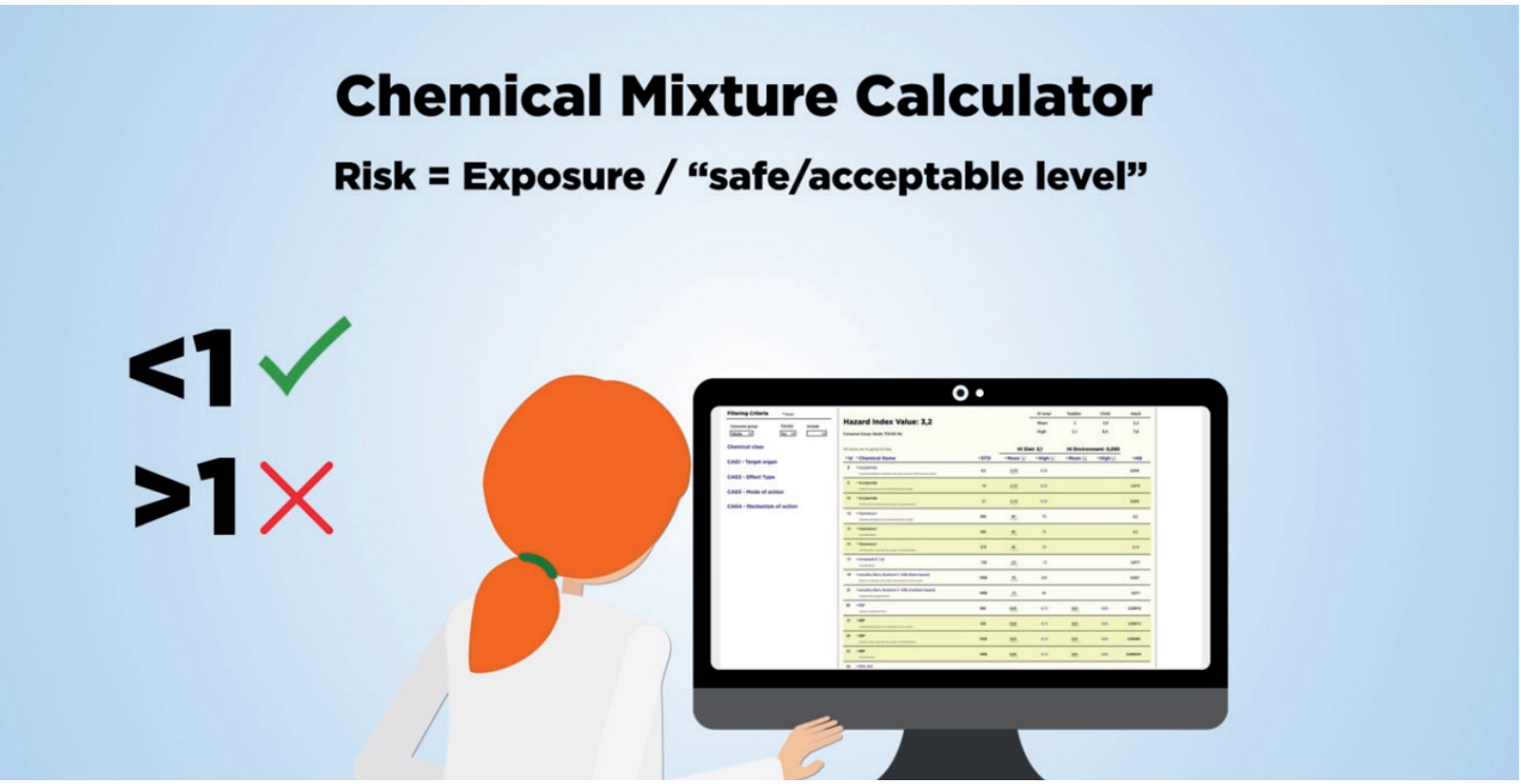
National Food Institute
Technical University of Denmark

MIraculiX

Strengthened risk assessment of chemicals



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Miraculix
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Indhold

Introduction

In April 2015 Fødevareforlig 3 regarding chemistry in food was adopted by the Danish Parliament. Fødevareforlig 3 included research projects on

- Analytical methodology for chemical screening and analyses in food surveillance,
- Strengthened risk assessment of chemicals, and
- Risk-benefit assessment of foods.

This report is for the Miraculix project - Strengthened risk assessment of chemicals.

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MIraculiX – Strengthened risk assessment of chemicals

Humans are continuously exposed to complex chemical mixtures from foods and the environment. Current regulatory approaches for assessing chemicals typically evaluate one chemical at a time, an approach that fails to take into account the human real-world scenario of low-dose exposures to multiple chemicals. Since a growing body of evidence now suggests that simultaneous exposure to many chemicals at doses not singularly causing any effects can add up to induce adverse outcomes (Boberg et al. 2019), the current regulatory approaches are inadequate. Due to the complexity of the issue, the implementation of methodologies for risk assessment of chemical mixtures remains a challenge, hindering the urgent need to improve chemical risk assessments.

At DTU, we have for many years been investigating mixture effects in various experimental systems that have provided us with extensive knowledge on how chemicals interact or cause combination effects, and how we can predict mixture effects. However, pragmatic tools to evaluate the combined risk of mixed chemical exposures have been more difficult to develop. In recent years, regulatory authorities across the world have made some progress towards developing pragmatic frameworks to deal with combined exposure to multiple chemicals for risk assessment purposes. These approaches require a high level of information about chemical exposures and toxicities, information that often is lacking. We see this data gap as delaying urgently needed improvements in chemical safety.

With MIraculiX, we have aimed at closing some of the data gaps that hinders an appropriate risk assessment of chemicals individually and in combination.

1.1 Overall aim

In MIraculiX we have addressed the above mentioned challenges and we have:

- Investigated ‘real-life’ exposures to mixtures of chemicals and evaluated the human risk
- Further developed a pragmatic tool for mixture risk assessment that includes a user interface for mixture calculations and a step-by-step framework that guides the risk assessor
- Used this tool for illustrating a case in which chemical exposure from a ‘healthy’ versus an ‘un-healthy’ diet has been compared
- Developed a first proof-of-concept of an alternative approach for evaluating the risk of a chemical to adversely affect male reproductive health, which is a first step towards a reduced use of animals
- Elucidated novel mechanisms of action of chemicals that may affect male reproductive health

1.2 Topic 1: Tools for calculating mixture effects of chemicals

Over the last decade, authorities and scientific expert committees such as the US-Environmental Protection Agency (US-EPA), the Agency for Toxic Substances and Disease Registry (ATSDR), the World Health Organisation (WHO), the non-food Committees of the

European Commission (SCCS, SCHER, SCENIHR), the European Chemicals Agency (ECHA), and the European Food Safety Authority (EFSA) have all made considerable progress towards developing pragmatic frameworks that are “fit for purpose” and tiered, to deal with combined exposure to multiple chemicals for risk assessment purposes. The assumption that chemicals act additively and behave as if they were a simple dilution of each other has resulted in the development of methods for cumulative risk assessment using various approaches (EFSA, 2019a; Boberg et al., 2019).

In some previous cases of mixture risk assessment (MRA), chemicals with similar mode or mechanism of action have been considered together. However, in many cases grouping on the basis of similar effects is considered relevant, as experimental evidence shows that dose-additive effects are seen with exposure to chemicals having the same types of effect, even when they acted via different modes and mechanisms of action (Christiansen et al., 2009; Conley et al., 2018).

Grouping chemicals by similarity of effect has turned out to be a practical grouping method (Nielsen et al., 2012). Toxicological data for adverse effects of chemicals can be applied for grouping substances in cumulative assessment groups (CAGs). This grouping can be performed at different levels, i.e. at target organ level (CAG 1), at specific effect level (CAG 2), at mode of action level (CAG 3), or at mechanism of action level (CAG 4), as presented by Nielsen et al. (2012). This grouping approach was adapted by the EFSA PPR Panel in their opinion on the identification of pesticides to be included in CAGs on the basis of their toxicological profile (EFSA, 2013). Here, the PPR Panel suggested a methodology for grouping of pesticides based on phenomenological effects and provided CAGs for the thyroid gland and the nervous system (EFSA 2013). A draft scientific report on CAGs for effects of pesticides on the nervous system was published for public consultation in 2018 (EFSA, 2018b).

Recently, EFSA has compiled the available knowledge on methodologies used to perform MRA for human health, animal health and ecological MRA (EFSA, 2018a). For component based MRA, EFSA presented a tiered approach in which the first tier is MRA of all components regardless of toxicological endpoint (EFSA, 2018a). At the next tier, a refined MRA takes into consideration that not all the components have the same adverse effect/target organ. For this purpose, a target organ toxicity dose is derived for each endpoint (EFSA, 2018a, EFSA, 2013). Likewise, ECHA has presented a tiered approach in their guidance document for MRA of active substances in biocidal products (ECHA, 2017). Here, tier 1 includes substance-by-substance risk assessment, while tier 2 involves MRA without consideration of target organs. At tier 3 target organ specific effects are considered, and if possible knowledge on mode of action is applied for subgrouping (ECHA, 2017).

In several previous projects, DTU Food has been leading the way by presenting MRA across different chemical classes and exposure sources. Examples include MRA of four phthalates (ECHA 2012), of 13 endocrine disrupting chemicals (Andersen et al., 2012), of 69 neurotoxic or endocrine disrupting chemicals to which children and pregnant women are exposed (Larsen et al., 2017), of pesticide residues in food on the Danish market (Jensen et al., 2015) and anti-androgenic pesticides in food (Müller et al., 2009). This work serves as stepping stones for the development of the Chemical Mixture Calculator in the current project.

1.2.1 Aim

Tools to assess the combined risk of mixed chemical exposures have been lacking. In recent years, however, regulatory authorities across the world have made considerable progress

towards developing pragmatic frameworks to deal with combined exposure to multiple chemicals for risk assessment purposes. Here, we present a tool to perform MRA using available toxicity data for grouping of chemicals.
Our pragmatic tool (designated the “Chemical Mixture Calculator”) is a web-based tool facilitating MRA. The first version of it was developed in a previous project for Fødevarestyrelsen, but has been updated during MIraculiX. This tool includes a database on exposure and toxicity data for a number of chemicals in food and environment. As a first tier, it is possible to perform MRA without consideration of different effect types. This first tier likely overestimates the risk, as it combines toxicity data from various endpoints. At following tiers grouping of chemicals is based on similar target organs (or target organ systems), similar effects, or similar mode or mechanism of action. Additionally, a database (the “Intake Calculator”) was developed enabling comparison of population groups with different dietary patterns. Three case studies were performed to evaluate the use of the developed tools.

1.2.2 Activities and results

Principles of mixture risk assessment

In the Chemical Mixture Calculator, MRA is performed on the basis of principles of dose-addition in a tiered approach. The equation below is applied to determine the hazard index (HI) of a mixture). For each component of the mixture, the ratio between an exposure estimate and a health-based guidance value (HBGV) is established for each chemical and designated the hazard quotient (HQ). The HI is the sum of the HQs of several compounds.

$$Hazard\ index = \sum_{i=1}^{n} \frac{Exposure\ estimate}{HBGV}$$

In this project, exposures from dietary and non-dietary sources are summed up for each chemical before calculation of the HQ for that chemical. The term HBGV describes a dose level that can be ingested over a defined time period (e.g. lifetime or 24 h) without appreciable health risk (EFSA, 2018) and includes tolerable daily intakes (TDIs) and acceptable daily intakes (ADIs) (see terminology in Appendix 1). This HI calculation is also the first step presented in our pragmatic approach for MRA (Boberg et al., 2019). Using TDI or ADI for MRA can be a crude first step, and the resulting HI may overestimate the actual risk, as TDIs and ADIs may be based on critical effects for different toxicological endpoints for different chemicals. Thus, MRA can be refined by grouping using *in vivo* data or alternative data such as QSAR predictions, *in vitro* data or omics output (Boberg et al., 2019).
To refine MRA by use of *in vivo* data, target-organ specific toxicity data can be applied, and chemicals can be grouped at different levels, i.e. at target organ level (CAG 1), at specific effect level (CAG 2), at mode of action level (CAG 3), or at mechanism of action level (CAG 4) (Nielsen et al., 2012). By using different CAGs for grouping, MRA can be performed at different levels of refinement.

Two fit-for-purpose databases developed

In this project, two tools were developed: the Chemical Mixture Calculator for MRA, and the Intake Calculator for comparison of chemical intake of populations with different diets.

The Chemical Mixture Calculator, a tool for mixture risk assessment

The Chemical Mixture Calculator is a web-based tool facilitating MRA and will be made available online in 2020. This tool includes a database on exposure and toxicity data for a number of chemicals in food and environment. As a first tier, it is possible to perform MRA without consideration of different effect types, i.e. MRA is performed based on the TDI/ADI of the single compounds. This first tier likely overestimates the risk, as it combines toxicity data from various endpoints. At following tiers grouping of chemicals is based on similar target organs (or target organ systems), similar specific effects, or similar mode or mechanism of action.
To enable the grouping of compounds, we selected six main target organs / tissues: thyroid gland, kidney, liver, haematological system, nervous system, and reproductive and developmental toxicity. The main CAGs within each of these target organs / tissues and principles for selecting these as relevant for MRA are described in a manuscript in preparation (Boberg et al., *in prep*).

Database setup
For the purpose of MRA, a database and a web-based user interface were developed. The requirements for MRA are 1) dietary or non-dietary exposure data, and 2) toxicity data including HBGVs and target-organ specific toxicity information.
In brief, exposure data were mainly based on Danish data pertaining to content in food, then the chemical intake of children and adults was calculated based on the Danish Dietary Survey (Petersen et al., 2013; Jensen et. al, 2019). For chemicals where Danish dietary data were not available, exposure data were collected from published reports from e.g. EFSA and ECHA. Non-dietary exposure data were also collected from published reports. For each chemical a mean and a “high” value was selected for three age groups (when possible): toddlers, children and adults.
Toxicity data were mainly collected from reports published by EFSA and ECHA. Health-based guidance values such as TDIs and ADIs were listed together with Derived Tolerable Doses (DTDs) for target-organ specific effects. First, we listed ADIs and TDIs for each compound. For the purpose of refined grouping, we defined the term DTD describing the dose level that can be ingested over a lifetime without appreciable risk of a specific effect on a selected organ system. The term DTD corresponds to the term Target-organ-specific dose (TTD) applied by EFSA (EFSA 2013).
In practice, all selected compounds exerting a toxicological effect on one of the six selected organ systems were allocated to a CAG Level 1. In the next step, toxicological information from scientific reports (EFSA, ECHA etc.) was scrutinized and compounds showing a common toxic effect on a phenomenological/specific effect basis in each target organ and tissue were grouped into CAGs at Level 2. Refined CAGs were established, when it could be demonstrated that the compounds actually possess the same mode of action (CAG Level 3) or mechanism of action (CAG Level 4).

User interface for mixture risk assessment
The user interface was designed to enable grouping of chemicals into different CAGs by filtering functions. Additionally, selection of exposure data for different age groups was possible. Thus, HI values can be extracted at different levels of refinement of MRA and for different populations.

The Intake Calculator, a database for comparison of populations having different dietary intake

For the purpose of enabling comparison of population groups with differential dietary patterns (case 1), a database was developed for chemical exposure calculation based on data from Denmark both with regard to consumption and content (Pedersen et al, 2015, Petersen et al., 2013). In this Excel tool, individual food intake is linked to information on contaminant levels in those food types. This enables the comparison of contaminant exposure of individuals in different age groups. The information is obtained from individuals participating in the Danish Dietary Survey for 2011-2013 (Pedersen et al. 2015), and chemical concentration data are from measurements in Danish foods in the period 2004-2011 (Petersen et al., 2013). Additionally, all individuals were ranked according to how well their dietary habits fulfil the Danish dietary guidelines (Knudsen et al., 2012). The database is available for use in future projects at DTU Food not only for calculating total exposures, but also for calculating exposures from specific foods or food groups.

Three cases studies

Three case studies were performed to evaluate the use of the developed tools. First, we compared two populations with different dietary intakes with respect to chemical intake (Case 1) and MRA (Case 2), and then we focused on a specific group of chemicals, the phthalates (Case 3).

Case 1: Comparing chemical exposures in populations with different dietary patterns

The first case study focused on comparison of two populations using information on their dietary intake patterns. The aim was to compare a “healthy” with an “unhealthy” diet with respect to chemical intake, as described by Petersen et al (2019). Using the database for chemical exposure calculation, two population groups with different degree of fulfilment of the Danish dietary guidelines were selected. The chemical intakes as well as the risk of adverse health effects for these two groups were compared and discussed for each individual chemical (Petersen et al., 2019).

This case study showed that consumers who have a diet more in compliance with the dietary guidelines have a higher exposure to contaminants than consumers whose consumption patterns are less in compliance with the guidelines (Petersen et al., 2019). However, large standard deviations indicate that the consumption patterns can be very different within each population group. As expected, children in general have a higher mean contaminant exposure per kg body weight than adults, probably due to a higher consumption of food per kg body weight. Higher HQs in the population with the highest scores for fulfilling dietary guidelines indicate higher risk of toxic effects. The main reason for the higher level of contaminants in the healthy diet was because of the fish contained in the healthy diet. As contaminant levels between fish varies to a great extent, the conclusion will not always hold true. Furthermore, the conclusion should be regarded with caution due to the large standard deviations in exposure values and to the limited number of chemicals for which we have data on their occurrence in foods. More importantly, nutritional benefits of a diet that fulfils the dietary requirements (e.g. containing much fish) may outweigh the possible concern related to higher risk of toxic effects of chemicals in the diet.

The developed Intake Calculator combining data on consumption and concentration was very useful for calculating exposures for selected consumer groups and is considered applicable for future projects comparing populations with different dietary patterns.

Case 2: MRA in comparison of population groups
In the second case study, the chemical intake data from the two population groups in case study 1 was used for MRA at different levels of refinement using the Chemical Mixture Calculator (Boberg et al., *in prep*).
Grouping of chemicals was performed by filtering the dataset, i.e. selecting a specific CAG in the Calculator tool. At the first tier, MRA was performed using ADI or TDI, thus no filtering for organ system was performed when HQs for all chemicals were summed. At the next tier, the same data were grouped by target organ, i.e. data were filtered at CAG level 1. This was followed by grouping at CAG level 2 as a next tier.
These data showed how refinement of grouping leads to reduction of HI values. The selection of level of refinement requires scientific insight and is crucial for the obtained conclusions. The highest HI values were seen when no grouping was applied, and mainly lead and dioxins/dioxin-like-PCBs contributed to this value. However, most other substances also contributed markedly, particularly for children.
In this case of comparing populations with different dietary patterns, this approach was useful for identification of the chemicals and food groups that are the main contributors to the overall risk.
We concluded that the Chemical Mixture Calculator can be used by risk assessors as a pragmatic tool for MRA or for identification of the chemicals that are main contributors to the overall risk in specific populations. The selection of level of refinement makes a large difference in results, and thus the use of the Chemical Mixture Calculator is preferable in conjunction with expert advice.

Case 3: MRA of phthalates
The third case study used the Chemical Mixture Calculator for MRA of phthalates at different levels of refinement. Grouping was performed first for phthalates alone, and then across all chemical classes included in the Chemical Mixture Calculator (Boberg et al., *in prep*).
HI values for phthalates alone exceeded 1 for highly exposed toddlers, indicating a potential risk. This was seen even with refinement of MRA to CAG levels 2 (specific effect) and 3 (mode of action). When evaluating HI values of all substances, the phthalates contributed markedly to the overall HI for toddlers, whereas for adults other substances (particularly dioxins and dioxin-like PCBs) contributed too. This approach also allowed comparison of HI values for different effect types.
The Chemical Mixture Calculator proved useful for MRA for a specific chemical group. This approach was useful for identification of how much a certain chemical group contributes to the overall risk, and whether it makes a difference to include other chemicals in MRA. Again, the selection of level of refinement makes a large difference in results.

1.2.3 Discussion & conclusion

Two tools were developed; one for MRA (the Chemical Mixture Calculator), and one for comparison of chemical intake of populations with different diets (the Intake Calculator).

The Intake Calculator was applied in a case study comparing populations with dietary patterns. The developed database combining data on consumption and concentration was very useful for calculating exposures for selected consumer groups, and is considered applicable for future advisory and research assignments comparing populations with different dietary patterns.
The Chemical Mixture Calculator is considered useful for MRA, both when comparing different diets (Case 2) and for evaluating the risk related to a specific chemical class alone and grouped with other substances (Case 3). This ability to perform MRA using existing data on numerous substances is an important step in meeting future requirements of performing MRA across different chemical classes, different target organs / tissues and exposure sources. Additionally, the Calculator could be a useful research tool. The collected knowledge and the developed tool for grouping of chemicals may in the future be applicable for use in other fields such as risk-benefit analyses or life cycle assessment.
The Chemical Mixture Calculator can be used by risk assessors as a pragmatic tool for MRA or for identification of the chemicals that are main contributors to the overall risk. The use of the tool is preferable in conjunction with expert advice, because the selection of tier (level of refinement) requires scientific insight and is crucial for the obtained conclusions. Future use of the tool in practice should be followed-up by evaluation of applicability in practice as well as needs for improvements.
The database contains information with some uncertainty, as is always the case in risk assessment of individual chemicals, and in MRA such uncertainties may be amplified. As stated by EFSA 2014, grouping based on effect rather than mode of action will lead to more uncertainties in prediction, but there will also be high uncertainty in excluding substances with little information on mode of action. Generally, the current approach is considered useful for enabling MRA of multiple chemicals, while still embracing the related uncertainty.
In the current form of the Chemical Mixture Calculator, the toxicity grouping and setting of DTDs are mainly based on experimental in vivo data summarized in scientific opinions by expert committees, predominantly EFSA. Future development of the tool can include the use of in vitro data or other alternative data (Boberg et al., 2019) for chemicals for which no experimental animal data are available. This may be possible by e.g. combining knowledge on toxicokinetics and relative potencies in vitro of different substances. The Chemical Mixture Calculator includes some information on human exposure from other sources than food, but for most substances, this information is difficult to obtain. Future development of the tool may include the use of human biomonitoring data, which represent the true integrated human exposure and therefore could contribute to more realistic MRA. However, in biomonitoring the exposure sources of chemicals are often unknown, which may hamper regulatory actions.
We consider it a strength of the tool that the principles behind are in good agreement with principles of e.g. EFSA and ECHA (EFSA, 2018, 2019, EFSA, 2013, ECHA, 2017). The future use of the tool in practice is expected to have international impact, as specific cases where the tool might be is used in advisory work (for the Danish Food and Veterinary Administration or Danish Environmental Protection Agency) will likely be of interest for e.g. EFSA and ECHA. Our presentations of the idea behind the tool at international meetings have led to expression of interest from regulatory bodies of other European countries, particularly the Nordic countries.

1.3 Topic 2: Realistic mixtures of chemicals

Usually, toxicological studies are performed at relatively high dose levels compared to actual human exposure levels. However, what is the risk when humans are exposed to a large number of chemicals at really low dose levels?

1.3.1 Aim

The aim of this work was to investigate potential adverse effects caused by low-dose exposure to chemical mixtures. The project focused on investigating chemical mixtures in male as well as female reproductive studies and aimed at characterizing some of the underlying molecular mechanisms for endocrine disrupting effects on fetuses.

1.3.2 Activities & results

The available literature on exposures to mixtures containing large numbers of compounds remains small. Most large mixture studies report on mixtures with closely related chemicals, which is done to study putative dose-addition effects. In our first study, we looked at female reproductive effects of a human relevant mixture of 13 endocrine disrupting chemicals with known anti-androgenic or estrogenic modes of action (Johansson et al. 2016). Our second study, however, was specifically designed to recapture more realistic human-relevant exposures, where 27 diverse chemicals were selected (Hadrup et al. 2016).
The first mixture study was a continuation of previous work (EU financed project called CONTAMED) looking into the combined effects of various chemicals known to act by anti-androgenic or estrogenic mode of actions. Thirteen chemicals were included in a developmental mixture toxicity study in rats, including phthalates, pesticides, UV-filters, bisphenol A, parabens and the drug paracetamol. The mixture ratio was chosen to reflect high-end human exposure scenarios. Two subgroups, anti-androgens (AAmix) and estrogens (EEmix) were designed and used for *in utero* exposure in rats, and a complete mixture (Totalmix) comprised all of the 13 chemicals. In this paper, we focused on potential female reproductive effects, as previous studies on the same mixtures have mainly looked at mixture effects on male reproductive endpoints (Christiansen et al. 2012; Isling et al. 2014; Axelstad et al. 2014). We found effects of chemical mixtures on female reproductive parameters in both prepubertal and adult female offspring following in utero exposure (Johansson et al., 2016). In TotalMix exposed rats, we observed irregular estrous cycling in the adults, but not in prepubertal animals, which could indicate premature senescence. We also observed reduced ovary weights in exposed rats, likely caused by a significant lower number of corpus lutea (large cysts formed after ovulation), again pointing towards accelerated rate of aging. Interestingly, it was the AAmix, rather than EEmix that seemed to adversely affect the ovaries, attesting to the importance of androgens in ovarian development and function (Johansson et al., 2016).

In the second study (Hadrup et al. 2016), the rationale for mixture design was different, as the aim was to capture a more realistic exposure scenario that included different chemicals with different modes of action. For this purpose, we designed a mixture comprising 27 human-relevant chemicals at doses approaching human exposure levels based on human biomonitoring data. The aim was to investigate whether this mixture could cause adverse health effects. Juvenile male rats were exposed to a mixture of metals, phthalates dioxins, bisphenol,

PCBs, etc. (Table in Appendix 2). The study included three exposure doses, with the lowest dose designed to obtain plasma levels in rats approaching human relevance.
We did not observe any adverse reproductive effects in this study, but the liver was significantly affected already in the low dose group, with clear signs of steatosis ('fatty liver'). In exposed animals, we saw increased liver weight and histopathological changes, as well as altered gene expression pattern for markers of hepatotoxicity. There were also signs of adverse effects on kidneys and the metabolome in the high-dose group. Notably, we performed temporal metabolomics analysis on blood samples in this study, which allowed us to look at responses to chemical insult over time. As exemplified by the PLS-DA plots (Appendix 2), we could clearly separate the metabolome between the three dose groups, but the variation between the three time points used for measurements (30, 60, 90 days) were not that great. More broadly, the effects that we observed in the livers indicate a stress-response to chemical exposure, with consequences such as increased carbohydrate metabolism, disturbance to cholesterol biosynthesis and bile acid synthesis. In addition, since these effects were observed in the low-dose group, most of the chemicals in the mixture known to cause such effects, including arsenic, bisphenols or PCB, were singularly present in doses well below doses previously reported to cause these effects. One exception, and a caveat to our results, was TCDD, which we estimated to have an accumulated dose 0.66 µg/kg bw after 3 months of exposure. This dose falls between previously reported liver toxicity doses (Kociba et al 1976). Hence, we cannot exclude that TCDD could cause some of the liver effects singularly, but it is most likely that it is the combined exposure of many chemicals at low dose that cause the adverse effects, effects that otherwise would not be observed in single-chemical exposure experiments.

In this project, we have carried out a pilot study to start assessing the potential risk associated with replacing known reproductive toxic phthalates with other plasticizers expected to be less toxic to the male reproductive system. The chemicals studied were DINCH and its main metabolite MINCH, and diisononylphthalate (DINP) and its main metabolite monoisononylphthalate (MINP), with the ultimate aim of assessing their influence on developing testis and potential for mixture effects. The study involved exposing testis explants to these four chemicals. The first ex vivo experiments were carried out to determine at what dose we could observe effects on the developing testis, and subsequently design low-dose mixtures below individual effect doses to see if we could provoke the same effects by dose-addition principles. Dose response experiments were conducted with changes to testosterone levels used as adverse effect read-out. As exemplified by DINCH in Figure 2 and MINCH in Figure 3, testosterone synthesis was not significantly affected at the lower doses. The significant effect on testosterone at the highest dose was caused by general toxicity, with severe tissue necrosis. Unfortunately, we experienced large variations between samples for DINP and MINP, which prevented us from concluding on these results; but again, there appeared not to be any significant effects on testosterone production even at high doses. Based on these preliminary findings, we have not continued the experiments with mixtures.

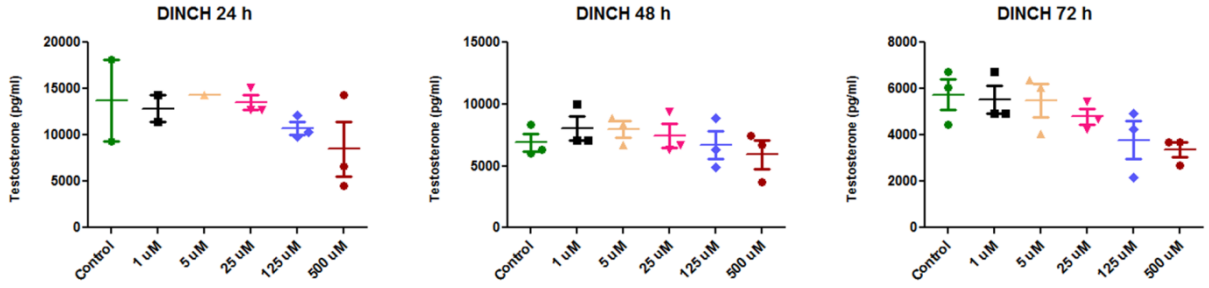

Figure 2: Ex vivo fetal rat testes exposed to increasing doses of DINCH. Testes were dissected from GD15 rat male fetuses and cultured in the presence of DINCH. Testosterone levels in medium were measured after 24, 48 and 72 h. In the 72 h measurement, a dose response decrease in testosterone levels was evident, whereas decreased testosterone was only observed at high dose at 24 and 48 h, where tissue toxicity was also observed.

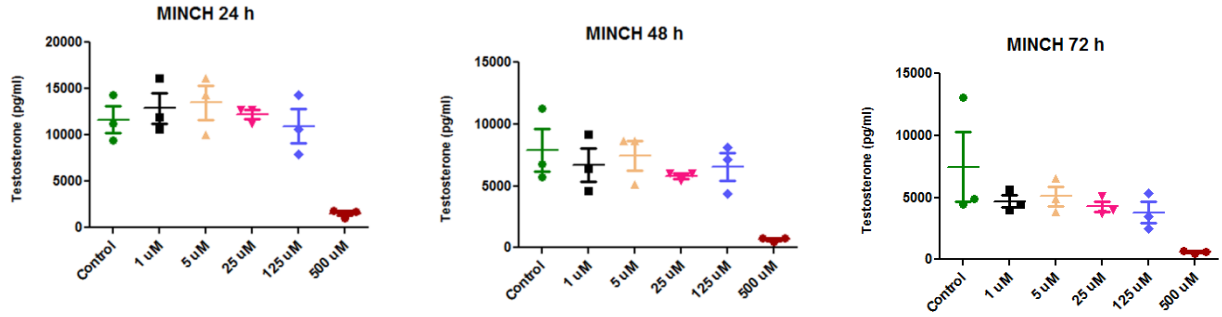

Figure 3: Ex vivo fetal rat testes exposed to increasing doses of MINCH. Testes were dissected from GD15 rat male fetuses and cultured in the presence of DINCH. Testosterone levels in medium were measured after 24, 48 and 72 h. No significant decrease in testosterone was evident at any stage apart from in the 500 µM exposure group, an effect that was due to significant tissue toxicity.

1.3.3 Discussion & conclusions

In this part of the project, we have reported three separate studies looking for mixture effects of environmental chemicals. The first study was a continuation of previous studies examining the effects of exposure to mixtures of known endocrine disrupting chemicals. As we previously have published male reproductive effects, the study included in Miraculix was looking at long-term effects for female reproduction. We found that chemical mixtures approaching human relevant doses can cause adverse reproductive effect in female offspring exposed in utero and postnatally. Ultimately, this could suggest that we do not have a safety margin of 100 when considering contaminants in foods and the environment. In other words, highly exposed individuals, especially women of the reproductive age, may not be sufficiently protected against the combined effects of chemicals capable of disrupting sexual development. This effect was most pronounced for the anti-androgenic compounds, a class of chemicals otherwise considered for their detrimental effects on male reproductive development.

In view of this potential underestimation of risk associated with exposure to chemical mixtures, it is clear that additional studies must be carried out. And new studies must account for human-relevant exposure scenarios and inter-species differences, including determination internal dose

levels in animal models and compare them to human biomonitoring data. Additionally, more sensitive endpoints for endocrine disruption should ideally be included, but this may rely on the development of new test methods.
In the second study, the chemical mixture was more complex and included chemicals of varied structures and known adverse effects outcomes. Individually, the chemical doses were set to represent low human exposure levels. However, a caveat was that certain chemicals were chosen to represent chemical classes, so that for instance BPA levels were higher to also account for other bisphenols. We assume same effects of these very similar chemicals, but a more realistic scenario would nevertheless be to include all of the actual chemicals, thus expanding the mixture well beyond the 27 compounds.
Although we looked at male reproductive endpoints in the 27 chemical mixture study, we only exposed male rats postnatally and not during development. It is well-known that the most sensitive period for male reproductive outcomes is during fetal life when the masculinization process takes place. Exposing pregnant rats to the same mixture could therefore result in effects not observed in the juvenile male rats, so that lack of effects in this study should not be understood as being safe with regard to reproductive parameters. We did observe effects on the liver, however, as well as on the metabolome. As discussed above, the dose of TCDD could in itself cause some of these effects according to some previously published studies, but the more likely event is that the mixture contributed towards the effects. It is very challenging to design and perform large mixtures studies, and our study is one of only a very few such studies that are currently available. We would therefore advocate for even more studies like this, where chemicals with different modes of action and effect outcomes are included in the same mixture, rather than what is more common; mixtures that contain chemicals with similar modes of action. Also, the possibility of including mixture prediction models in such projects would be warranted.
In future studies, it will be important to gain knowledge on internal dose levels in reproductive and developmental in vivo toxicity studies and compare those to human biomonitoring data. This is essential if we are to make sound judgements on the relative risk of exposure to the human population. But for mixture studies this is both technically challenging and expensive. However, if internal dose levels are known from single exposure studies this can be extrapolated and used a reference for mixture designs. Our recommendation is therefore to design new mixture studies based on human biomonitoring data and select chemicals for which we can calculate actual internal dose levels relevant for specific endpoints.
In future studies with realistic mixtures we would like to include even more sensitive endpoints when more sensitive methods have been developed for the identification of endocrine disrupting effect in vivo.
Moreover, knowledge of low-dose effects in vivo including knowledge of internal doses may be useful for comparison with the in vitro active doses.

1.4 Topic 3: Mechanisms of action in male reproductive health

AGD has been used for decades as a biomarker to distinguish male from female offspring in various animals (e.g. rodents, kittens) and the presumption has always been that the level of androgens during fetal life determines the length of the AGD. In other words, a long AGD is the result of fetal testosterone (as in males) and a short AGD is the result of very little testosterone (as in females). A short male AGD is therefore a result of too little androgen action and a sign of feminization. Consequently, AGD measurements are used in reproductive toxicity studies, where a short male AGD is considered an adverse effect, and are mandatory in various new and enhanced OECD test guidelines (OECD TG414, TG421/422 and TG443).
In view of the androgen hypothesis mentioned above, it would be reasonable to assume that AGD is directly proportional to androgen action (or conversely, directly proportional to concentrations of plasma anti-androgenic compounds). In other words, exposure studies to anti-androgenic chemicals would most often result in clear dose-response relationships. However, this is not always the case. Many studies have reported disparate results, including shallow dose-response curves. Additionally, female AGD may be affected in both directions.

1.4.1 Aim

The overall aim of this project was to improve on our knowledge on the use of AGD as a biomarker for endocrine disruption. To get a better overview of the available data, we wanted to collect all relevant data on AGD measurements from relevant exposure scenarios, and compile an extensive list to serve as a resource for further studies. Not least with the aim of improving risk assessment of chemicals for effects on male reproductive health. Finally, the project had a strong focus on characterizing mechanisms causing EDC-induced shortening of AGD in male offspring.

1.4.2 Activities & results

Literature review of effects on anogenital distance

We have completed a comprehensive literature review of studies involving AGD measurements (Schwartz et al 2019a). This is the first review that covers this topic across animal toxicity studies and will be a very valuable resource for researchers, regulatory bodies and the industry. Our synthesis includes a large catalogue of data concerning measurements of AGD as a read-out for endocrine disruption and discuss the utility of AGD in a toxicological setting. The review contains two extensive data tables providing an overview of available reproductive toxicity studies using AGD as an endpoint (Appendix 3).
Overall, we found that around 24 chemicals were published to have an effect on AGD, half of which were phthalates and half of which were either pesticides, drugs, or environmental chemicals.

Table 3.1 (Appendix 3) lists the phthalates that have been tested in rat toxicity studies and reveals that this group of chemicals generally shows relatively clear monotonic dose-response relationships. The higher the dose of anti-androgenic phthalate, the shorter the AGD in male offspring without discernible effects on female AGD.

Table 3.2 (Appendix 3) lists all the other chemicals that have been tested in similar rat toxicity studies, and do not always show steep dose-response curves for effects on AGD. Although

exposure to known anti-androgenic compounds such as finasteride, linuron and acetylsalicylic acid have resulted in markedly shorter male AGD at high doses, many compounds only cause ~15% shorter AGD even if the dose is increased further. Such shallow dose-response curves are puzzling and indicate that not only androgen action may be responsible for determining AGD. Even more puzzling are the effects observed for azole fungicides, with many of them causing longer male AGD as well as effects on female AGD. With epoxiconazole, the picture is less clear with only weak indications that it may affect male AGD, but longer female AGD is seen in several studies. For prochloraz, the effects on AGD are conflicting between studies, but longer female AGD is often seen, whereas exposure to 50 mg/kg of the potent drug ketoconazole results in shorter AGD in both sexes.

Having compiled available data on AGD measurements following in utero exposure to various chemicals, it is apparent that the phthalates generally act as anti-androgens in rats with predicted dose-response relationships. With other chemicals such as some conazole fungicides, bisphenol A or butylparaben, however, the picture remains less clear and suggests that there are other regulatory pathways involved. It is likely that disrupted androgen signaling is the main regulatory pathway affected when male AGD is short, but that there are other mechanisms that act either in parallel, or at the very least within a complex regulatory network, so that their disruption can modify the effects seen on AGD. Elucidating these mechanisms thus becomes necessary for proper testing of suspected EDCs, with the end goal being to construct complete AOPs for AGD effects and associated diseases.

Mechanisms involved in development of the anogenital tissue

To characterize novel regulatory pathways that can be involved in AGD effects, we performed molecular analyses of the perineal region of male rat fetuses that have shorter AGD after exposure to chemicals. We induced short male AGD in rat offspring by several chemicals (as described in Appendix 3 in this report) and isolated appropriate tissues for molecular profiling. The first chemical we used was finasteride (FIN), specifically for its known effect of inhibiting 5-alpha Reductase, which leads to lower levels of DHT and less AR activation. We would based on earlier studies on FIN (Christiansen et al. 2009) predict that FIN exposure would result in significant shorter male AGD. It turned out to be the case (37% shorter than control males) (Appendix 3).

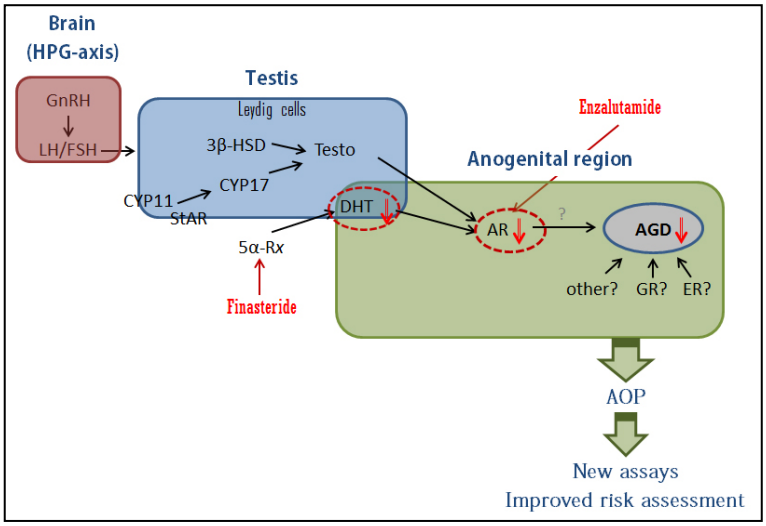

Figure 4: A diagram of the androgen sensitive pathway believed to be the main driver of fetal male masculinization, particularly external genitalia and the anogenital region. ENZ is like VIN and PRO anti-androgenic and targets AR. FIN targets mainly 5α-reductase but also AR. Red circles indicate target molecules of the selected drugs to be used for in utero exposure in the in vivo studies.

Since we were looking for novel pathways affected when male AGD is shortened by *in utero* exposure to chemicals, we performed a whole-genome gene array analysis to cover all possible genes that can be regulated in rat tissues. We isolated the perineal tissue (region defining AGD) in GD21 fetuses, including control tissues from both male and female fetuses, and in FIN-exposed male fetuses with a significantly shorter AGD. From this tissue, we isolated RNA and performed the Gene Array analysis. To our knowledge, this is the first gene array ever reported for this tissue, and will thus serve as a valuable resource both in biology and toxicology.

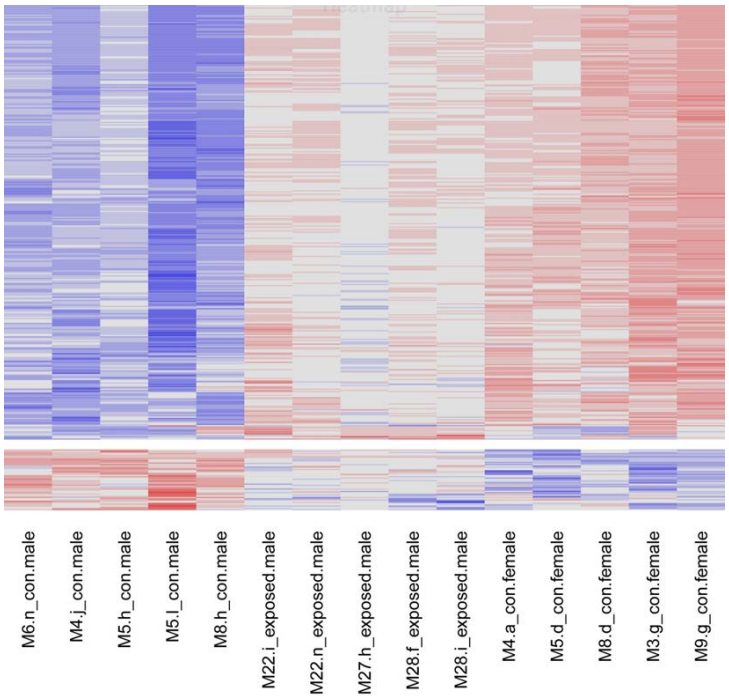

Figure 5: A heat-map cluster of approximately 350 genes that are up- or down-regulated in males exposed to finasteride relative to control animals. The exposed male animals, presenting with significantly shorter AGD, have an expression profile lying between male and female controls (Schwartz et al 2019b).

Gene expression analysis revealed clusters of genes that were dysregulated in exposed male offspring relative to male controls, totalling 350 regulated genes (Appendix 3). The expression profile was intermediate from male and female controls. Of the many regulated genes, we identified four genes we believe to be of particular interest: *Esr1*, *Padi2*, *Wnt2* and *Sfrp4*, and thus verified their expression profiles by RT-qPCR analysis (Appendix 3). The regulation of these genes, and the fact that they proved to be expressed in the male foetuses with short AGD at levels between males and females, strongly suggests that both estrogens and Wnt-signaling play key roles in the sexually dimorphic development of the perineum, and that chemicals affecting these pathways can be speculated to also affect AGD (Schwartz et al 2019b).

A stated aim for this work was to gain new knowledge that will enable us to refine Adverse Outcome Pathways (AOPs) where AGD is the effect endpoint. With mechanistic knowledge gathered from the studies described above, including on-going investigations using techniques such as tissue explants, preliminary steps can be included in existing AOP frameworks. We have included both ER and WNT signalling events, which can help explain some of the disparate AGD data observed in rat toxicity studies, as well as effects on female AGD.

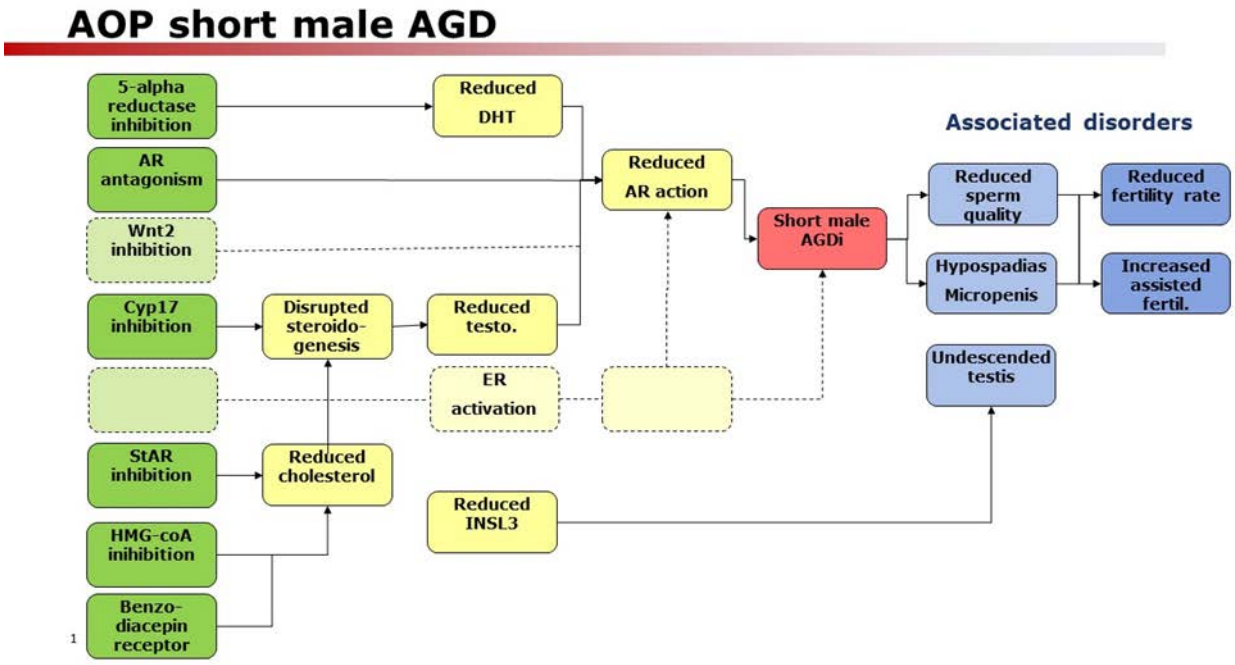

Figure 6: Suggested amendments to Adverse Outcome Pathway (AOP) for short male anogenital distance (AGD). A molecular initiating event (green boxes) leads to downstream events at cellular or organ levels (yellow boxes) that ultimately caused the AGD of male offspring to be shorter than normal (red box). A short male AGD is associated with several adverse reproductive outcomes in males (blue boxes), both in rodents and humans. AR = androgen receptor; ER = estrogen receptor, DHT = dihydrotestosterone; AGDi = AGD-index.

1.4.3 Discussion & conclusions

AGD has been used as a marker of masculinization in animals for a very long time. First as a simple way of determining the sex of newborn animals such as cats and rodents, but later also in rodent developmental and reproductive toxicity studies looking at fetal programming of masculine traits. AGD has also been associated with exposure to environmental chemicals and male reproductive disorders in humans, so that AGD measurements are now included in OECD test guidelines for Reproductive toxicity to better cover endocrine disruption. Several OECD guidance documents states that "*A statistically significant change in AGD that cannot be explained by the size of the animal indicates effects of the exposure and should be considered in setting the NOAEL (No Observed Adverse Effect Level)"*

Over the years many studies have reported on effects on AGD in rodent studies, but both study designs and reporting have varied in detail and quality, making it difficult to draw up a clear picture of what exactly AGD effects measure. Having synthesized much of the available data on early life exposure and perinatal AGD in rat, our recent review article reveals that phthalates as a group act as anti-androgens in a dose-responsive manner. Other chemicals such as pesticides and drugs also have clear effects on male AGD, but with sometimes more shallow dose-response relationships.

Especially with regard to some conazole fungicides effects on the AGD can vary. In many cases female AGD is also increased after conazole exposure, which is a clear sign of masculinization,

or androgenic effects. This effect can be hypothesized to be secondary to the expected increase in progestagens that is seen for many conazoles. Whether the males are affected or not may be a matter of potency of the pesticides.
Although it is clear that androgens are the main driver of masculinization, including making the AGD longer in males than in females, disparate data also suggest that there are other signaling pathways involved. Therefore, this projects specifically aimed at finding additional 'masculinization factors'. Our gene array analysis of perineal tissues in male rat offspring presenting with short AGD have given us several clues as to what other mechanisms can influence the AGD, and in extension also other reproductive effects. Most interestingly, estrogen signaling was found to be different in this tissue between males and females, and importantly, in feminized males. Estrogen signaling has been proposed to be involved in penile development, but until now it has not been shown to be directly involved in perineal development, and thus AGD. Another important developmental signaling pathway, WNT, was also found to be affected in the feminized male perinea. These findings opens up new avenues to be explored with regard to the effects that environmental chemicals can have on development and disease. This knowledge can also be directly used to construct more complex AOPs, which ultimately will help us make more informed decisions when assessing the likely impact of chemicals on human health. We are currently continuing work from this project to further characterize the complex associations between AGD, chemical exposure and other reproductive disorders.

1.5 Topic 4: A novel approach for predicting male reproductive toxicity of chemicals

1.5.1 Aim

The majority of chemicals in current use have not been tested for endocrine disrupting effects and there is a great need for developing new animal-free tools that can be used for this purpose. The aim of this subproject was to further develop a new approach to be used for prioritizing chemicals for in vivo testing for male reproductive health disorders. Based on information from in vitro experiments and physio-logically-based kinetic modelling (PBK) modelling, we have investigated the applicability of the tool for predicting effects on AGD, which was presented in the last chapter as a unique marker of male reproductive health disorders in animals and humans.
In addition, our aim was to advance the application of alternative test methods in chemical risk assessment by incorporating metabolism into in vitro assays.
These activities have been made possible due to funding by the Danish EPA of other projects dealing with the same topics.

1.5.2 Activities & results

Exposure of the human male fetus to endocrine disrupting chemicals contributes to the increasing incidences of reproductive health disorders, such as poor sperm quality and malformation of sex organs at birth. To test whether chemicals are safe for humans, novel methods without animal tests are urgently needed. We developed an approach that combines in vitro assays and in silico modelling for predicting effects that could also be measured in animals. As a readout, we used AGD – a unique and lifelong marker for male reproductive health disorders in humans and animals (Fig 7).

Prediction model

Information on the hazards of single chemicals was gathered by using a panel of in vitro assays. The focus was on endpoints that are related to or can be used as predictors for reproductive toxic effects such as antagonism of the androgen receptor and inhibition of testosterone synthesis.
Next we developed PBK models for a range of pesticides (Table 1) in collaboration with Brunel University. These models were used for predicting the fetal levels of pesticides that would cause a reduced AGD in male rat pups. The in vitro data (IC_{50} or IC_{20} values) served as the input of the models.

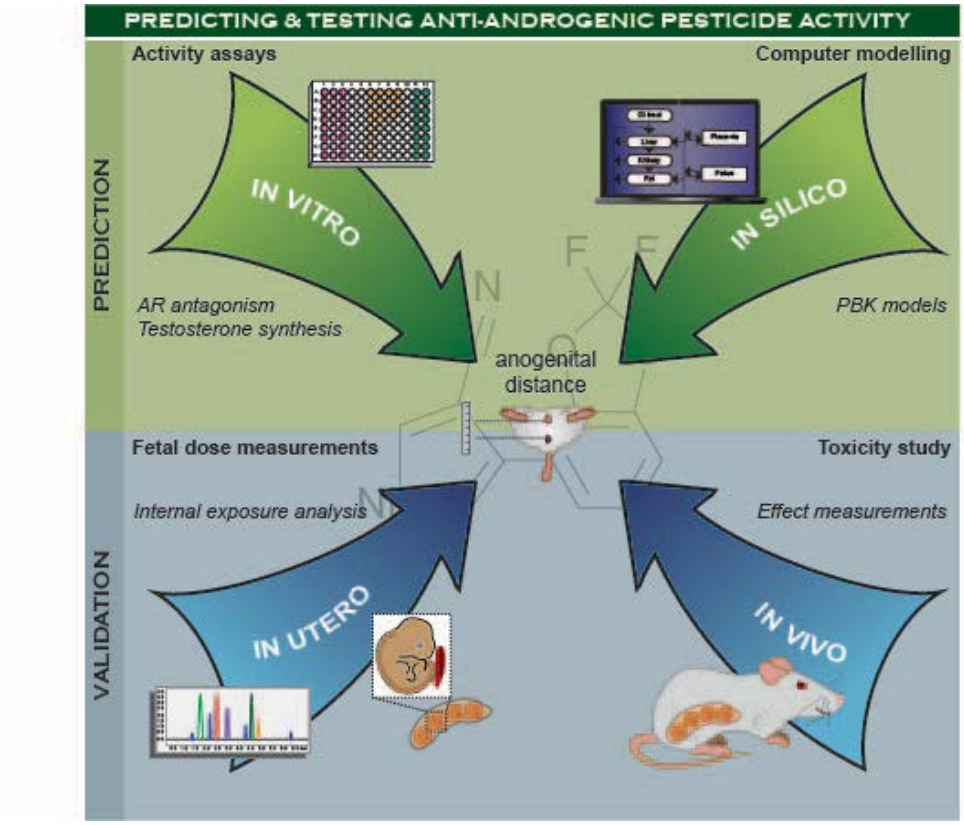

Figure 7: Graphic illustration of our strategy for predicting and validating male reproductive health effects of chemicals. A) The upper left part illustrates the *in vitro* profiling for relevant mechanistic endpoints. **B)** *In vitro* responses were fed into the PBK models by 'reverse dosimetry' for predicting the foetal concentration and external dose leading to androgen insufficiency in the fetus. **C)** The lower part illustrates how we validated our predictive model. The predicted active doses of pesticides were dosed to pregnant dams from gestational day 7 (GD7) until GD21. **D)** Fetuses were taken out by Caesarean section on GD21 or allowed to give birth. Pesticide levels including their metabolites were measured in foetal blood, amniotic fluid and in the dams on GD21 and compared to the simulated levels and the predicted *in vitro* active concentrations. E) AGD was measured as a morphometric readout for androgen insufficiency in fetuses or the newborn pups.

Validation of predictions

In order to validate our predictions, in vivo studies in pregnant rats were performed at doses that did not cause maternal toxicity. The animals were exposed to pesticides in utero from GD7 to GD21 (or GD 13-21 for linuron) which includes the sensitive foetal masculinization window GD15-19. In the fetuses that were retrieved by Caesarean section, the pesticides and potential active metabolites were measured in fetal blood and amniotic fluid as well as in the blood from dams. At GD 21 AGD was measured. This validation was performed in order to evaluate the accuracy of the predictions and in order to get confidence in our prediction model.

We have in this and previous projects collected in vitro, in vivo and PBK modelling data for seven pesticides as seen in Table 1 below. The results indicate that pesticide levels in fetuses generally can be predicted very well by PBK modelling and that the measured levels for these six pesticides are within a factor of 5 from the predicted levels.

Importantly, the validation studies showed that our predictions for effects on AGD seemed to be successful in predicting actual in vivo effects. The measured in vitro activities for anti-androgenic action are in general within the range of the measured fetal levels, which supports the hypothesis that these mechanisms of action are responsible for the effect on AGD. Overall, we find a relatively good predictive power of our alternative approach to predict AGD effects in vivo, although more work is needed to test and refine the approach for more chemicals. This work has now been submitted as a paper (Taxvig et al. 2020, submitted).

Table 1: Validation of in vitro to in vivo extrapolations
In vitro profiling for anti-androgenic effects (ICs for AR antagonism and IC_{50} for testosterone inhibition) and simulated average fetal plasma levels (GD15 - GD18, 2-6 hr after dosing) for the in vivo active AGD dose in comparison to the actual measured pesticide levels in male fetal plasma GD21, and the doses causing a significant effect on AGD.

	In vitro data			**Simulated levels by PBK modelling**	**In vivo data**
Compound	IC_{20} for AR antagonism (µM)	IC_{50} for AR antagonism (µM)	IC_{50} for Testosterone inhibition *in vitro* (µM)	Foetal levels (µM)	Plasma levels in male fetuses* (µM)
Procymidone		0.2	6	7.5	7.8
Vinclozolin		0.2	NE	-	ND
M1		2.0[c]		8.2	18.0
M2		0.2[c]		2.7	3.4
Linuron	1	3	27	1.2	1.8
M1	2	4	NE		5.9
M2 (main metabolite)	9	22	5		11.8
M3	4	12	ND		ND
p,p-DDE		0.6[d]	ND	1.5	3.5
Fludioxonil	0.8[a]	0.7	10	3.3 - 5.4	2.3 - 4.3
Cyprodinil	15.1[a]	28[b]	24	11.5 - 18.3	5.5 – 7.6
Dimethomorph	0.3[a]	0.9[b]	ND	1.0 – 1.5	1.3 – 1.4

[a] Orton et al. 2011; [b] Kugathas et al. 2016; [c]In-house unpublished data; [d]Li-Chun Xu et al. 2006; [e]Li You et al. 1998; NE: No effect; ND: could not be determined; * Foetal blood from male pups was pooled for each litter and analyzed for pesticide levels.

Incorporation of metabolism into in vitro assays

It is evident that in some cases, the metabolites – and not the parent compounds - turn out to be the active antiandrogenic compounds. One limitation of *in vitro* assays is that many cell lines in general have a low or no metabolic capacity and this means that we may overlook effects as we in many cases are only testing the parent compounds. In order to address this limitation, we have in the InVita project funded by the Danish EPA evaluated and applied a method to incorporate primarily Phase I metabolism in our *in vitro* assays. This was done by pre-incubating test compounds with rat liver enzyme fractions called S9, in order to generate a 'soup' of metabolites. This 'metabolite soup' was tested in our *in vitro* assays for antiandrogenic effects and the response was compared to that of the parent compound. The results can be seen in figure 9 and figure 10.

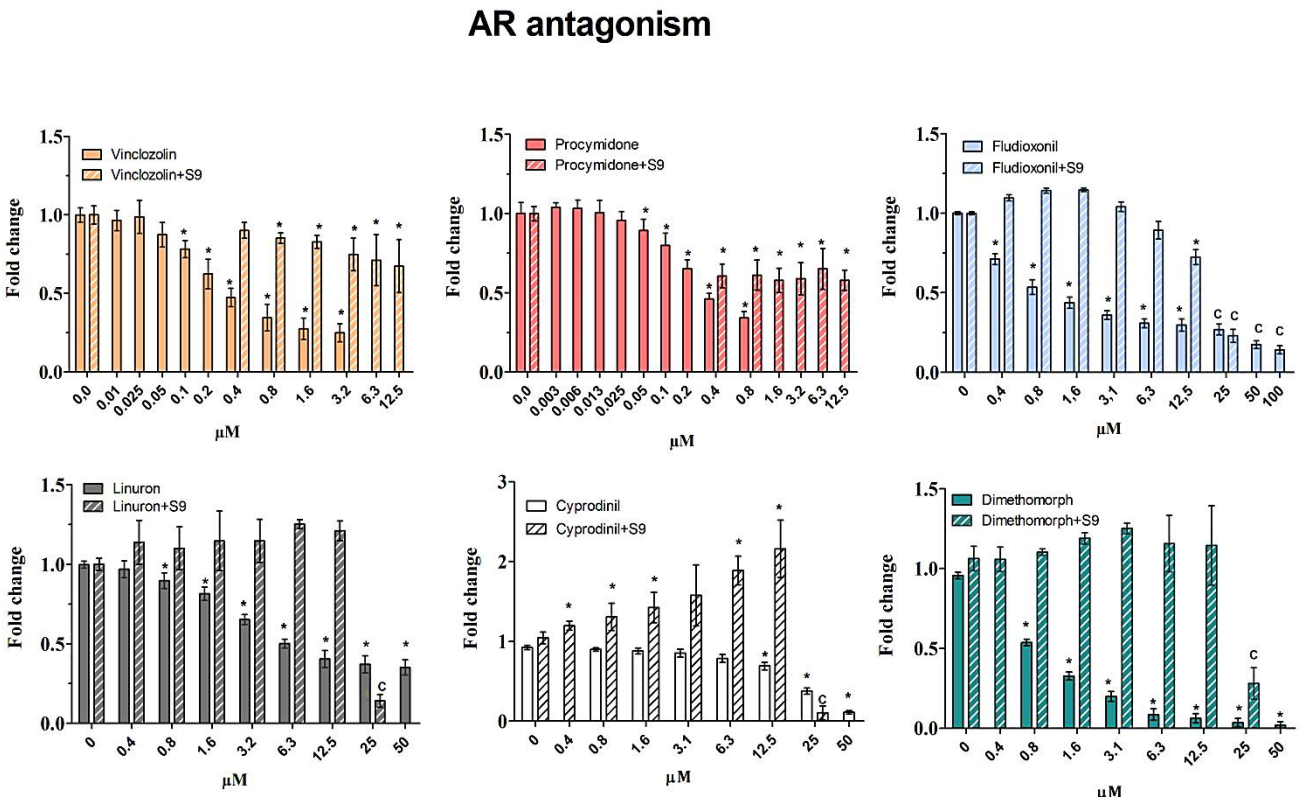

Figure 9. AR antagonistic effects of test compounds with and without S9
The effects of six test compounds (vinclozolin, procymidone, fludioxonil, linuron, cyprodinil, and dimethomorph) in the AR antagonist assay alone and following incubation with rat S9. Data represents mean ± SD. * indicates statistically significant from the background control. C: cytotoxicity.

Six pesticides were tested for AR antagonistic effects alone and following incubation with S9. As expected, all six parent compounds showed AR antagonistic effects with varying potency.
With the addition of S9 the AR antagonistic effects decreased dramatically or disappeared altogether suggesting that it is the parent compounds and not the metabolites that are the most potent AR antagonists.
It was somewhat unexpected that an increase in the AR antagonistic response was not seen for vinclozolin following incubation with S9. Several studies have reported that not only vinclozolin, but also the two main metabolites referred to as M1 and M2 exhibit AR antagonistic activity *in vitro.* Metabolite M2 has been reported to be the most potent antagonist followed by vinclozolin itself and the metabolite M1 (Molina-Molina et al. 2006; Wong et al. 1995). It is known that vinclozolin is unstable in aqueous solutions. In a study by Bursztyka et al., 2008 it was reported that after 2 hours incubation of vinclozolin in the cell culture medium, vinclozolin was almost totally hydrolyzed into around 20% M2 and 80% M1.
Taking this information into consideration, it is hypothesized that when we test vinclozolin alone (as parent compound) without S9 in our cell assays, the majority of vinclozolin is likely present as its major metabolites M1 and M2.
An explanation for why the AR antagonistic effect in the AR assay, meaning the concentration-response curves are not more alike with and without S9, could be that without S9 there might be more of the more potent M2 present compared to what is formed following incubation with S9. Additionally, it could also be that with the addition of rat S9 and the co-factors used, vinclozolin

is metabolised in a greater extent to less active metabolites than M1 and M2. In rats, it has been demonstrated that M1 and M2 are not the end products of the biotransformation of vinclozolin. In rats, vinclozolin and M2 are quickly biotransformed by dihydroxylation of the vinyl group and by further conjugation to glucuronic acid, and the activity of these metabolites toward the AR remains to be established (Bursztyka et al. 2008).

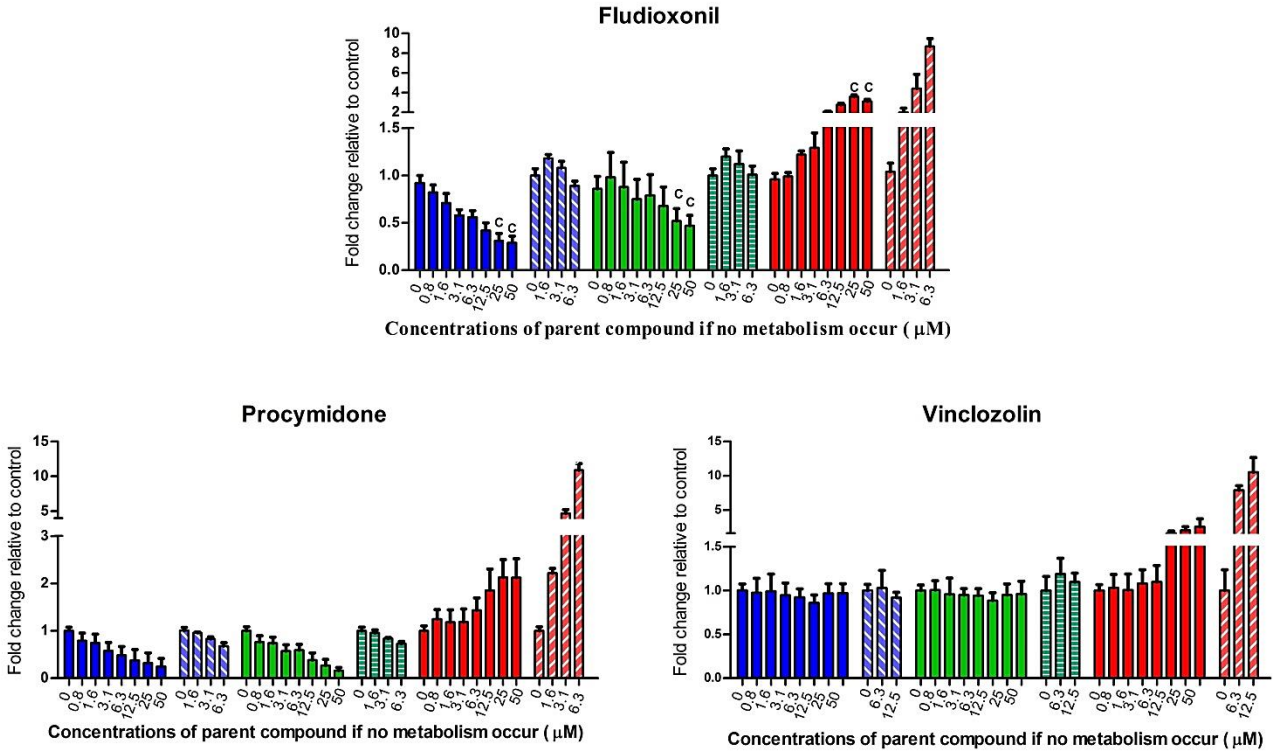

Figure 10. Effects on steroid hormone production in the H295R steroidogenesis assay
The pesticides fludioxonil, procymidone and vinclozolin were tested alone or following incubation with S9 for their effects on steroid synthesis. The bars show the effect on the production of testosterone (blue), androstendione (green) and estradiol (red). Data represents mean ± SD. C: cytotoxicity

Fludioxonil is known to inhibit synthesis of testosterone and androstendione, but this effect more or less disappeared after metabolizing the compound with S9. This indicates that it is the parent compound that is active on androgen inhibition. In contrast the significant increase in estradiol seen without S9 was even more pronounced with S9 (Fig. 10). This more pronounced increase in estradiol following incubation with S9 was also seen for procymidone and vinclozolin. Overall, for fludioxonil, procymidone and vinclozolin a more marked increase in estradiol production was observed following incubation with S9. This suggests that some metabolites are able to affect estradiol production more potently than the parent compounds.

1.5.3 Discussion & conclusion

Still the majority of the approx. 350 pesticides within the EU have not been tested for sensitive endocrine disrupting endpoints. We aimed at developing an alternative approach for ranking of

chemicals for their adverse effects on male reproductive health, focusing on AGD as an effect biomarker. A large number of the currently used pesticides show the potential to act as anti-androgens in vitro and the combination with kinetic modelling applied in this study proved very valuable for predicting in vivo effects, as well as serum dose levels in fetuses.
We successfully predicted adverse effects for six pesticides, three of which are current-use pesticides. We conclude that we have developed a first proof-of-concept of an approach that allows us to predict adverse effects of chemicals on male reproductive health. We did not only validate the predicted fetal levels and the adverse effect on the AGD, but were also able to show evidence that the observed effect on the AGD is due to disturbed androgen signaling tested in vitro. With this, we provide evidence that for these compounds mechanism-based in vitro assays can predict adverse health outcomes. Importantly, our approach shows that in vitro concentrations and in vivo levels can be correlated. although we at this point cannot predict the exact dose (but rather a dose range) that will result in a lowest-observed adverse effect level on AGD in vivo. Our approach has in the future the potential to be used to prioritize pesticides for in vivo testing on male reproductive effects, to optimize design of future animal tests, or to predict intake dose ranges that most likely will produce non-toxic in vivo responses. However, we still need to verify that the approach is valid for a larger range and number of chemicals. Our approach holds promise to become a novel animal-free tool for risk assessment of chemicals for male reproductive health disorders, although this will require more work in the future. Notably, the approach has a long-term potential as a concept for risk assessing chemicals for adverse effects on male reproductive disorders.

1.6 Overall conclusions

The MIraculiX project has provided new knowledge and better tools for testing and assessing chemicals for potential endocrine disrupting effects. This contributes to putting Denmark at the forefront with regard to endocrine disrupting effects of chemicals individually and in combination.

The research results include:

• Knowledge showing that the presumed safety margin of 100 between chemical exposure and human safety is inadequate.

• Further development of a user-friendly tool that brings together existing knowledge about the effects of chemicals and human exposure. This tool can be used by regulators specifically to calculate mixture effects of chemicals based on the current knowledge. Updating of the tool with new hazard and exposure data in the future is warranted.

• Knowledge that there are additional mechanisms of action involved in the endocrine disrupting effects of chemicals than those already determined; mechanisms of action that are currently not being investigated for and which we should focus more on in the future.

• The first steps on a method to predict the endocrine disrupting effect of chemicals in males with the use of fewer animals. This method indicates that computer models can relatively accurate predict the level of chemicals in the fetus. Along with data on mechanisms of action of hazards for chemicals, effects from in vitro models, the method may have a potential in future risk assessment of chemicals, although this will require more work in the future.

1.7 Perspectives

The results of the MIraculiX project support the work of adapting legislation of chemicals to take into account mixture effects. Among other things, the project points out that in the future there will be a need for a strengthened chemical risk assessment by:

• generating human-relevant data on hazardous effects and exposure for the many thousands of chemicals we lack data for. Such data are needed for calculating the risk of chemical mixtures

• generating more knowledge on the mechanisms of action of endocrine disruptors. This knowledge should be used to expand and improve the risk assessment of chemicals

• further developing more alternative methods based on cell and computer models to assess individual chemicals. Such methods are necessary to obtain a more humane-relevant risk assessment of chemicals.

• working politically within the EU to increase cooperation across legislations. Various chemicals are regulated under various legislations and risk assessment of mixtures should be encouraged by support from the different legislations.

Dissemination

The work performed within the MIraculiX project has been published in a number of peer-reviewed paper (see references in bold). More papers will be submitted during the next months. The MIraculiX project and results obtained herein has been presented more than 10 times at national and international meetings, workshops and conferences.

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Appendix 1: Terminology for mixture risk assessment

Acceptable daily intake (ADI)	An estimate of the amount of a substance in food or drinking water that can be consumed over a lifetime without presenting an appreciable risk to health. This term applies to chemical substances such as food additives, pesticide residues and veterinary drugs. (EFSA, 2018)
Component-based versus whole mixture approach	In a component-based approach the risk of a mixture is assessed based on exposure and effect data of its individual components. In contrast, in a whole mixture approach the mixture is treated as a single entity, similar to single chemicals, and so requires dose–response information for the mixture of concern or a (sufficiently) similar mixture. (EFSA, 2018)
Cumulative assessment group (CAG)	Group of substances that could plausibly act by a common mode of action, not all of which will necessarily do so (EFSA, 2013).
Dose addition and concentration addition	A component-based model in which the components are treated as if having a similar action. The components may vary in toxic potency. Components contribute to the mixture effect relative to the ratio between their concentration and toxic potency. Dose is the exposure metric used in human and animal health risk assessment. Concentration is the exposure metric used as a proxy for dose in in vitro studies and ecological risk assessment. (EFSA, 2018)
Hazard index (HI)	The HI is the sum of HQs for individual components of a mixture or in a cumulative assessment group. In its simplest and most conservative form, the HI sums up HQs for substances with different modes of action, whereas refined approaches to mixture risk assessment sums up HQs for substances with the same target organs or the same modes of action. (EFSA, 2018)
Hazard quotient (HQ)	The HQ is the ratio between exposure and HBGV for a given substance. (EFSA, 2018)
Health based guidance value (HBGV)	A numerical value derived by dividing a point of departure (a no observed adverse effect level, benchmark dose or benchmark dose lower confidence limit) by a composite uncertainty factor to determine a level that can be ingested over a defined time period (e.g. lifetime or 24 h) without appreciable health risk (EFSA, 2018; WHO, 2009). In the food safety area, HBGVs include the Acceptable Daily Intake (ADI) for food additives and pesticides, the Tolerable Daily Intake (TDI) for contaminants and chemicals in food contact materials and, for acute effects, the Acute Reference Dose (ARfD) (EFSA, 2013).
Mixture	Any combination of two or more chemicals that may jointly contribute to real or potential effects regardless of source and spatial or temporal proximity. A simple mixture is a mixture whose components are fully chemically characterized, e.g. a group of defined substances with the potential to have combined effects, in contrast to complex mixtures for which not all constituents are characterized. (EFSA, 2018)

Mode of action versus mechanism of action	Biologically plausible sequence of key events in an organism leading to an observed effect, commonly supported by robust experimental observations and mechanistic data. It refers to the major steps leading to an adverse health effect following interaction of the compound with biological targets. It does not imply full understanding of mechanism of action at the molecular level, which is a detailed explanation of the individual biochemical and physiological events leading to a toxic effect (EFSA, 2018; EFSA, 2013).
Tolerable daily intake (TDI)	An estimate of the amount of a substance in food or drinking water which is not added deliberately (e.g contaminants) and which can be consumed over a lifetime without presenting an appreciable risk to health. (EFSA Glossary)

Appendix 2: Realistic mixtures of chemicals

Table 2.1 showing the composition of the chemical mixture for the animal experiment and the doses of each chemical in the low dose group (Hadrup et al. 2016):

Chemical (or common) name	**Concentration measured in humans (reference)**	**Low-dose (µg/kg bw/day)**
Acrylamide	5.4 nmol/L plasma [10]	4
Benzophenone-3 /oxybenzone	22.9 µg/L urine [10]	2.6
Bisphenol A	2.64 µg/L urine [10]. Alternatively a plasma concentration in the Hong Kong population was 0.95 µg/L [12]	10
Triclosan	13 µg/L urine [10]	5
Ortho-phenylphenol	0.5 µg/L urine [10]	0.06
trans-nonachlor	0.11 ng/g serum [10]	0.35
p.p-DDE / Dichlorodiphenyl-dichloroethylene	1.54 ng/g serum [10]	13
2.4.6-trichlorophenol	2.85 µg/L urine [10]	10
Chlorpyrifos	1.77 µg/L urine of the metabolite TCPγ. [10]	0.4
3-phenoxybenzoic acid	0.29 µg/L urine [10]	0.01
Arsenic	8.3 µg/L urine [10]	1.2
Barium	1.5 µg/L urine [10]	0.13
Cadmium	0.41 µg/L blood [10]	0.1
Cesium	4.4 µg/L urine [10]	0.48
Cobalt	0.38 µg/L urine [10]	0.05
Lead	0.80 µg/L urine [10]	16
Mercury	0.44 µg/L urine [10]	3.5
Thallium	0.18 µg/L urine [10]	0.3
PFOS/Perfluorooctanesulfonic acid	20.7 µg/L serum [10]	0.9
PFNA/Perfluorononanoic acid	1.0 µg/L serum [10]	0.2
Mono-n-butyl phthalate	24.6 µg/L urine [10]	62
AHTN / 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline	No data on human tissue concentrations. Values are based on intake HERA (2004) data [13]	6.2
PCB 153 (covering the PCBs) Polychlorinated biphenyl 153	0.17 ng/g serum [10] An alternative number is reported by Bakker *et al.* [14]. Here seven indicator PCBs are present at 0.36 ng/g serum	20
TCDD (Dioxines) / 2,3,7,8-Tetrachlorodibenzo-p-dioxin	2.6 ng/L serum (sum of four dioxins: HpCCD 155 fg/g serum; HxCCD 105 fg/g serum; OCDD 2230 fg/g serum; HpCDF 62 fg/g serum) [10]	0.034
Benzo[a]pyrene (PAHs)	6.3 µg/L urine (a sum of 10 PAHs: 2-hydroxyfluorene 304 ng/L; 3-hydroxyfluorene 134 ng/L; 9-hydroxyfluorene 267 ng/L; 1-hydroxynaphtalene 2680 ng/L; 2-hydroxynaphtalene 2470 ng/L; 1-hydroxyphenantrene 140 ng/L; 2-hydroxyphenantrene 54 ng/L; 3-hydroxyphenantrene 105 ng/L; 4-hydroxyphenantrene 23 ng/L; 1-hydroxypyrene 89 ng/L) [10]	0.4
PHIP / 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine	0.41 ng/ L (mean of 13 subjects, 24 h urine set to 1 L) based on Wakabayashi *et al.* [15]	0.1
MeIQx/2-Amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline	22.5 ng/ L (mean of 13 subjects, 24 h urine set to 1 L) based on Wakabayashi *et al.* [15]	0.05
Total dose		**160**

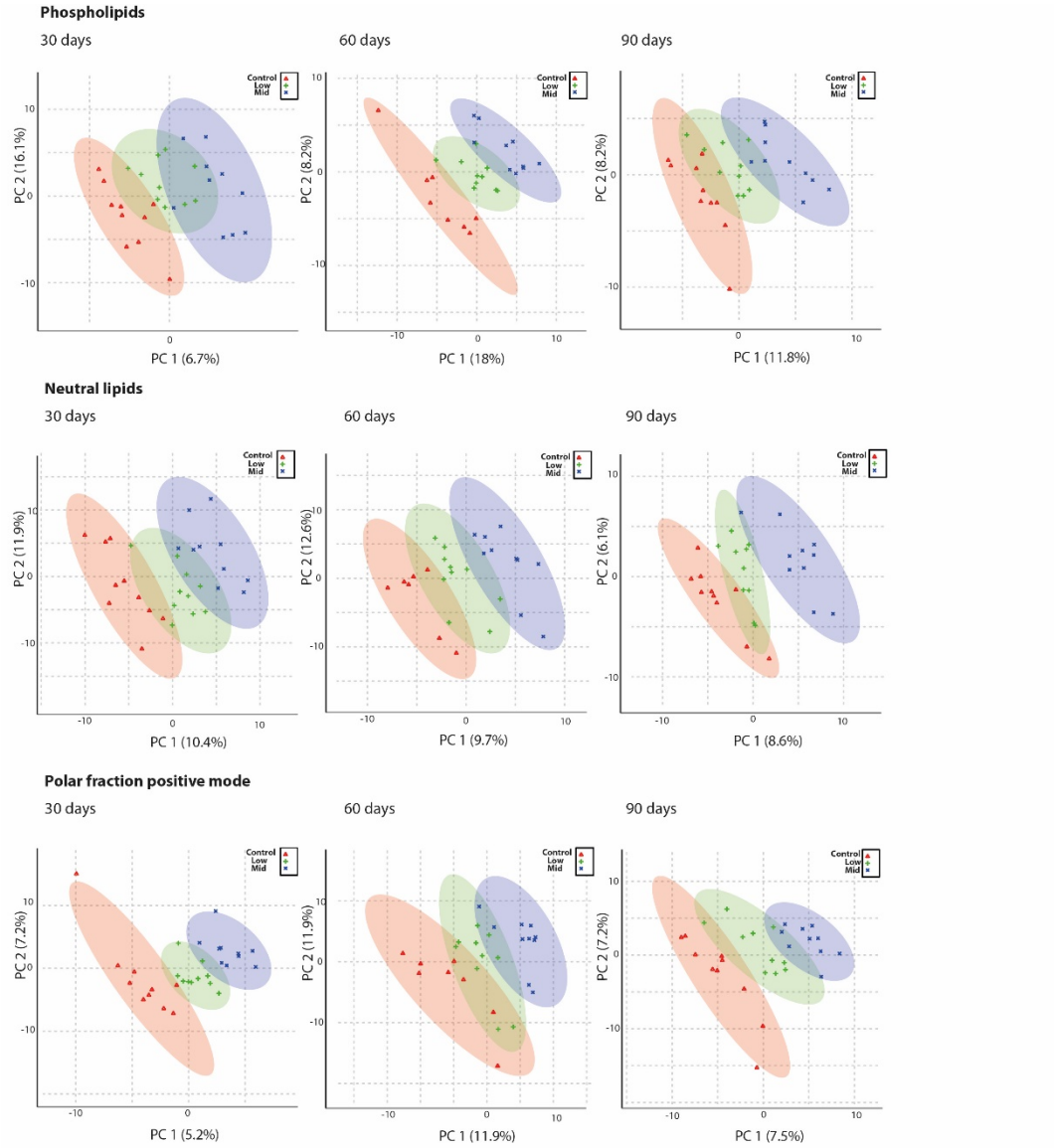

Figure 2.1. PLS DA plots of metabolomics data show that the Low-dose has an overall effect on the metabolome. Plasma from rats administered Low- and Mid-dose for 30, 60 or 90 days was separated into a phospholipid, a neutral lipid and a polar fraction. These were separated by HPLC and analyzed by MS using the positive ionization mode (positive mode) and for the polar fraction also the negative mode (Hadrup et al. 2016).

Appendix 3: Mechanisms involved in AGD development

In vivo study

In addition to the *in vivo* rat studies described in section 3, we have carried out an additional GD21 study (Sprague Dawley time-mated rats) with the compounds finasteride (FIN) and enzalutamide (ENZ). These compounds were specifically selected based on *a priori* knowledge about mechanisms of action. Moreover we predicted that exposure to these compounds would result in significant shorter male AGD at the chosen doses, allowing for follow-up mechanistic studies. Each *in vivo* study included six pregnant rats per group exposed orally from GD 7-GD 21 and both compounds resulted in significantly shorter AGD in male offspring at GD 21 caesarean sections (Figure 3.1). In addition, few male fetuses in the Finasteride exposed group showed cryptorchidism at GD21. At GD 17, a subset of the animals was also collected at GD17 (n=2-4 litters) for chemical analysis of e.g. Blood and amniotic fluid (see figure 3.2).

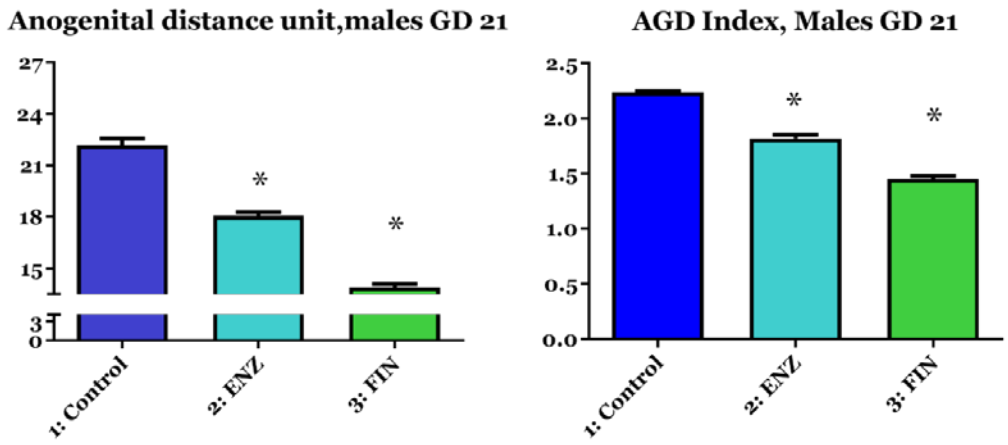

Figure 3.1: Compared to control fetuses (Sprague Dawley), male anogenital distance (AGD) was significantly shorter in GD21 offspring following in utero exposure GD 7-21 to Enzalutamide (ENZ) (19%) or Finasteride (FIN) (35%)(both compounds 10 mg/kg bw/day, N=6 litters/group). Here AGD units is shown on the left (statistics made with body weight as covariate) and on the right, the AGD index (calculated by dividing AGD by the cubic root of the body weight) is seen. These results are consistent with previous studies showing several adverse effects on male reproduction after fetal exposure to Finasteride (ENZ data are published in Schwartz et al. 2019b).

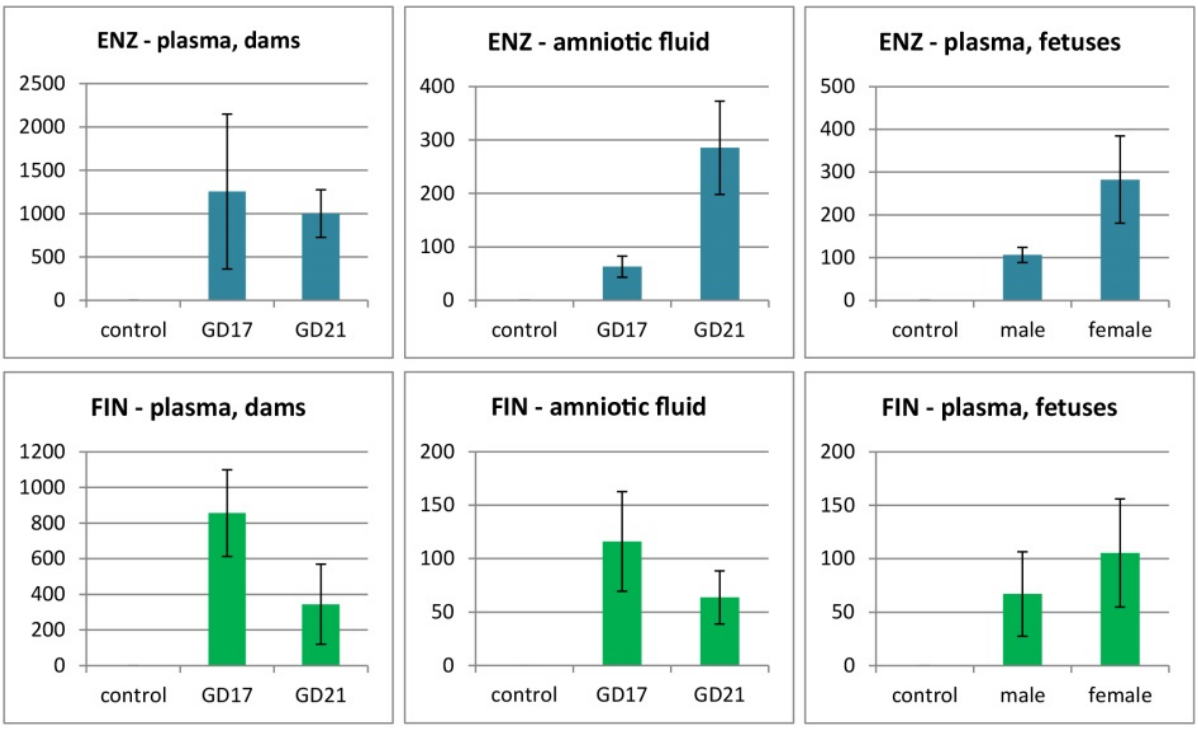

Figure 3.2: Concentrations of ENZ (blue) and FIN (green) at gestational day (GD) 17 and 21 in dams' plasma, amniotic fluid or in male and female fetal plasma, respectively are shown (n=2 litters/group). A tendency towards higher levels of ENZ at GD21 and at GD17 for FIN are seen. Even if FIN fetal levels were lower it produces a more marked effect on AGD (see fig 3.1) (FIN data are published in Schwartz et al. 2019b, and ENZ data has just been submitted for publication).

Table 3.1: Summary of rat toxicity studies reporting on AGD measurements following gestational exposure to phthalates. AGD data after *in utero* exposure to various phthalates and the dose at which maximum shorter mean AGD was observed. In many instances, percentage shorter AGD was estimated from published graphs, as raw data was not available (Schwartz et al. 2019a).
X = Not assessed; **n.e.** = no effect; ↑ = Longer female AGD or AGDi; ↓ = Shorter female AGD or AGDi.

Abbreviations: ***DMP*** *(dimethyl phthalate),* ***DEP*** *(diethyl phthalate),* ***DBP*** *(dibutyl phthalate),* ***MBuP*** *(monobutyl phthalate),* ***DiBP*** *(di-isobutyl phthalate),* ***DEHP*** *(diethylhexyl phthalate),* ***DHP*** *(di-n-hexyl phthalate),* ***DCHP*** *(dicyclohexyl phthalate),* ***BBP*** *(benzyl butyl phthalate),* ***MBeP*** *(monobenzyl phthalate),* ***DnHP*** *(di-n-hexyl phthalate),* ***DHPP*** *(di-n-heptyl phthalate),* ***DiHP*** *(di-isoheptyl phthalate),* ***DnOP*** *(di-n-octyl phthalate),* ***DOTP*** *(dioctyl terephthalate),* ***DiNP*** *(di-isononyl phthalate),* ***DUDP*** *(diundecyl phthalate),* ***DTDP*** *(ditridecyl phthalate).*

	Substance	Dose at max effects (mg/kg bw/day)	male AGD max effect (% shorter)	male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
Increasing chain length (descending order)	**DMP**	750	n.e.	n.e.	n.e.	(Gray et al. 2000)
	DEP	750	n.e.	n.e.	n.e.	(Gray et al. 2000)
	DiBP	250	n.e.	5	x	(Saillenfait et al. 2017)
		600	14	9	↑	(Borch et al. 2006)
		625	22	x	n.e.	(Saillenfait et al. 2008)
	DBP	500	n.e.	x	x	(Scott et al. 2007)
		500	9-14	12(Martino-Andrade)	n.e.Martino-Andrade	(Barlow et al. 2004; Howdeshell et al. 2007; Martino-Andrade et al. 2009; Wolf et al. 1999)
		500	20-28	21(de Mello Sanotos)	n.e.Saillenfait	(Carruthers and Foster 2005; de Mello Santos et al. 2017; Mylchreest et al. 1999; Saillenfait et al. 2008; Scott et al. 2008; Wolf et al. 1999)
		~640	11	10	x	(Clewell et al. 2013)
		~650	43	26(AGD/BW)	n.e.	(Ema et al. 1998)
		~700	19Increases at other doses	x	n.e.	(Lee et al. 2004)
		750	x	9	x	(Jiang et al. 2007)
		750	20-24	x	n.e.Mylchreest	(Mylchreest et al. 1998; van den Driesche et al. 2017)
		750	36	x	x	(Van den Driesche et al. 2012)
		850	20	x	x	(Jiang et al. 2011; Liu et al. 2016)
		850	x	6	x	(Jiang et al. 2015b)
		900	27	x	x	(Li et al. 2015)
		1500	48	26(AGD/BW)	n.e.	(Ema et al. 2000)
	MBuP	750	39	29	n.e.	(Ema and Miyawaki 2001)

	Substance	Dose at max effects *(mg/kg bw/day)*	male AGD max effect *(% shorter)*	male AGDi max effect *(% shorter)*	Female AGD	References
Increasing chain length (descending order)	**DEHP**	30	X	n.e.	n.e.	(Christiansen et al. 2009)
		150	n.e.	n.e.	n.e.	(Martino-Andrade et al. 2009)
		300	X	5	n.e.	(Nardelli et al. 2017)
		500	10	x	x	(Howdeshell et al. 2007)
		500	18	18	x	(Saillenfait et al. 2009b)
		750	17-18	17(Kita)	x	(Jarfelt et al. 2005; Kita et al. 2016; Lin et al. 2009)
		750	30-34	x	n.e.Gray	(Gray et al. 2000; Wolf et al. 1999)
		900	14	x	x	(Christiansen et al. 2010)
		1000	30	11(AGD/BW)	x	(Li et al. 2013)
		1500	27	x	n.e.	(Moore et al. 2001)
	DHP	500	20	23	x	(Aydoğan Ahbab and Barlas 2015)
	DCHP	~350	6	7	n.e.	(Hoshino et al. 2005a)
		500	27	26	x	(Aydoğan Ahbab and Barlas 2015)
		750	17	13	n.e.	(Saillenfait et al. 2009a)
	BBP	500	8-13	x	↑Nagao	(Hotchkiss et al. 2004; Nagao et al. 2000)
		750	9	x	n.e.	(Tyl et al. 2004)
		750	30	x	n.e.	(Gray et al. 2000)
		1000	38	29	n.e.	(Ema and Miyawaki 2002)
	MBeP	375	30	29	n.e.	(Ema et al. 2003)
	DnHP	500	18	18	x	(Saillenfait et al. 2009b)
		750	35	31	↓	(Saillenfait et al. 2009a)
	DiHP	~500	15	x	n.e.	(McKee et al. 2006)
	DHPP	1000	11	10	n.e.	(Saillenfait et al. 2011)
	DnOP	1000	n.e.	n.e.	x	(Saillenfait et al. 2011)
	DOTP	750	n.e.	n.e.	n.e.	(Gray et al. 2000)
	DiNP	750	n.e.	n.e.	n.e.Gray	(Clewell et al. 2013; Gray et al. 2000)
		900	8	6	n.e.	(Boberg et al. 2011)
		~1165	n.e.	x	n.e.	(Masutomi et al. 2003)
	DUDP	500	n.e.	4	n.e.	(Saillenfait et al. 2013)
	DTDP	1000	n.e.	n.e.	n.e.	(Saillenfait et al. 2013)

Table 3.2: Summary of rat toxicity studies reporting on AGD measurements following gestational exposure to compounds other than phthalates. AGD data after *in utero* exposure to various substances and the dose at which maximum shorter mean AGD was observed. In many instances, percentage shorter AGD was estimated from published graphs, as raw data was not available (Schawartz et al. 2019a)
***** = non-monotonic (low-dose) effect; **x** = Not assessed; **n.e.** = no effect; ↑ = Longer female AGD or AGDi; ↓ = Shorter female AGD or AGDi.
Abbreviations: ***DDE*** *(DDT metabolite, dichlorodiphenyl- dichloroethylene),* ***TCDD*** *(2,3,7,8-tetrachlorodibenzo-p-dioxin),* ***HBM*** *(2-Hydroxy-4-Methoxybenzone),* ***OMC*** *(Octyl Methoxycinnamate)*

	Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
	Acetylsalicylic acid	400	38	x	n.e.	(Gupta and Goldman 1986)
	Aniline	*93*	*x*	*20*	*x*	(Holm et al. 2015) (mouse study)
	Paracetamol	150	9*	10.5*	x	(Kristensen et al. 2011)
		150	*x*	*15*	*x*	(Holm et al. 2015) (mouse study)
		350	8	9	x	(van den Driesche et al. 2015)
		360	x	n.e.	n.e.	(Axelstad et al. 2014)
	Dexamethasone	0.1	10	x	x	(Van den Driesche et al. 2012)
	Finasteride	0.1	x	9	x	(Christiansen et al. 2009)
		100	33	x	x	(Bowman et al. 2003)
	Flutamide	16-20	44[Kita]	41-42	x	(Hass et al. 2007; Kita et al. 2016)
		50	16-53	x	x	(Foster and Harris 2005; McIntyre et al. 2001)
		100	33-55	x	x	(Mylchreest et al. 1999; Scott et al. 2007; Welsh et al. 2007)
	Ethinyl estradiol	(0.00-0.05)	n.e.	n.e.	(↑)[Mandrup]	(Ferguson et al. 2011; Howdeshell et al. 2008; Mandrup et al. 2013)
	Ketoconazole	(50)	n.e.	x	x	(Wolf et al. 1999)
		50	8	11	↓	(Taxvig et al. 2008)
	Epoxiconazole	3.75	5*	5*	↑	(Hass et al. 2012)
		15	7[(PND0)*]	10[(GD21)*]	↑	(Taxvig et al. 2007)
		50	n.e.	n.e.	n.e.	(Taxvig et al. 2008)
	Myclobutanil	145	12 [Increased]	x	x	(Goetz et al. 2007)
	Prochloraz	(0.01-35)	n.e.	x	(↑)[Melching, Hass]	(Christiansen et al. 2009; Hass et al. 2012; Melching-Kollmuss et al. 2017; Vinggaard et al. 2005)
		150	x	12	↑	(Laier et al. 2006)
		250	6	x	↑	(Noriega et al. 2005)

	Propiconazole	50	n.e.	n.e.	n.e.	(Taxvig et al. 2008)
		~158	7[Increased]	x	x	(Goetz et al. 2007)
	Tebuconazole	12.5-50	n.e.	n.e.	(↑)[Hass]	(Hass et al. 2012; Taxvig et al. 2008)
		100	n.e.	10[Increased (only at GD21)]	↑	(Taxvig et al. 2007)

	Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
	Triadimefon	~114	3[Increased]	x	x	(Goetz et al. 2007)
	Mancozeb	25	n.e.	n.e.	n.e.	(Hass et al. 2012)
	Vinclozolin	12	n.e.	n.e.	n.e.	(Colbert et al. 2005)
		50-60	21[Matsuura]	9-21	n.e.[Matsuura]	(Christiansen et al. 2009; Matsuura et al. 2005a)
		~100	28	22	x	(Schneider et al. 2011)
		100	28	x	x	(Ostby et al. 1999)
		160	x	35	x	(Hass et al. 2007)
		200	46-56	x	(↓)[Gray]	(Gray et al. 1994; Wolf et al. 2004)
	Procymidone	50	10	9	n.e.	(Hass et al. 2012)
		100	24	n.e.	x	(Wolf et al. 1999)
		150	x	37	x	(Hass et al. 2007)
	Linuron	50	8[Not sig. in 2000]	x	n.e.[2002]	(McIntyre et al. 2002; McIntyre et al. 2000)
		75-100	25-31	x	x	(Hotchkiss et al. 2004; Wolf et al. 1999)
	p,p'-DDE	100	6-9	x	x	(Wolf et al. 1999)
		50-200	x	11 [(AGD/crown-rump length)]	x	(Loeffler and Peterson 1999)
	Fenitrothion	25	16	x	n.e.	(Turner et al. 2002)
	Lindane	~16	n.e.	n.e.	(↓)	(Matsuura et al. 2005b)
	Methoxychlor	~82	n.e.	x	n.e.	(Masutomi et al. 2003)
	Benzophenone	(~130)	n.e.	n.e.	↓	(Hoshino et al. 2005b)
	HBM	(~3250)	x	n.e.	n.e.	(Nakamura et al. 2015)
	OMC	(1000)	n.e.	n.e.	n.e.	(Axelstad et al. 2011)
	Butylparaben	500	7	6	↓	(Boberg et al. 2016)
		600	n.e.	n.e.	n.e.	(Boberg et al. 2008)
		1000	16	x	x	(Zhang et al. 2014)
	Bisphenol A	0.25	7	x	↓	(Christiansen et al. 2014)

		(0.0025-50)	n.e.	n.e.(Ferguson, Tinwell)	n.e.Ferguson, Tinwell	(Ferguson et al. 2011; Howdeshell et al. 2008; Tinwell et al. 2002)
		(5-385)	n.e.	x	n.e.	(Takagi et al. 2004)
	Nonylphenol	(~250)	n.e.	n.e.	↓	(Takagi et al. 2004)
	Genistein	~67	n.e.	x	n.e.	(Masutomi et al. 2003)
	TCDD	0.1	6-12Not sig. when BW or CR length taken into account	x	x	(Bjerke and Peterson 1994; Gray et al. 1995)

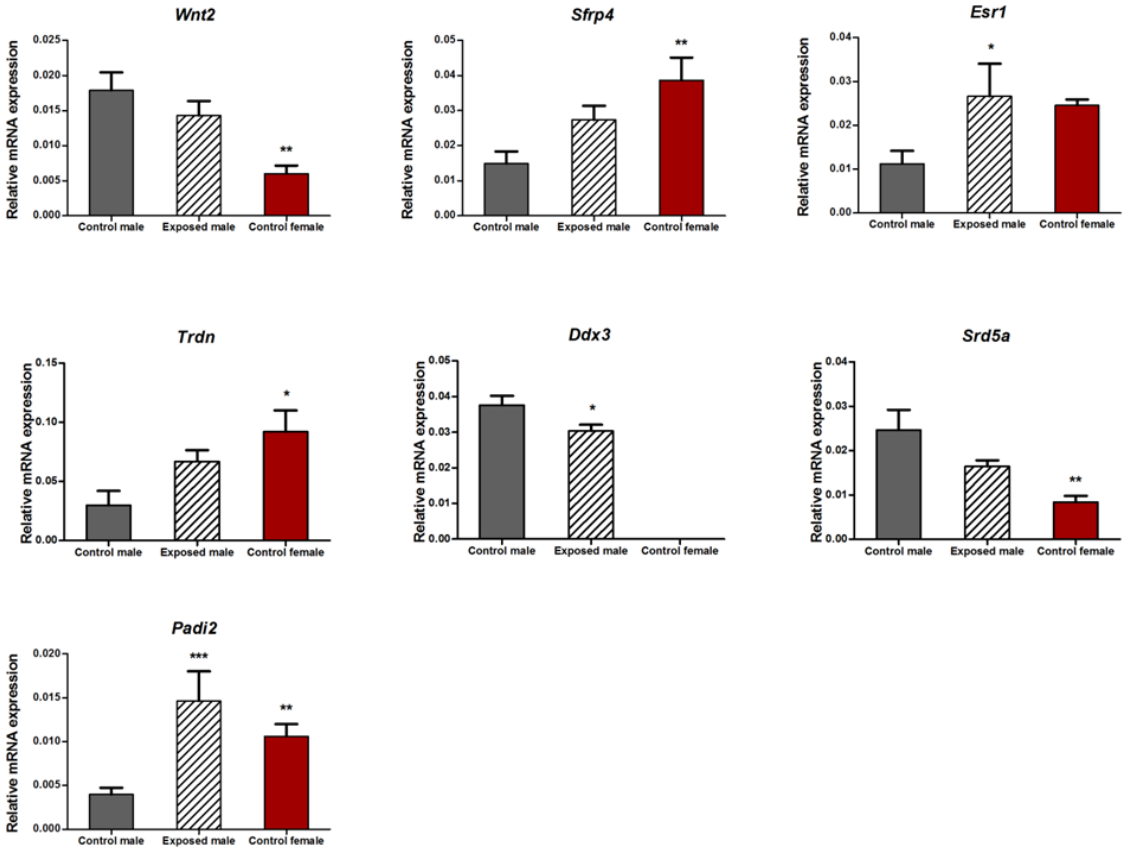

Figure 3.3: RT-qPCR validation of the gene array. The data corroborates the gene array findings. In addition, several genes confirm that the male pups exposed to Finasteride have an expressional profile intermediary to that of the control male and control female, indicating a feminization of these animals.

Appendix 4: Predictive approach for evaluating compromised male reproductive health

In vivo studies

Several in vivo studies were conducted in order to obtain knowledge on chemicals' effects on AGD levels and for obtaining knowledge on fetal exposure levels. Such data were needed in order to validate the predictions that were made based on the alternative methods. The studies were made with time-mated pregnant Sprague Dawley rats from which fetuses were taken by Caesarean section for analytical chemistry (blood and amniotic fluid) and measurements of AGD at GD 21. In general, the pregnant dams were exposed orally to the pesticides *in utero* from Gestation day (GD)7 to GD21, which includes the sensitive fetal masculinization window GD15-19. At GD21, fetuses were retrieved by Caesarean section and analysed for changes in AGD and other clear signs of toxicity. In some cases, a subset of animals was also collected at GD17 (n=2-4 litters) or GD19 (n=2 litters) for retrospective molecular analyses depending on results obtained from the GD21 experiments, as well as additional blood sampling from the dams for chemical kinetics analysis. At GD 17 and GD 19 it was not possible to assess AGD or collect blood from the fetuses. The AGD data from GD 21 for vinclozolin and procymidone are shown in fig 4.1 below and AGD data for linuron in fig 4.2.

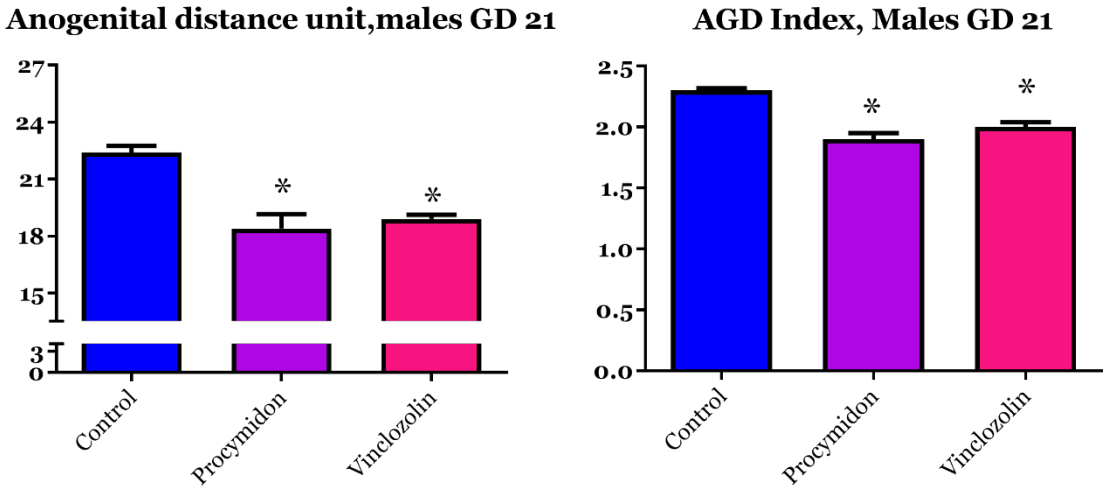

Figure 4.1: Compared to control fetuses (Sprague Dawley), male Anogenital distance (AGD) was significantly shorter in GD21 male fetuses following in utero oral exposure GD 7-21 to Procymidone (PRO) (17%) or Vinclozolin (VIN)(15%) (40 mg/kg bw/day both groups, n=6 litters, *p-value <0.001). Here AGD units is shown on the left (statistics made with body weight as covariate) and on the right, the AGD index (calculated by dividing AGD by the cubic root of the body weight) is seen. No significant effects on female AGD was observed (data not shown). For PRO and VIN similar findings were seen in previous studies with these compounds with AGD measured at PND 1 and in another rat strain (Wistar).

An in vivo study on linuron has been performed with three dose levels and a control (25, 50 or 100 mg/kg/day). However, some maternal toxicity was seen and therefore the highest dose

(100 mg/kg/day) had to be lowered to 75 mg/kg/day. The time-mated Sprague Dawley rats were exposed from GD 13-21 as maternal toxicity has been described in the published literature in earlier parts of gestation. The sensitive fetal masculinization window GD15-19 is still covered. Notably, we have seen shorter AGD in our exposed male offspring (Fig 4.2) and the levels of linuron and its metabolites have been measured in fetal blood and amniotic fluid (Table 7.1).

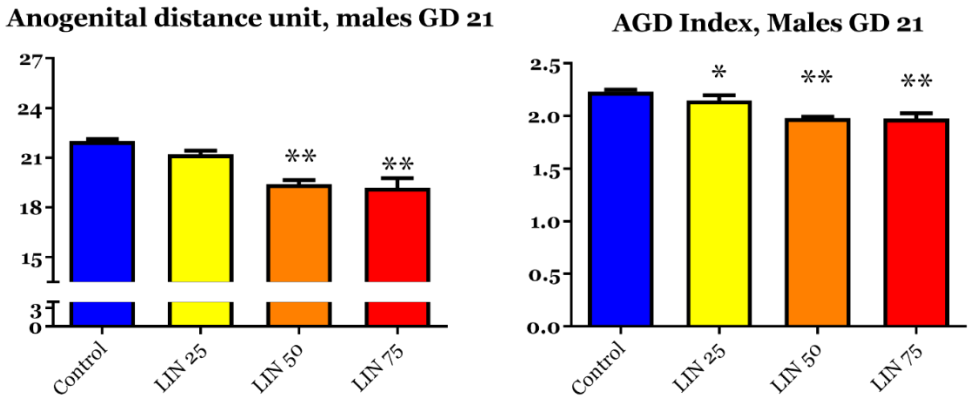

Figure 4.2: Compared to control fetuses (Sprague Dawley), male Anogenital distance (AGD) was significantly shorter in GD21 fetuses following in utero exposure GD 13-21 to Linuron from 25 mg/kg bw/day and above. Here AGD units is shown on the left (statistics made with body weight as covariate) and on the right, the AGD index (calculated by dividing AGD by the cubic root of the body weight) is seen. No significant effects on female AGD at GD 21 were observed; N=4 litters/group, *=p<0.05, **=p<0.01

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