Many more studies are in the pipeline, but already these new studies have reinvigorated the TDS area as they have shown that fetal (MPW) origins of human male reproductive disorders are remarkably common. On the more general conceptual level an important impact of these developments has been to validate the TDS hypothesis, the more so because the animal studies have shown a close inter-relationship between the occurrence of focal testicular dysgenesis, impaired testosterone production in the MPW, reduced AGD and occurrence of downstream TDS disorders – more or less exactly as predicted in the original hypothesis based on clinical observations.

Another impact that results from identification of the MPW is that it has sharpened up the process of hazard identification and risk assessment, because in relation to TDS disorders (which comprises the commonest male reproductive disorders in humans) it has pinpointed the time period for concern about exposures to exogenous factors such as endocrine disrupting chemicals. For the same reasons it has altered the approach of certain epidemiological studies concerning TDS disorders in humans by showing that these need to account in particular for exposures during the first trimester of pregnancy.

Two other major outcomes of our experimental animal studies have yet to cause impact because they are so recent (one about to be published, the other as yet unpublished), but they are both expected to have

considerable impact. First, the identification of how DBP (and other phthalates) affect fetal testicular steroidogenesis will have considerable impact on research as well as on risk assessment; identification of a mechanism of effect provides a much sharper focus for evaluating if similar effects occur in the human fetal testis (and our results suggest the answer is No). There will also be impact because the identified mechanism is completely novel and unexpected – in many respects it is the antithesis of what researchers had been looking for - and is clearly vulnerable to effects of factors other than DBP. The identified mechanism also reconciles a number of otherwise disparate findings in the literature relating to targeting of SF1-dependent genes (but no effect on SF1 itself), and species-specific differences in effects of phthalates. The second outcome which is expected to perhaps have as wide an impact as identification of the MPW, has been the demonstration that deficiency in fetal androgens (during the MPW, but perhaps also later in pregnancy as well) results in altered proliferation/numbers of the progenitor cells for adult Leydig cells and consequences for adult Leydig cell function. This adds a new and fundamentally important dimension to the TDS story because of the potential ramifications of this observation. It implies that adult testosterone levels, which have an intimate relationship to risk of “Western” diseases, are fundamentally influenced by fetal events, in particular by fetal androgens. Thus this may be a new way via which fetal events can shape adult risk of these disorders.

To study the potential effect of phthalates on steroidogenesis we used three different in vitro models in animals and the human, from the fetus to the adult. The three systems showed direct anti-androgenic effects of phthalates in the rat fetal testis, and anti-androgenic effects in humans. However, the sensitivity and the mechanism of action DEHP and MEHP appear to differ from one model to another.

Indeed, we have shown that DEHP (10-5M) produced a pro-androgenic effect in the rat fetus while it significantly inhibited testosterone production in both human models. Biotransformation experiments in the three models revealed that the fetal rat testis, under our culture conditions, does not process DEHP into MEHP, whereas the cell line and the human adult testis were able to process DEHP into MEHP, and further into 5OH-MEHP. We have also demonstrated that the mechanism of action of MEHP could be model-dependent. Indeed, in the fetal rat testis MEHP specifically blocks the 17,20 lyase activity of CYP17, whereas in the TEXAS, MEHP inhibited all testosterone precursor production. These discrepancies between species are due to the specificity of the cyp17 lyase activity. The enzyme promotes the Δ^5 pathway in humans, whereas it promotes the Δ^4 pathway in the rat.

Last but not least the fact that we demonstrate that mild analgesics can exert anti-androgenic effects and to inhibit testicular prostaglandin production in the rat fetal testis should lead to new research on the possible endocrine disruption induced by these drugs, notably during gestation in humans or at different stages of pubertal development.

In conclusion, in vitro studies provided evidence that phthalates directly affect steroidogenesis in all three models studied. Some discrepancies were revealed between the models, and notably between the rat and human models. Indeed, the results confirm that endocrine disruptor effects demonstrated in animals should be carefully transposed to human and it is thus mandatory that experimentation is also performed in human model-systems.

The main conclusion is that phthalates should be considered as endocrine disruptors in humans, at least when adult steroidogenesis is considered.

In this way, partner 5 has communicated information about the project with the scientific community but also the general public. Indeed, one article about the effect on human steroidogenesis was published in collaboration with partner 8 (Desdoits-Lethimonier et al., 2012), and both partners participated at different international congresses (see dissemination part). Moreover, some information was disseminated in a strictly controlled manner to the general public as press releases in national newspapers or as TV reports.

Theme 2

Implementation of the in vivo model to study the impact of early and late environmental exposures on reproductive maturation and obesity in rats has resulted in significant advancement of our knowledge of the physiological basis of the bidirectional interactions between hormonal and metabolic influences modulating the development and function of the neuroendocrine networks controlling energy homeostasis, puberty onset and reproductive function; it has also provided novel mechanistic evidence for their eventual disruption by different environmental, hormonal or metabolic insults occurring at different maturational stages. While this knowledge is mechanistic and preclinical, it may provide the scientific basis for a better understanding of endocrine and metabolic disruption of puberty and related functions (e.g., bodyweight and energy homeostasis).

Dissemination of results has been implemented via publication of original papers in international, peer-reviewed journals (in most cases of high impact in the first quartile of the area of Endocrinology and Metabolism), as well as invited reviews also in international journals. In addition, results from this study have been presented in numerous international scientific meetings, via invited presentations. Additional dissemination of results of this study has been conducted via local scientific and other meetings, and interaction with local media (in some cases direct to the lay public).

Our striking results from the DEER project showing much earlier puberty nowadays among Danish girls were disseminated through numerous interviews in major international news media including New York Times, The independent and BBC as well as in national newspapers and TV programs (TV avisen, DR2). Results have been presented as lectures and poster presentations at international scientific meetings (ECE Istanbul 2009, ESPE New York 2010, ESPE Glasgow 2011, COW 2009 and COW 2011). Furthermore, data have been presented on several occasions to the Danish EPA, as well as at public meetings for lay people and journalists at our hospital.

We have described a worrying and hitherto unexplained downwards trend in age at pubertal onset in healthy Danish children. Clearly, obesity, insulin resistance or single endocrine disrupting compounds could not explain our findings. This is not surprising as we are exposed to hundreds of endocrine disrupting compounds which may act in concert (cocktail effect) on different genetic backgrounds in each child. Therefore, complicated systems biology approaches combining genetic polymorphisms, lifestyle factors and exposure to hundreds of chemicals are needed to unravel the true exposome and its effects on sexual maturation. Thus, our report of a new puberty epidemic has caused a lot of concern and given rise to numerous questions from professionals as well as from the public. We have tried our best within the DEER consortium to explain the myths and facts in an open and honest way. But the truth is that inasmuch as we - as researchers and clinicians within the DEER project - have gained new information

and knowledge on the altered timing of puberty – we are still left with many unanswered questions. Certainly, we do not have any simple explanations for this trend. Something in the environment and/or lifestyle influences our child population and affects their sexual maturation. This is worrying in itself. And it remains to be seen whether or not the altered pubertal timing will have long term consequences for risk of certain diseases. Regardless of this, we cannot just watch this peculiar phenomenon influencing the development of children of today, but have to continue to monitor pubertal timing, and to study potential determinants of early puberty, and hereby identify possible risk factors and compounds affecting puberty. Only then will we be able to take preventive measures in the future.

Theme 3

For the perfluorinated compounds PFOS and PFOA, very little is known about their endocrine disrupting properties in humans. Our studies are some of the first to document associations between the degree of exposure and testicular function. Notably the findings were done in a population of healthy men with normal exposure patterns. Such findings indeed call for further examination of the perfluorinated compounds and call for greater awareness about the exposure to, and the biological consequences, of this large group of compounds.

For phthalates, multiple studies, both animal and epidemiological studies, point towards endocrine disrupting, especially anti-androgenic, effects of this compound group. Our studies take this knowledge one step further, so to say. It answers to some degree the question as to why some people are more susceptible towards exposure compared to others. The answer is the heterogeneous genetic background. Knowledge about susceptibility is a key to ensure the right direction of precautionary actions. And to understand the biology behind differences in vulnerability.

The knowledge gained in our studies has been presented at various levels. Within the scientific community, presentation of our data at national and international conferences ensures that the conclusions can be implemented in future studies. The results have indeed also been communicated to the wider public at national open information meetings, as well as to the media.

Results from the study in which we investigated the consumption of beef on human semen quality are somewhat reassuring, since levels of current beef consumption in upstate New York, appeared to be unrelated to men's semen quality. Moreover, no traces of hormones known to be in US beef were detected in their serum. Nonetheless, they do not rule out associations for high beef consumption.

Theme 4

Identifying the combined effects of genetic and environmental factors in a study cohort is extremely complex and the risk assessment of chemicals is in most cases multi-factorial and likely to be influenced by pathways, genomic variations and biological perturbations. Meta-analyses of large-scale association studies typically use only one data type and therefore only a fraction of the relevant evidence is exploited. With the development of an integrative systems biology approach, which enables the combination of heterogeneous data from chemogenomics, GWAS studies, protein-protein interactions data, linkage

studies and gene expression experiments, into a multi-layered evidence network, identified candidate genes associated to reproductive disorders can be prioritized and subjected to further experimental analysis with greater confidence. It provides also the possibility to analyze the chemical exposure in a complex environment and to identify genes and pathways involved in reproductive health. With the rapid advance in technologies to develop heterogeneous and complementary data, such as next generation sequencing, metabolomics and epigenetics, integration of systems biology should help in the decision of the etiology framework of a variety of diseases, including the reproduction disorders and in evaluation of the risk assessment of environmental chemicals.

Through the valuable set of new human chemical exposure data generated, covering a wide range of target chemicals and a high number of individuals characterised in terms of internal dose, the present project have undoubtedly contributed to provide new and large scale useful information to contribute to risk assessment. The inclusion of some “historical” persistent chemicals (dioxins / PCB) may now enable the characterisation of temporal trends from previous available data, while data generated for more emerging substances, including very rarely monitored compounds (HBCD, BDE 209), provides a unique opportunity to investigate the possible impact of these substances in humans, which might be useful from a risk assessment perspective for European and/or member states food safety agencies. These data are also expected to contribute to better information for consumers and possibly to support public authorities in terms of risk management. Metabolomic activities initiated in the frame of this project may be considered as a new basis for multiclass multi-residue assessment in toxicological studies, also with direct potential impact for regulatory purpose in the context of REACH. The further integration of the generated data on an even larger scale is also expected to contribute significantly to progress in this integrative biology area, and to refine the contribution of this environmental component to the studied health outcomes.

List of Websites:

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