PROJECT FINAL REPORT

Grant Agreement number: 212502

Project acronym: CONTAMED

Project title: Contaminant mixtures and human reproductive health – novel strategies for health impact and risk assessment of endocrine disrupters

Funding Scheme: Collaborative project

Period covered: **from** 01/05/2008 **to** 31/10/2012

Name, title and organisation of the scientific representative of the project's coordinator[1]:

Name: Dr. Frances Orton

Organisation: Institute for the Environment, Brunel University

Tel: +44(0)1895 266208

Fax: +44(0)1895 269761

E-mail: frances.orton@brunel.ac.uk

From 1 January 2013 onwards:

Name: Prof Andreas Kortenkamp

Organisation: Institute for the Environment, Brunel University

Tel: +44(0)1895 266525

Fax: +44(0)1895 269761

E-mail: andreas.kortenkamp@brunel.ac.uk

Project website address: http://www.contamed.eu/

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the grant agreement

Contents

Executive Summary 4
Project context and objectives 5
Background 5
The state of the science before CONTAMED 6
Motivation and aims of CONTAMED 6
Main S&T results/foregrounds 8
Bioassay-directed fractionations of human tissue extracts and identification of new anti-androgenic pollutants 8
Method development 9
Detection of fractions with anti-androgenic activity 10
Identification of novel candidate compounds in active fractions 10
Development of biomarkers for internal exposure to anti-androgens 12
Dealing with toxic masking and endogenous androgenicity 12
Making fractions comparable – procymidone equivalents 12
Investigating new EDC-relevant modes of action and new candidate chemicals – suppression of prostaglandins and AR antagonistic pesticides 12
Targeted chemical analyses for multiple EDCs in human tissues and estimation of human exposures 14
Assessing the joint effects of EDC mixtures in experimental studies modelled on high-end human exposure scenarios 15
Selection of chemicals for inclusion in the mixture 15
Selection of doses and experimental design 15
Observations of combined effects in the rat 15
Further developmental toxicity studies in the rat 16
Development of the brain 17
In vitro experiments with combinations of endocrine disrupters 18
Epidemiological studies of the role of chemical exposures in contributing to cryptorchidisms and hypospadias 18
Mild analgesics and cryptorchidisms 18
Associations between single EDCs and congenital malformations 18
Associations between biomarkers of internal exposure to AR antagonists and congenital malformations 19
Interpretation of results 19
Further work 19
Implications for chemical regulation and health impact studies 20

Potential impact and main dissemination activities 21
Potential impact 21
Scientific impact 21
Impacts on chemical regulation in the EU 22
Alerts to health care professionals and the general public 22
Dissemination activities 23
Cited literature 24
Project public website 26

Executive Summary

The CONTAMED project was established to investigate associations between chemical exposures and male reproductive disorders such as cryptorchidisms and hypospadias and to study the combined effects of antiandrogenic chemicals. The project mobilised the resources of three European mother-child cohorts, Generation R Rotterdam, ALSPAC Bristol and INMA Granada. Several key observations were made:

The use of mild analgesics such as paracetamol during weeks 12-20 of pregnancy was associated with cryptorchidisms, but not hypospadias. Drug use was established on the basis of prenatal questionnaires. Attempts of ascertaining user status by analysis of urinary paracetamol levels produced the surprising finding that the drug was present in all urine samples, independent of drug usage. This points to as yet unidentified sources for background exposures and is the topic of on-going investigations. Possibly due to these background exposures, the presence of paracetamol in urine could not be shown to be associated with cryptorchidisms.

Urinary levels of multiple EDCs with anti-androgenic effects, including phthalate metabolites, parabens, bisphenol A, and o-phenyl phenol were determined, but none of these single chemical exposure markers was associated with cryptorchidisms or hypospadias. The effect of cumulative exposures to these chemicals is currently under investigation.

A biomarker based on *in vitro* androgen receptor (AR) antagonism and representative of cumulative internal exposures to AR antagonists was developed. Increased activity in non-polar fractions of placenta extracts from mother-child cohorts, encompassing lipophilic persistent pollutants, was associated with cryptorchidisms and hypospadias. There were also statistically significant associations between the concentrations of parabens and bisphenol A in the placentas and cryptorchidism / hypospadia case status. The findings with placenta material suggest that environmental chemicals with anti-androgenic activity play a role in the risk of developing cryptorchidisms and hypospadias.

In-depth analyses of the non-polar placenta fractions that were associated with higher risks of cryptorchidisms / hypospadias led to the discovery of previously unrecognised chemical structures with potential anti-androgenic activity. Of note are certain polycyclic aromatic hydrocarbons found in smoked food, so-called abientrienes.

The CONTAMED project has led to the identification of *in vitro* anti-androgenic properties of nine pesticides in fairly wide-spread use in the EU. Many of these pesticides are also capable of suppressing prostaglandin synthesis *in vitro*, some even at nanomolar concentrations, in the range of the potency of pharmaceuticals designed to interfere with these processes.

The effects of mixtures composed of anti-androgens and estrogenic EDCs on hallmarks of male sexual differentiation and on female reproductive and developmental endpoints were studied. These mixtures were modelled on high-end human exposure scenarios. At doses 100-150-times higher than high human exposures, developmental toxicity on male and female offspring was observed. This suggests that highly exposed population groups, especially women of reproductive age, may not be protected adequately against the effects of EDCs affecting the hormonal milieu required for proper sexual differentiation. These studies also demonstrated that different types of mixtures of EDCs can exert a profound effect on gene expression in the developing brain.

Project context and objectives

Background

In the context of male reproductive health and exposure to endocrine disrupting chemicals (EDCs) three groups of adverse health outcomes are commonly considered together: 1) Declines in reproductive function, as manifested by reduced semen quality and compromised male infertility; 2) disruption of male foetal development resulting in congenital malformations of the urogenital tract - non-descent of testes (cryptorchidism) and abnormal positioning of the opening of the urethra (hypospadia); and 3) testicular germ cell tumours.

Diagnosis of these disorders is complicated by the use varied classification criteria, and this makes it difficult to come to valid judgements about disease trends. However, studies that have adopted similar classification strategies and case ascertainment protocols have shown that several European countries (England, Denmark, The Netherlands, Lithuania) have experienced increases in incidence during recent years (Main et al. 2010). Countries with a high prevalence of cryptorchidism also exhibit high rates of hypospadias and poor semen quality, and vice versa. Furthermore, these three health endpoints correlate with the incidence of testicular germ cell cancers (European Science Foundation 2010).

Ten years earlier, similar findings have led Skakkebaek et al. (2001) to suggest that cryptorchidism, hypospadia, poor semen quality and testis cancer all share a common aetiological origin and constitute a syndrome, termed ***testicular dysgenesis syndrome*** (TDS). The TDS hypothesis derives from the foetal origin of cryptorchidisms, hypospadias, testicular germ cell cancers and poor semen quality. It proposes that its constituent disorders go back to diminished androgen action in foetal life, with a negative impact on the proper functioning of Sertoli cells (the cells supporting germ cells) and Leydig cells (where androgen synthesis takes place). Accordingly, it proposes a strong environmental component, and more specifically, exposures to anti-androgenic chemicals as the aetiological factor.

Thus far, the evidence supporting an involvement of single chemicals at current environmental exposure levels in the genesis of TDS is weak (reviewed by Kortenkamp et al. 2012). Some associations with fetal exposure to DES or polybrominated diphenyl ethers (PBDE) have come to light, but links with other chemicals including PCBs or certain organochlorine pesticides are inconclusive.

However, several epidemiological studies have given indications of cumulative effects of chemicals, although associations with individual chemicals either did not become apparent or were considerably weaker. For example, the statistically significant links between cryptorchidisms and a sum parameter of PBDE levels in mothers' milk found by Main et al. (2007) were not evident when the analysis was based on individual congeners. Similarly, there were no significant relationships between individual persistent pesticides and cryptorchidisms in the study by Damgaard et al. (2006), but the sum of the eight most prevalent pesticides was significantly associated with the condition. The combined estrogenicity of placenta extracts was strongly related to the likelyhood of cryptorchidisms (Fernandez et al. 2007). These observations echo the "something from nothing" phenomenon that has emerged from experimental studies with endocrine disrupter mixtures where

EDC were shown to act together in combination although they were present at concentrations that individually did not induce observable effects.

The state of the science before CONTAMED

At the beginning of the CONTAMED project the state of the science of elucidating the aetiology of TDS could be characterised as follows:

Epidemiology had explored fairly narrow hypotheses about the link between chemical exposures and adverse health outcomes, mostly focusing on single, highly persistent and bioaccumulative chemicals, with little regard for their anti-androgenicity. There were indications of cumulative effects, but these were not assessed systematically. Progress with investigating the role of chemical exposures was hampered by the following factors:

- Epidemiological concepts and approaches geared towards assessing the effects of cumulative exposures were missing
- Biomarkers capable of capturing internal cumulative exposures to endocrine disrupting chemicals with anti-androgenic activity were not developed
- Data about endocrine disrupters that occur together in human tissues were scarce, and knowledge about what might constitute environmentally relevant mixtures of EDCs, particularly anti-androgens, was fragmentary
- Too little was known about the full spectrum of chemicals with (anti-androgenic) endocrine disrupting properties and relevance to human exposures
- Although concepts for the evaluation of mixture effects in experimental studies with laboratory animals were well developed, data about the effects of combinations with relevance to human exposure scenarios were missing, and there was no information about the potential combination effects of different classes of EDCs (e.g. estrogens and anti-androgens)

Motivation and aims of CONTAMED

It was the motivation of the CONTAMED project to make a contribution to filling these knowledge gaps. This was only possible through the concerted efforts of epidemiologists, developmental toxicologists, in vitro toxicologists, mixture toxicology experts and analytical chemists with specialisations in metabolomics and human biomonitoring.

Several inter-related strands of work were followed:

A great deal of the investigations focused on developing biomarkers for cumulative exposures to anti-androgenic chemicals which were evaluated in the context of three European mother-child cohorts, Generation R in Rotterdam, the Netherlands, ALSPAC in Bristol, United Kingdom, and INMA in Granada, Spain. In each of these cohorts, cases of cryptorchidisms and hypospadias were identified according to common diagnostic criteria, and each of these cohorts provided different tissue samples.

Bioassay-directed fractionations of tissue extracts were conducted with the aim identifying fractions with anti-androgenic activity. These fractions were then analysed further by using metabolomics approaches to pinpoint new chemicals with antiandrogenic properties.

Mixtures of endocrine disrupting chemicals at exposures modelled on high-end human exposure scenarios were evaluated in a rat developmental toxicity model, with a multitude of endpoints relevant to both male and female sexual differentiation.

Targeted chemical analyses for multiple endocrine disrupters were conducted in tissue samples from the three cohorts. The data provide the basis for epidemiological investigations of associations between exposure markers and cryptorchidism and hypospadias.

In vitro studies were undertaken to investigate the effects of very large mixtures, composed of up to 30 components, and to screen for new chemicals with anti-androgenic effects.

Main S&T results/foregrounds

The work plan for the CONTAMED project involved detailed investigations of different aspects of related problems, spread over several work packages. For the sake of clarity, the main S&T results of the CONTAMED project will not be reported here according the work package structure of the project, but rather in terms of key achievements, across work packages, as follows:

- Bioassay-directed fractionations of human tissue extracts from the three mother-child cohorts and the identification of new anti-androgenic pollutants (effect-directed analysis)
- Development of biomarkers for internal exposure to anti-androgens
- Investigating new EDC-relevant modes of action and new candidate chemicals
- Targeted chemical analyses for multiple EDCs in human tissues and estimation of human exposures
- Assessing the joint effects of EDC mixtures in experimental studies modelled on high-end human exposure scenarios
- Epidemiological studies of the role of chemical exposures in contributing to cryptorchidisms and hypospadias
- Implications for chemical regulation and health impact studies

Bioassay-directed fractionations of human tissue extracts and identification of new anti-androgenic pollutants

The environmental health sciences have traditionally focused on a fairly narrow range of pollutants. These research efforts are not necessarily driven by any sense of priority in terms of human health relevance, but by what is already well researched. A recent bibliometric analysis of the environmental health literature has identified a striking mismatch between what several regulatory agencies worldwide regard as priority pollutants, and what is the topic of scientific investigations (Grandjean et al. 2012). Similar tendencies are noticeable in the area of male reproductive health, where research has concentrated on persistent bioaccumulative pollutants (Kortenkamp et al. 2012). Current-use chemicals such as phthalates or perfluorinated compounds have only recently come into focus. With several tens of thousands of chemicals in commercial use, and an estimated 2000 substances with anti-androgenic potential (Vinggaard et al. 2008), it was clear that greater efforts were necessary to search for anti-androgenic chemicals.

The topic can be approached in different ways, e.g. by screening of chemicals or by applying quantitative structure-activity relationships (QSAR). The CONTAMED has not only pursued these approaches, but also chosen to investigate the issue by using bioassay-directed fractionations. This methodology has long been exploited for drug discovery in the pharmaceutical sciences. It involves preparing suitable extracts from the material to be analysed and then fractionating the extracts. All extracts are then screened by using a suitable bioassay. The fractions that exhibit activity in the chosen assay are subjected to further fractionation followed by detailed chemical analyses with the aim of detecting candidate chemicals (“active principles”). In the environmental sciences, the method has been called toxicity identification and evaluation (TIE) or effect-directed analysis (EDA) and was instrumental in identifying estrogenic chemicals in surface waters. It is widely used for the study of environmental samples, but has not found application to the analysis of pollutants present in human tissues. The analysis of EDCs with anti-androgenic properties had not been attempted before.

Method development

In approaching the task of applying bioassay-directed fractionations to the analysis for anti-androgenic chemicals in human tissues, the CONTAMED project had to address a number of issues:

- Which tissues or body fluids should be used for extraction and fractionation?
- Which *in vitro* bioassays are suitable for the analysis of extracts and fractions?
- What is an optimal extraction and fractionation method that can capture a wide range of pollutants with differing physico-chemical properties?
- Is there interference from toxic chemicals present in the extracts, and how can this be controlled?
- Do endogenous agents interfere with the bioassays used for screening?
- Is there interference from endogenous agents during the identification of chemicals, and how can this be dealt with?

From the three cohorts assembled in the CONTAMED project, placenta homogenates, serum and urine samples were available. Screening of fractionated extracts with *in vitro* bioassays for androgen receptor antagonism revealed that the amount of anti-androgenicity was largest in placenta extracts. For this reason, it was decided to refine a method for placenta homogenates. Given the similarity of this tissue with blood and serum, the developed methods could be applied to serum also.

Three *in vitro* bioassays for AR antagonism were utilised, one based on yeast cells, the yeast androgen screen (YAS), and two based on mammalian cells, the AR-lux and PALM assays. Comparative analyses showed that the PALM assay detected the largest number of active fractions, and therefore was chosen for routine work and epidemiological studies.

Considerable efforts were spent on developing an optimal extraction and fractionation method. Extraction with acetonitrile followed by hexane gave satisfactory results, and fractionation by normal phase HPLC achieved optimal separation of endogenous steroidal hormones from other constituents.

Many chemicals with AR antagonistic properties are also toxic, and toxicity (like antagonistic activity) induces a diminution of the assay signal, potentially leading to erroneous assignments of anti-androgenicity ("toxic masking"). Toxic masking was dealt with by testing carefully chosen serial dilutions of extracts (to "dilute out" toxicity).

A similar problem became apparent due to the presence of endogenous steroidal hormones with androgenic activity which somewhat diminished the AR antagonistic effects of fractions. This issue was dealt with by separating out the fractions in which the steroids were prevalent.

High levels of lipids present especially in the non-polar fractions of the placenta extracts were found to interfere with the *in vitro* assays by suppressing the signal. This also led to problems with the identification of the chemicals present in the non-polar fractions. The issue was dealt with by diluting the fractions for biotesting and by developing a clean-up method for the removal of lipids prior to mass spectrometric analysis of the fractions.

Detection of fractions with anti-androgenic activity

Extensive profiling of fractionated placenta extracts from control subjects revealed several zones of activity (Figure 1). The most pronounced AR antagonistic activity resided in the non-polar fractions (2-4) which eluted first, with comparatively little variation between subjects. These fractions contained persistent lipophilic chemicals and fatty acids. This was followed by an array of fractions (5-11) with intermediate AR antagonistic activity, which varied considerably between subjects. These fractions also showed androgenic activity; they contained endogenous steroidal hormones. Lastly, intermediate AR antagonistic activity was identified in fractions 21-24 composed of polar chemicals.

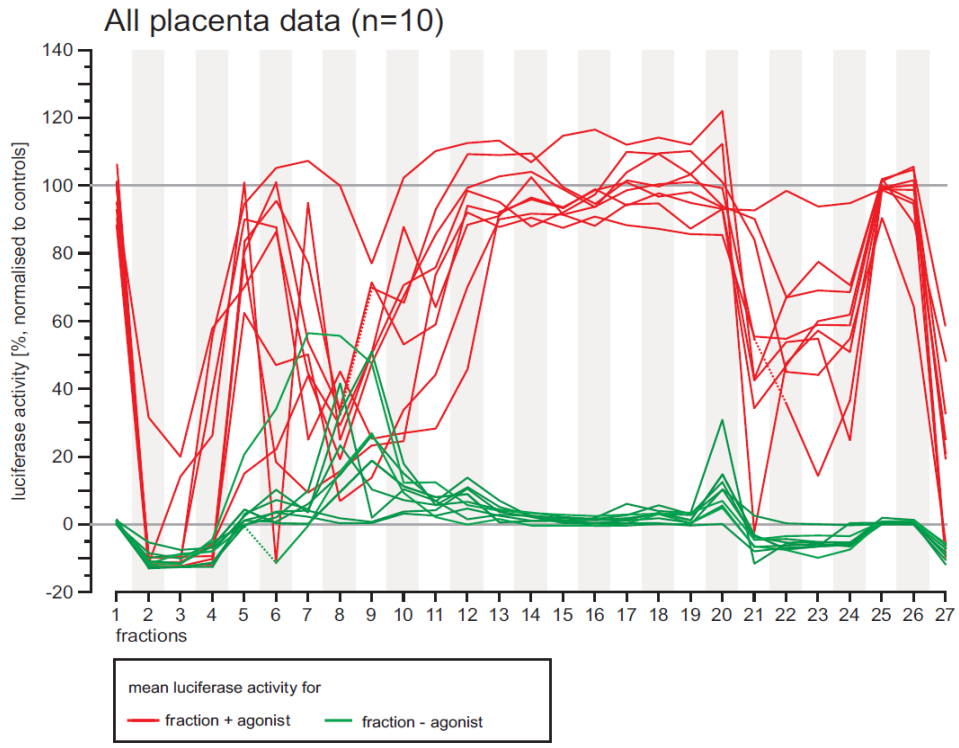


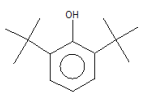
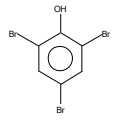
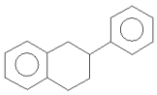
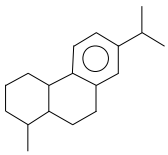
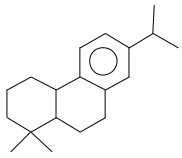
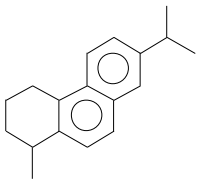
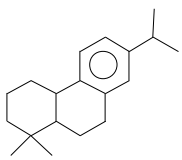
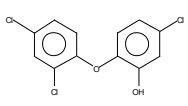
Figure 1: Anti-androgenic (red) and androgenic (green) activity in fractionated placenta extracts from 10 control subjects. Results from tests with the PALM assay.

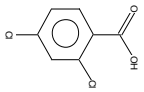
Identification of novel candidate compounds in active fractions

Because fractions 2-4 exhibited the most pronounced anti-androgenic activity, they were selected for detailed chemical structural analyses by gas chromatography coupled with mass spectrometry (GC-MS). At least 10 contaminants were detected in samples from cases and controls from the INMA Granada cohort. These contaminants were present at sufficiently high levels to enable structural identification (Table 1).

Of note are a number of polycyclic aromatic hydrocarbons which included a series of abientrienes, environmental contaminants found in lacquers and smoked food. Two bisnorabieta-8,11,13-trienes were present at significantly elevated concentrations in extracts from case subjects. The structures are currently not commercially available for testing for androgen receptor activity, but similar compounds have been reported as showing antagonistic activity comparable to flutamide (Hawliczek et al. 2012). Preliminary calculations of the total levels of abientrienes present in the extracted fractions indicate that they could account for 10-20% of the total anti-androgenic activity.

Table 1: Contaminants identified in non-polar fractions of case-control placenta extracts

Identity	**Putative structure**	**Formula**	**Retention time**	**m/z ions of derivatised compound**	**Number of samples detected (out of 20)** ***Mean±SD¶ approximate concentrations ng/g***	
Fraction 2.					controls	cases
2,6-bis(1,1-dimethylethyl)-phenol.		$C_{14}H_{22}O$	9.94	73,263,278	18 *372.0±331.2*	19 *403.8±258.6*
tribromophenol		$C_6H_3Br_3O$	11.48	228,308,389,404	6 *0.5±0.3*	2 *3.6±4.3*
1,2,3,4-tetrahydro-2-phenyl-naphthalene*		$C_{16}H_{16}$	11.70	78,104, 208	19 *55.2±32.4*	17 *70.2±29.4*
10,18-bisnorabieta-8,11,13-triene*		$C_{18}H_{26}$	14.25	143,227,242	20 *28.8±17.4*	19 *43.2±25.2**
1-methyl-10,18-bisnorabieta-8,11,13-triene*		$C_{19}H_{28}$	14.48	157,241,256	19 *6.0±3.6*	18 *9.0±4.2**
10,18-bisnorabieta-5,7,9(10),11,13-pentaene		$C_{18}H_{22}$	14.76	181,223,238	18 *8.4±6.69*	17 *13.2±9.0*
methyl-10,18-bisnorabieta-8,11,13-triene (isomer)		$C_{19}H_{28}$	15.35	157,241,256	18 *11.4±9.6*	18 *16.2±12.0*
Unidentified aromatic hydrocarbon			17.95	91,117,194, 207, 312	19 *179.4±86.4*	18 *240.5±96.6**
Fraction 3						
Triclosan		$C_{12}H_7Cl_3O_2$	14.98	200, 310,345,347, 360,362	*14 2.9±1.4*	16 *2.4±3.7*
Pool 2						

2,4-dichlorobenzoic acid		$C_7H_4Cl_2O_2$	10.04	145,273,247	2 out of 14 samples *3.9*	3 out of 12 samples *14.1±3.3*

Development of biomarkers for internal exposure to anti-androgens

On the basis of the extensive profiling work conducted with fractionated placenta extracts it was possible to develop biomarkers for internal exposure to anti-androgens that could be used for case-control epidemiological studies. Due to the competing AR-antagonistic and AR-agonistic effects found in the various fractions (see Figure 1), it was clear that testing of un-fractionated, crude placenta extracts would not provide a clear picture of the total "anti-androgenic load" in the investigated subjects. For the purpose of developing meaningful biomarkers of internal exposure that could be used in epidemiological studies it was necessary to separate out various fractions. On the other hand, the obtained profiles suggested that testing of all 27 fractions would not be necessary. Instead, it was decided to focus on the lipophilic fractions 2, 3 and 4, and to test aliquots of a pool of fractions 5-11 (where the endogenous steroid hormones resided) and a pool of fractions 21-24 with quite polar constituents.

Dealing with toxic masking and endogenous androgenicity

As discussed above, some fractions contained toxic components which interfered with the identification of AR-antagonistic properties of some fractions. To assess a valid measure of activity, these fractions had to be diluted appropriately, such that toxicity was kept to a minimum. Concomitant androgenicity in some fractions was measured by testing in the absence of the androgen agonist (R1881) that was used to prime the cells. This made it possible to distinguish fractions with "pure" AR antagonistic activity from those with accompanying cytotoxicity or androgen agonism.

Making fractions comparable – procymidone equivalents

The precautions necessary for dealing with toxic masking and androgenicity meant that some fractions had to be diluted more than others. To correct for the effect of dilutions, and to introduce a uniform quantitative measure of AR antagonistic activity, the observed effects were expressed in terms of procymidone equivalents and then adjusted for the respective dilutions. The scheme that was ultimately developed – full fractionation of all placenta extracts, followed by testing of fractions 2, 3 and 4, pool 5-11 and 21-24 made the large-scale testing of case and control placentas manageable. It meant that analyses of placentas from 89 subjects after extraction, fractionation and pooling would require the biotesting of around 440 samples. The results of these analyses are described in the section dealing with epidemiological studies.

Investigating new EDC-relevant modes of action and new candidate chemicals – suppression of prostaglandins and AR antagonistic pesticides

Over the last ten years it has become apparent that prostaglandins (PGs), and especially PGD2, have an important function in male sexual differentiation through the regulation of Sertoli cells. This has led to initiatives in the DEER project, one of the sister projects of the NECTAR cluster of projects devoted to investigating human reproductive disorders, to assess the consequences of suppressing PGD action in terms of the TDS.

PGs are synthesised from arachidonic acid precursers by cyclisation through cyclooxygenase enzymes (COX). COX isoforms are the target of non-steroidal antiinflammatory drugs (NSAIDs), some of which are widely used over-the counter drugs. A study published in 2010 (Kristensen et al. 2011) addressed the effects of male fetal exposure to NSAIDs and their association with reproductive disorders. Based on the knowledge of PGD2's involvement in normal testicular development, it was investigated whether exposure to PG synthesis inhibiting compounds during early pregnancy could interfere with male sexual differentiation and contribute to TDS disorders. In this work across the cluster projects CONTAMED and DEER the widely used analgesic paracetamol was identified as inducing changes in the anogenital distance in male offspring from female rats exposed during pregnancy. This suggested that paracetamol interfered with androgen action during the process of male sexual differentiation.

These findings provided the stimulus for investigating analgesic use and its association with congenital malformations (see section on epidemiology) and for studying a wider range of chemicals in terms of their ability to suppress PGD2 synthesis *in vitro*.

For this purpose, an *in vitro* assay based on mouse Sertoli cells developed by Kristensen and Leffers in Copenhagen's Rikshospitalet was implemented. By using this assay, 15 new pesticides in current use in the EU could be identified as capable of suppressing PGD2 synthesis.

In descending order of potency, these pesticides were: o-phenyl phenol, cypermethrin, cyprodinil, imazalil, linuron, chlorpropham, boscalid, tebuconazole, pyrimiphos-methyl, imidacloprid, methiocarb, fenhexamid, pyrimethanil, iprodione and fludioxonil. The first five of these chemicals inhibited PGD2 synthesis at nanomolar concentrations. It is suggested that these five (o-phenyl phenol, cypermethrin, cyprodinil, imazalil, linuron) are prioritised for *in vivo* testing in appropriate developmental toxicity assays.

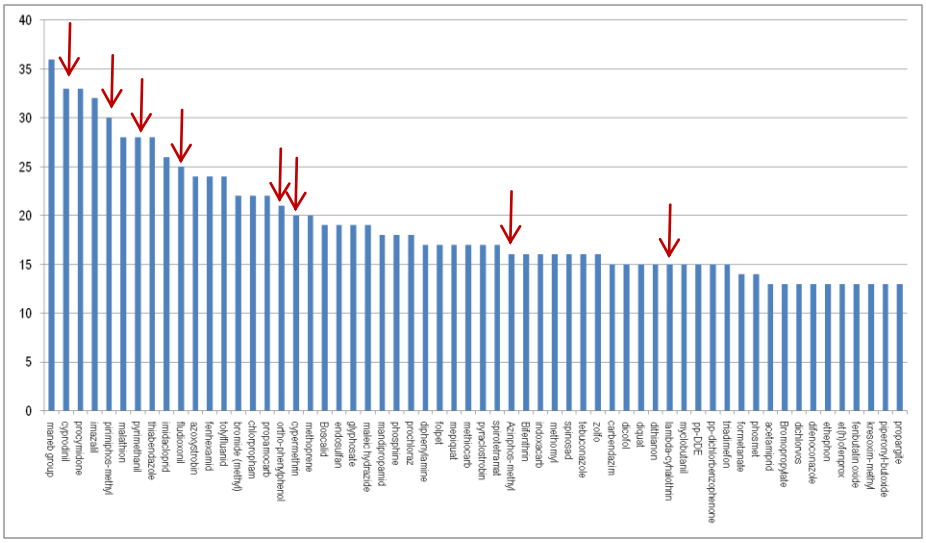


Figure 2: Rank order of pesticides authorised for use in the EU according to usage and exposure criteria, with 8 of the 9 newly identified AR antagonists highlighted by arrows (Orton et al. 2011).

Preceding this work, an effort was made to assemble a list of EU authorised pesticides according to criteria of widespread usage (Figure 2). It was striking that a large number of these pesticides had never been investigated for anti-androgenicity. It was therefore deemed important to screen these

pesticides for AR antagonistic properties. These efforts led to the identification of 9 previously untested pesticides as AR antagonists (dimethomorph, fenhexamid, quinoxyfen, cyprodinil, λ-cyhalothrin, pyrimethanil, fludioxonil, azinphos-methyl, pirimiphos-methyl). Due to their potency, their current use and estimated exposure, it was strongly recommend that dimethomorph, fludioxonil, fenhexamid, imazalil, *ortho*-phenylphenol and pirimiphos-methyl be tested for anti-androgenic effects *in vivo* (Orton et al. 2011).

Targeted chemical analyses for multiple EDCs in human tissues and estimation of human exposures

In the past, diverse chemicals have been measured in human tissues and body fluids, but because these efforts have targeted distinct chemicals only one-by-one, in different samples, it was difficult to assess which chemicals, and at what levels, co-occurred in one and the same sample. These data gaps present a formidable hurdle to assessing cumulative exposures. The CONTAMED project has made a concerted effort to fill this data gap by analysing multiple chemicals in samples drawn from the mother-child cohorts assembled in the project. The analytical work focused on urine samples from the Generation R and ALSPAC cohorts, and on placenta homogenates from the INMA Granada cohort. For the first time, urinary paracetamol levels were measured in Generation R and ALSPAC samples. These efforts yielded a striking result: paracetamol was detected in all analysed samples, even from subject who had not taken the drug. This raises important questions as to the source of these background exposures and is the topic of ongoing research among CONTAMED partners.

Table 2: Comparison of estimated daily phthalate intakes (DI) for ALSPAC and GenerationR cohorts with selected published values from other cohorts

Parent phthalate	DEP	BBzP	DnBP	DiBP	DEHP	DINP
ALSPAC median DI (µg/kg/d)	5.23	0.24	2.1	0.45	1.9	0.32
ALSPAC 95th perc DI (µg/kg/d)	32.5	1.28	18.5	2.66	12.1	2.33
GenR median DI (µg/kg/d)	3.74	0.16	0.73	0.67	1.72	0.42
GenR 95th perc DI (µg/kg/d)	40.7	1.95	3.90	4.89	7.45	2.7
NHANES 2005 median DI (µg/kg/d)	4.7	0.37	0.61	0.18	3.8	1.0
NHANES 2005 99th perc DI (µg/kg/d)	193	3.0	5.3	1.5	185	34
Koch et al 2003 median DI (µg/kg/d)		0.88	1.5			
Koch et al 2003 95th perc DI (µg/kg/d)		4.0	7.2			

The CONTAMED project has assembled a data base describing urinary levels of multiple EDCs and their break-down products, including dichloro-anilines (metabolites of imidazole pesticides),

phthalate metabolites, bisphenol A, triclosan, o-phenylphenol and various parabens. On the basis of the urinary levels of phthalate metabolites, and with knowledge about their toxicokinetics, it was possible to estimate human intakes by "reverse dosimetry". These efforts showed that the phthalate exposures of subjects of the mother-child cohorts were within the range estimated previously for other populations (Table 2).

Targeted analyses for parabens, benzophenones and bisphenol A were conducted for placenta samples from the INMA Granada cohort.

All these data were utilised to investigate associations with cryptorchidism and hypospadias in a case-control format. The results of these analyses are described in the section on epidemiological findings.

Assessing the joint effects of EDC mixtures in experimental studies modelled on high-end human exposure scenarios

A series of studies on perinatal exposure to a human relevant environmental contaminant mixture were performed in rats with the aim of investigating the possible role of mixtures of chemicals with various modes of action in affecting early markers of endocrine disruption and producing long-lasting delayed reproductive effects.

Selection of chemicals for inclusion in the mixture

The components in the mixture were carefully selected according to the following criteria: For chemicals qualifying for inclusion in the mixture, *in vivo* data about developmental effects targeting the male reproductive system had to be available. Since the aim was to compose mixtures representative of high human exposures, data about human intakes were needed. Chemicals that did not fulfill these criteria could not be included in the mixture. 13 chemicals were chosen, including phthalates, pesticides, UV-filter substances, bisphenol A, parabens and the drug paracetamol. There were anti-androgenic and estrogenic chemicals in the mixture.

Selection of doses and experimental design

The doses of each chemical (and thus the mixture ratio) were chosen to reflect high end human intakes. To make decisions about the dose levels for studies in the rat, the Point of Departure Index (PODI) approach was used. The PODI is an application of the mixture assessment concept of dose addition. It sums up ratios between the doses of each chemical in the mixture and no-observed-adverse-effect-levels (NOAEL) for developmental effects with relevance to the anti-androgenicity of the mixture components. For the 13 selected chemicals a PODI of 0.016 was calculated before the experiment began. Since only a PODI exceeding 1 was expected to lead to observable effects in the rat, it could be anticipated that a 62-fold higher mixture dose should induce responses at the borderline of detectability. Considering the high uncertainty of this estimate and the statistical power of the chosen experimental design, it was expected that combined doses 150 times higher than high end human intake estimates should give no or only borderline effects. Doses 450 times higher should, however, produce significant responses.

Observations of combined effects in the rat

Experiments indeed showed clear developmental toxicity of the 450-fold dose in terms of increased nipple retention (NR) and reduced ventral prostate weight. The 150-fold dose group already exhibited significantly increased NR (Figure 3). Considering that normally an uncertainty factor of

100 is required for extrapolation doses in the rat to human exposures, this finding is of significance. The observations in this study therefore suggest that highly exposed population groups, especially women of reproductive age, may not be protected sufficiently against the combined effects of chemicals that affect the hormonal milieu required for normal male sexual differentiation (Christiansen et al. 2012).

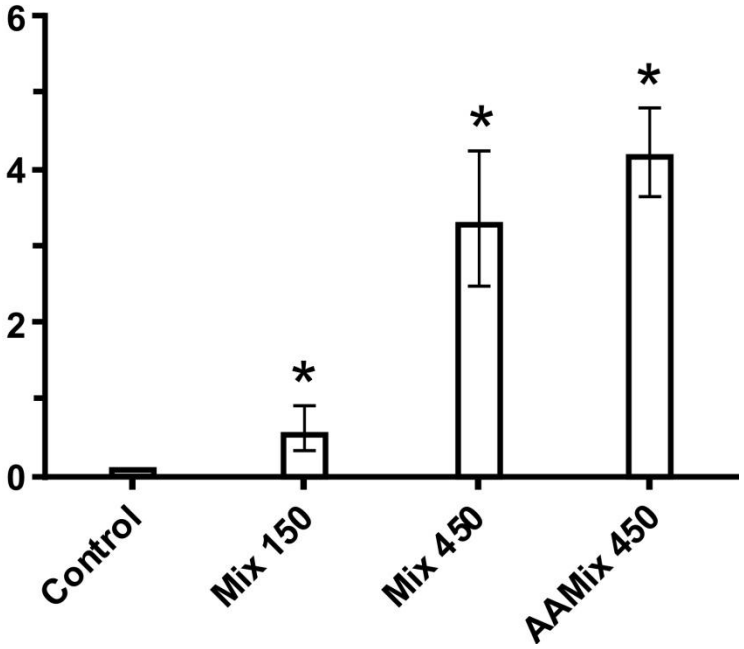


Figure 3: Number of retained nipples in male offspring of rats dosed with mixtures of 13 endocrine disrupting chemicals (Christiansen et al. 2012). Starred bars denote responses statistically significantly different from controls.

Further developmental toxicity studies in the rat

Since the initial exploratory mixture study included antiandrogens, estrogens and paracetamol, it was possible to create combinations composed of subcomponents of the mixture, i.e. antiandrogens (Amix) and estrogens (Emix) with the aim of comparing the effects of these smaller mixtures to those of the entire combination of 13 chemicals ("TotalMix"). These experiments showed that the estrogens present in TotalMix (the UV filters 4-MBC and OMC, and bisphenol A and butylparaben) did not make a contribution to the effects of the entire combination on hallmarks of male sexual differentiation, including changes in anogenital index and retained nipples.

In the final study, exposure levels for the TotalMix were 100-, 200- and 450-fold "high human exposure levels". Early markers of endocrine disruption were investigated in prepubertal male pups, i.e. changes in male anogenital distance, nipple retention and changes in reproductive organ weights in prepubertal males. Early postnatal examination of the developing brain was conducted at postnatal day 6. Further endpoints measured included puberty timing, estrous cyclicity and sexually dimorphic behavior. The studies showed clear effects on early markers for endocrine disrupting effects, such as AGD, nipple retention, reproductive organ weight in male offspring, mammary gland outgrowth in female offspring as well as profound effects on gene expression in the developing brain. Long-lasting delayed adverse reproductive effects were seen as effects on reproductive aging of females, reduced epididymal sperm count, changes in patterns of prostatic aging towards

hyperplasia at one year of age and behavioural effects in adult male offspring. These effects were mainly attributed to the anti-androgenic compounds in the mixture.

For the majority of affected endpoints, pronounced and significant effects were observed at the highest dose level of the TotalMix and Amix, i.e. the 450-fold human exposure levels, but adverse effects were also seen at the lower mixture doses. These effects included decreased AGD and increased nipple retention at 150-200 fold human exposure levels, increased numbers of females with irregular oestrus at 200-fold, and decreased ovary weights at all doses of the total mixture, including the 100-fold. An antiandrogenic effect on male anogenital distance also appeared to be present in the group exposed to the 100-fold human exposure. The dose levels of the single chemicals present in the 100- and 150-fold mixtures were considerably lower than the reported NOAELs for these chemicals. Overall, these observations suggest that highly exposed population groups, especially women of reproductive age, may not be protected sufficiently against the combined effects of chemicals that affect the hormonal milieu required for normal male sexual differentiation.

Development of the brain

The mixture studies demonstrated that different types of mixtures of EDCs can exert a profound effect on gene expression in the developing brain. It was possible to characterize gene expression patterns resulting from developmental exposure to different types of mixtures of EDCs, by a combination of transcriptomics and real time RT PCR.

These expression patterns may result from estrogenic or anti-androgenic activities of the mixture components, or from a combination of both in the case of TotalMix; interactions of some mixture components with thyroid hormones may also have contributed. However, the possibility of non-endocrine effects of mixture components should also be considered, in particular with regard to effects of the anti-androgenic mixture, which contained pesticides, and of the total mixture containing paracetamol. In order to define core expression patterns, e.g., estrogenic-type or anti-androgenic-type patterns, comparative studies with different mixtures of either estrogenic or anti-androgenic chemicals should be conducted.

Expression patterns differed between brain regions. In consequence, the characterization of patterns requires a very precise and highly reproducible location of individual brain regions. Since in a given region and sex, the LOAEL is reached at different doses for different genes, expression patterns caused by the same EDC mixture are to be expected to change with dose. This may be particularly important for extrapolations to the low dose range. The concept of prior genome-wide microarray analysis followed by microarray-based selection of individual mRNA species for further analysis by quantitative real time RT PCR, proved to be a very valid approach because it is free of biased selection of genes for investigation.

In the present study, this procedure disclosed important main target processes of the EDC mixtures. The preponderance of genes involved in synaptogenesis is in line with the developmental stage studied. Very interesting, however, is the strong focus on genes involved in formation and function of excitatory glutamergic synapses. The effect of the anti-androgenic mixture in early postnatal female brain, at a time of supposedly low androgen levels, is unexpected. It would be important to clarify the part possibly played by other types of actions of components of Amix on developing brain tissue.

In vitro experiments with combinations of endocrine disrupters

Numerous mixture experiments utilising *in vitro* assays sensitive to EDCs were conducted. The *in vitro* assays included the AR-lux assay which captures AR antagonists and the H295R assay for measuring the effects on steroid synthesis. These experiments were designed to analyse whether combination effects would arise between the newly identified AR antagonistic pesticides (Orton et al. 2012), and these pesticides and mixtures of established *in vitro* AR antagonists. The work led to the examination of one of the largest mixtures ever studied (30 components) and showed combination effects largely in line with concentration addition. In this mixture, all components were present at concentrations far below their NOECs.

The effects seen in the H295R assay involved chemicals utilised in the *in vivo* rat studies and produced results also in good agreement with concentration addition.

Epidemiological studies of the role of chemical exposures in contributing to cryptorchidisms and hypospadias

Mild analgesics and cryptorchidisms

Motivated by findings from Danish cohorts of associations between maternal analgesics use during preganacy and the occurrence of cryptorchidisms in boys (Kristensen et al. 2010, Jensen et al. 2010), a similar study was undertaken in the Generation R cohort. As in the Danish studies, the use of mild analgesics was assessed in prenatal questionnaires in pregnancy, resulting in four periods, namely, periconception use, use during the first period (0-12 weeks of gestation), use during the second period (12-20 weeks of gestation) and use during the third period (18-30 weeks of gestation). Odds ratios of cryptorchidism/hypospadia were estimated by using logistic regression models.

The use of mild analgesics during the second period of pregnancy (12-20 weeks) increased the risk of congenital cryptorchidism (adjusted OR 2.23; 95%CI 1.21-4.13), primarily due to paracetamol (adjusted OR 1.99; 95%CI 1.04-3.78). In total, 29.9% of the mothers in the Generation R study population used mild analgesics during pregnancy. Of the mothers of cryptorchid sons, 33.8% reported (23 of 68) the use of mild analgesics during pregnancy, versus 31.8% (7 of 22) of mothers with a boy with hypospadia, and 29.9% (926 of 3094) of mothers with healthy boys. These results suggest that intrauterine exposure to mild analgesics during the second period in pregnancy (12-20 weeks of gestation), when male sexual differentiation takes place, increases the risk of developing cryptorchidisms, but not hypospadias (Snijder et al. 2012).

Associations between single EDCs and congenital malformations

Nested case-control studies were conducted within three birth cohort studies: ALSPAC – Bristol, Generation R – Rotterdam, and INMA-Granada. Three different studies were conducted using urine samples (Generation R, ALSPAC), plasma samples (ALSPAC), and placenta material (INMA-Granada). Cases were defined by the presence of cryptorchidism or hypospadias, since both congenital malformations are specifically linked to the potential endocrine disruption. Controls were randomly selected from all newborn boys.

In the nested case-control study with urine samples (Genration R and ALSPAC), cases (n=70) were matched with controls (n=70) on age of mother and a 1:1 ratio was used. In both cohorts, urine

samples of pregnant women were collected 3 times during pregnancy. In these urine samples the following markers of EDC exposure with anti-androgen potency were determined: paracetamol, dichloro-anilines, phthalate metabolites, bisphenol A, triclosan, o-phenylphenol, and parabens.

In the nested case-control study on plasma samples (ALSPAC), cases of cryptorchidism (n=18) were matched with controls (n=18) and a 1:1 ratio was used. Plasma samples were collected from the second and third trimester of pregnancy.

In the nested case-control study on placenta material (INMA-Granada), cases of cryptorchidism and hypospadias (n=29) were matched with controls (n=60) on age of mother and a 1:2 ratio was used.

The studies showed the abundant presence of various endocrine disrupting chemicals in tissue specimens and body fluids of pregnant women. The nested case-control study with urine samples of pregnant women could not demonstrate clear associations between markers of exposures to individual EDCs and the occurrence of cryptorchidism and hypospadias.

In placenta material from INMA Granada higher concentrations of methylparaben, propylparaben, butylparaben, and bisphenol A were associated with case status of cryptorchidism of hypospadias.

Associations between biomarkers of internal exposure to AR antagonists and congenital malformations

A case-control study on placenta material from INMA-Granada showed that for cases with cryptorchidism and hypospadias the anti-androgenic activity in fraction 2 was statistically significantly increased (OR=2.56). Fraction 2 contained non-polar contaminants (see Figure 1). In contrast, statistically significantly decreased activity was observed for fraction 21-24 of the placenta material (OR=0.13).

The nested case-control study on plasma material from the ALSPAC cohort showed that procymidone-equivalent concentrations for fraction 21-24 and for all fractions had increased odds ratios for cryptorchidism, but due to small numbers did not reach statistical significance.

Interpretation of results

The three nested case-control studies used different biological material and showed different results. In urine samples it was not possible to demonstrate clear associations between individual chemical markers of EDC exposure and the occurrence of cryptorchidism and hypospadias. The complexity of human biology makes it very difficult to establish a relationship between EDC exposure and male congenital malformations.

This raises the question as to the best biological material to determine internal EDC exposure. It could be hypothesized that EDC measures in placenta material are most relevant, since it is derived from an environment closest to the developing foetus. The placenta study clearly suggests that environmental chemicals with anti-androgenic activity play a role in the risk of cryptorchidism and/or hypospadias.

Further work

The data base on urinary levels of multiple EDCs and their metabolites will be analysed further by exploiting concepts of mixture toxicology. Having established that single chemical exposure markers do not show associations with congenital malformations, measures of cumulative exposure will be

assessed. Since the analytical results of the urine samples became available only at the end of the project, these analyses could not be completed during the lifetime of the CONTAMED project.

The apparent discrepancy between the questionnaire-based study of the effects of exposure to mild analgesics (Snijder et al. 2012) with the lack of associations with the analytically determined urinary paracetamol levels requires careful analysis. It may turn out that questionnaire-based analyses are more reliable than chemical analyses, because of the unexpected background exposures to paracetamol and the strong inter-individual variations.

A third area for further analysis will be the unexpected discovery of elevated levels of polycyclic aromatic hydrocarbons in the placentas from cryptorchidism and hypospadias cases. It will be necessary to examine the smoking status of these subjects before firm conclusions can be drawn.

Implications for chemical regulation and health impact studies

The CONTAMED project has provided clear evidence that European citizens are exposed to multi-component mixtures of EDCs from different sources and via multiple routes. Furthermore, CONTAMED has shown that quite reliable assessments of cumulative risks can be derived from the assumption of a dose additive action of different EDCs.

The options for implementing this scientific knowledge into regulatory strategies for improved assessment of health risks from combined exposure to multiple EDCs were examined. First, an overview on the existing regulatory framework for the assessment of mixtures of chemicals in the EU has been prepared and needs for the advancement of regulatory strategies have been identified. Second, a detailed comparative examination of available options for the regulatory assessment of mixture toxicity and cumulative risks in terms of data requirements and reliability of results has been conducted. And finally, further research needs have been summarized.

The analysis shows that basic tools for performing cumulative risk assessments are available, but the existing framework of chemical legislation in the EU does not allow their consistent and effective implementation. Further advancements of both the regulatory strategies and the scientific basis for cumulative risk assessments should be brought forward.

Potential impact and main dissemination activities

Potential impact

The impact of the CONTAMED project is at several levels. There are impacts on science and further research, on chemical regulation in the EU and in terms of alerts to health care professionals and the public.

Scientific impact

By conducting bioassay-directed fractionations, the project was able to develop valuable leads for new, previously unrecognised, contaminants with anti-androgenic activity, most notably polycyclic aromatic hydrocarbons derived from lacquers, smoked food and wood burning. Further efforts aimed at identifying chemical structures in other fractions of the placenta material are ongoing. These discoveries will necessitate further testing of chemically pure substances in the appropriate bioassays, but this is currently complicated by the lack of availability of some synthesised compounds. The novelty of this work is in its application to human tissues which was never attempted before. It opens up the possibility for future systematic searches for novel EDCs present in human tissues and will be invaluable for establishing more comprehensively the range of environmental anti-androgenic exposures of human populations.

The project has also led to the development of biological markers based on reporter gene assays sensitive to AR antagonism. These markers are representative of internal exposures to complex mixtures of anti-androgenic contaminants. There are associations with cryptorchidism/hypospadias case status. They will be of great value in future epidemiological studies aimed at establishing more holistic markers of cumulative exposures. It will open up the possibility of searching for further chemical structures with anti-androgenic effects and will facilitate targeted analyses of such substances.

Surprising was the discovery that paracetamol residues were present in all analysed urine samples. This provokes questions as to the sources of this background exposure and has already spawned research efforts aimed at identifying sources and routes of exposure.

The project has led to the identification of AR antagonistic activities of a number of current-use pesticides which were never tested before for their anti-androgenicity. These observations provide guidance for future *in vivo* testing of some of these substances in developmental toxicity assays. They also enrich our understanding of structure-activity relationships predisposing to AR antagonist status and continue to be useful in the refinement of QSAR models.

CONTAMED has added to the list of chemicals that are capable of suppressing PGD2 synthesis by identifying a series of pesticides, some of which have an *in vitro* potency comparable to that of some NSAID drugs that were designed to interfere with COX enzymes. These observations will trigger further mechanistic research into the importance of PDG2 in the regulation and control of proper male sexual differentiation. They highlight the importance of modalities beyond estrogenic, (anti)-androgenic and thyroid disrupting (EAT) that have dominated endocrine disrupter research for so long.

The mixture experiments *in vivo* and *in vitro* that were conducted in CONTAMED have enriched our understanding of the predictability of combination effects of EDCs and of the usefulness of the dose

addition concept in making such predictions. In investigating mixtures that emulated human exposures as far as this was possible, the project has added a further dimension to mixture toxicology by identifying those chemicals in the mixtures that contributed most to a joint effect.

The epidemiological studies conducted in CONTAMED will have an impact on the selection of the most appropriate tissue material in future epidemiological studies of male reproductive disorders. It was striking that the analyses of the placenta material yielded the strongest indications that cumulative exposure to EDC can influence cryptorchidism/hypospadia case status. This may have something to do with the proximity to the developing fetus, but further work will be necessary to substantiate this idea.

The work also underlines the importance of taking account of cumulative exposures. In the urinary exposure markers, no single EDC was associated with cryptorchidisms / hypospadias. It is now timely to investigate whether aggregation of these single chemical exposure markers will yield higher risk estimates. This work is currently ongoing – due to the completion of the chemical analyses towards the end of the project, it was not possible to conduct these assessments earlier.

Impacts on chemical regulation in the EU

The results of the CONTAMED project will have an impact on the debate about combination effects and cumulative exposures that is currently ongoing in the EU. The project has demonstrated that the tools and concepts needed for a scientific evaluation of combination effects are available and that their utility is high. The observation that chemicals from different regulatory domains – pesticides, industrial chemicals, cosmetics ingredients, pharmaceuticals – can work together highlights serious shortcomings of the current European chemical regulatory framework. This framework lacks the instruments for considering the possibility of combination effects from chemicals that belong to different regulatory domains. Regarding risks to the developing fetus, it is not sufficient to take account of one regulatory domain in isolation (e.g. pesticides), when CONTAMED experimental results show clearly that pesticides work together with industrial chemicals in causing anti-androgenicity.

The project also has an impact on the ongoing debate about criteria for regulating endocrine disrupters in important pieces of EU chemicals regulations. The demonstration of the importance of PGD2 in male sexual differentiation shows that too narrow a focus on estrogens, (anti)-androgens and thyroid disrupting chemicals runs the risk of overlooking important ED relevant modalities. There is a case for beginning validation processes for the mouse Sertoli PGD2 assay so that this modality can enter regulatory testing.

Alerts to health care professionals and the general public

The CONTAMED project has highlighted the issue of exposure to mild analgesics and its possible associations with congenital malformations. This issue is of great importance and sensitivity. Mild analgesics such as paracetamol are the only painkillers that women can use during pregnancy; other drugs are contraindicated. If substantiated by careful analysis, it will become necessary to raise awareness among health care professionals about the risks of mild analgesics, especially over-the-counter drugs, to pregnant women and their unborn baby boys. The CONTAMED partners will take great care to communicate these observations in a balanced way to the public.

Dissemination activities

Several key observations made by the CONTAMED project have already been published in the peer-reviewed literature. Some of these observations, e.g. the discovery of previously unrecognised AR antagonistic pesticides, have attracted interest in the print media. Further papers and communications with a similarly high impact are forthcoming.

CONTAMED partners have also disseminated key findings at major international conferences, including the Gordon Research Conferences on Environmental Endocrine Disrupters. The 2010 conference was chaired by the CONTAMED coordinator and has given the project a good platform. The 2014 conference will take place in Italy, and the CONTAMED coordinator will take steps to organise special events to publicise CONTAMED scientific findings and their societal implications.

Several of the CONTAMED findings will be publicised at the forthcoming 2013 Copenhagen Workshop on endocrine disrupters.

Cited literature

Christiansen, S., Kortenkamp, A., Axelstad, M., Boberg, J., Scholze, M., Rosenskjold Jacobsen, P., Faust, M., Lichtensteiger, W., Schlumpf, M., Burdorf, A., Hass, U. (2012) Mixtures of endocrine disrupting contaminants modelled on human high end exposures – an exploratory study in rats. Internat J Androl , doi:10.1111/j.1365-2605.2011.01242.x

Damgaard IN, Skakkebaek NE, Toppari J, Virtanen HE, Shen HQ, Schramm KW, Petersen JH, Jensen TK, Main KM. 2006. Persistent pesticides in human breast milk and cryptorchidism. Environmental Health Perspectives 114:1133-1138

Fernandez MF, Olmos B, Granada A, Lopez-Espinosa MJ, Molina-Molina JM, Fernandez JM, Cruz M, Olea-Serrano F, Olea N. 2007. Human Exposure to Endocrine-Disrupting Chemicals and Prenatal Risk Factors for Cryptorchidism and Hypospadias: A Nested Case-Control Study. Environmental Health Perspectives 115:8-14.

Grandjean P, Eriksen M, Ellgard O, Wallin JA. (2011). The Matthew effect in environmental Science publication. Environ Health, 10: 96

Koch HM, Gonzalez-Reche LM, Angerer J. 2003. On-line clean-up by multidimensional liquid chromatography-electrospray ionization tandem mass spectrometry for high throughput quantification of primary and secondary phthalate metabolites in human urine. J. Chromatogr. B Analyt Technol Biomed Life Sci, 784 (1): 169-182 (2003)

Kortenkamp A, Martin O, Orton F, Faust M, Evans R, Rosivatz E, et al. 2012 State of the art assessment of endocrine disrupters. European Commission DG Env, Project Contract Number 070307/2009/550687/SER/D3

Kristensen DM, Hass U, Lesne L, Lottrup G, Jacobsen PR, sdoits-Lethimonier C, Boberg J, Petersen JH, Toppari J, Jensen TK, Brunak S, Skakkebaek NE, Nellemann C, Main KM, Jegou B, Leffers H. 2011. Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. Hum Reprod. 1: 235-244.

Main KM, Kiviranta H, Virtanen HE, Sundqvist E, Tuomisto JT, Tuomisto J, Vartiainen T, Skakkebaek NE, Toppari J. 2007. Flame retardants in placenta and breast milk and cryptorchidism in newborn boys. Environ Health Perspect 115:1519-1526.

Main KM, Skakkebaek NE, Virtanen HE, Toppari J. 2010. Genital anomalies in boys and the environment. Best Practice & Research Clinical Endocrinology & Metabolism 24:279-289.

Orton F, Rosivatz E, Scholze M, Kortenkamp A (2011) Widely used pesticides with previously unknown endocrine activity revealed as *in vitro* anti-androgens. Environ Health Perspect. 119, 794-800

Orton F, Rosivatz E, Scholze M, Kortenkamp A (2012) Competitive androgen receptor antagonism as a factor determining the predictability of cumulative antiandrogenic effects of widely used pesticides. Environ Health Perspect 120: 1234-1242

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

Snijder, C.A., Kortenkamp, A., Jaddoe, V.W.V., Hofman, A., Hass, U., Burdorf, A. (2012) Intrauterine exposure to mild analgesics during pregnancy and the occurrence of reproductive disorders in the offspring. The Generation R Study. Human Reproduction 27, 1191-1201

Vinggaard AM, Niemela J, Wedebye EB, Jensen GE. 2008. Screening of 397 chemicals and development of a quantitative structure-activity relationship model for androgen receptor antagonism. Chemical Research in Toxicology 21(4): 813-823.

Project public website

The project's website can be found at

http://www.contamed.eu/